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Molecular Characterization of the Horizontal Gene Transfer between Mycoparasite, *Parasitella parasitica*, and its Host, *Absidia glauca*

Dissertation

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2) Burmester A., **Karimi S**., Wetzel J. Wöstemeyer J. (2013) Complementation of a stable *Met2-1* mutant of the zygomycete *Absidia glauca* by the corresponding wild-type allele of the mycoparasite *Parasitella parasitica*, transferred during infection. *Microbiology* 159: 1639–1648.

Summary

Parasitella parasitica is a zygomycetous mycoparasite with several hosts in Mucorales. During the infection process, it transfers nuclei and other cellular components to its hosts through biotrophic fusion. Gene transfer was proven by the formation of para-recombinants after infection with the genetic background of the host plus some additional traits from the parasite. In this thesis, natural horizontal gene transfer system has been studied for the first time at the molecular level with respect to chromosomally located genetic markers. For this purpose, a *P. parasitica* adenine auxotrophic mutant was employed as DNA donor, whereas a methionine auxotroph *A. glauca* served as recipient. The resulting methionine prototrophic hybrids were selected and analyzed for their properties:

1) Inability of the *A. glauca* auxotroph to convert homoserine to homocysteine in feeding experiments led us to identify the genes for homoserine O-acetyltransferase (*Met2-1*, *Met2-2*) as most probable point of mutation. These genes also identified in *P. parasitica*.

2) Hybridization patterns and nucleotide sequences of the *A. glauca* mutant revealed a large insertion in the *Met2-1* gene in comparison with its wild type strain.

3) Transformation experiments with constructs containing the *Met2-1* gene as selective marker could recover methionine prototrophic growth, while the *Met2-2* gene could not. Beside *Met2-1*, *GFP* gene was applied as the reporter gene.

4) Hybridization of para-recombinant DNAs with *Met2-1* and *Met2*-2 probes of *A. glauca* and *P. parasitica* showed that recombinants had acquired part of the *Met2-1* gene from *P. parasitica* and a duplicated part of *A. glauca Met2-2* gene in extrachromosomal form that enabled them to grow in absence of methionine.

This way of foreign DNA establishment is a common feature in mucorales and regarded as the main reason for observed mitotic instability during subsequent sporulation cycles that discussed by possible defense mechanisms, directed against invading DNA.

Zusammenfassung

Parasitella parasitica ist ein zu den Zygomyceten gehörender Mycoparasit, der etliche Wirte aus der Ordnung Mucorales infizieren kann. Während des Infektionsverlaufs transferiert dieser Pilz Kerne und in der Folge auch genetische Information in seine Wirte. Der Transfer von Kernen, anderen zellulären Komponenten und schließlich von DNA ist die Konsequenz eines Fusionsereignisses zwischen Parasit und Wirt an den Infektionsstrukturen. Wegen dieser Eigenschaft hat man diesen Infektionstyp als biotrophen Fusionsparasitismus beschrieben.

Der Gentransfer wurde anhand der Bildung von Rekombinanten (Para-Rekombinanten) nach der Infektion bewiesen. Diese weisen im Wesentlichen den genetischen Hintergrund des Wirts zuzüglich einiger zusätzlicher Eigenschaften des Parasiten auf. In dieser Arbeit wurde der natürliche horizontale Gentransfer (HGT) zum ersten Mal für chromosomal lokalisierte Elemente auf molekularer Ebene analysiert. Zu diesem Zweck wurde eine Adenin-auxotrophe Mutante von *P. parasitica* als DNA-Donor verwendet, während eine *A. glauca* Mutante mit einem Defekt in der Methionin-Biosynthese als Rezipient diente. Para-Rekombinanten wurden anhand ihrer Fähigkeit selektiert, auch in Abwesenheit von Methionin prototroph zu wachsen. Die resultierenden genetischen Hybride wurden auf die folgenden Eigenschaften untersucht:

1) Die genetische Basis des Defekts in der Methionin-Biosynthese in der auxotrophen *A. glauca*-Mutante wurde mit Fütterungsexperimenten aufgeklärt. Die Unfähigkeit dieser Mutante, Homoserin in Homocystein umzuwandeln, wies auf das Gen für Homoserin-O-acetyltransferase (*Met2-1*, *Met2-2*) als wahrscheinlichsten Ort für die zugrunde liegende Mutation hin. Die entsprechenden Gene wurden ebenfalls in *P. parasitica* identifiziert.

2) Die Hybridisierungsmuster und Nukleotidsequenzen der Methionin-defizienten *A. glauca* Mutante ließen eine große Insertion im *Met2-1* Gen der Mutante im Vergleich zum Wildtyp erkennen.

3) Transformationsexperimente mit Konstrukten, die das *Met2-1* Gen als selektive Marke enthielten, ermöglichte uns, Methionin-prototrophe Derivate zu erhalten, während das *Met2-2* Gen in Transformationsexperimenten nicht in der Lage war, die Mutation zu komplementieren. Neben *Met2-1* als selektiver Marke wurde das Gen für das Grün-Fluoreszierende-Protein (*GFP*) als Reportergen verwendet. Damit wurde es möglich, solche Transformanten auch mikroskopisch zu analysieren.

4) Die Identifikation von *Met2-1* als verantwortliches Gen für die Komplementation der Methionin-Auxotrophie in der *A. glauca*-Mutante verhalf uns zu einem verlässlichen Werkzeug, die genetischen Ereignisse in Para-Rekombinanten zu verfolgen. Hybridisierungsexperimente mit DNAs aus Para-Rekombinanten mit Proben für die *Met2-1* and *Met2-2* Gene aus *A. glauca* sowie aus *P. parasitica* zeigten, dass diese Rekombinanten einen Teil des *Met2-1*-Gens aus *P. parasitica* aufgenommen hatten. Das neu erworbene *Met2-1*-Gen aus *P. parasitica* erlaubte den Para-Rekombinanten Wachstum in Abwesenheit von Methionin. Dieses Phänomen wird von der Duplikation eines Teils des *Met2-2*-Gens von *A. glauca* in extrachromosomaler Form begleitet. Die Funktion von *Met2-2* in *A. glauca* bleibt jedoch unklar.

Die Resultate der Experimente sowohl mit Para-Rekombinanten als auch mit Transformanten zeigen klar, dass neu erworbene fremde DNA immer extrachromosomal auf Plasmiden propagiert wird. Diese Art der DNA-Etablierung in den Rezipienten wird als Grund für die mitotische Instabilität der neu erworbenen Eigenschaften interpretiert, wie sie immer während aufeinander folgender Sporulationszyklen beobachtet wird. Extrachromosomale Etablierung ist eine generelle Eigenschaft von Transformationssystemen in allen Pilzen der Ordnung Mucorales. Dieses Phänomen, das in dieser Form charakteristisch für *Mucor*-artige Pilze ist, wird als möglicher Verteidigungsmechanismus gegen aufgenommene DNA diskutiert, sei es gegen DNA-Aufnahme aus biotrophen Fusionsparasiten oder gegen virale DNA.

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1. Literature Review

1.1. Horizontal Gene Transfer (HGT)

Transmission of genetic information from ancestors to descendants is naturally associated with vertical reproduction by sexual or asexual processes. The offspring of two individual parents in reproducing sexually have more diversity in comparison to an asexual process with a single parent. The substantial differences between meiosis in sexual and mitosis in asexual reproduction cause not only novel genotypes in sexually produced heterokaryones, but also the possibility for genetic rearrangement during meiotic division. Consequently variation in sexual recombinants is higher than among mitotic descendants. Here the major source of genetic diversity is mutation.

In addition to parental inheritance and mutations, horizontal gene transfer is a source of diversity in organisms. Horizontal gene transfer refers to any process in which genetic information from an organism is transferred to another one with or without similar genetic background; therefore the recipient is not necessarily the offspring of the donor organism(s). Horizontal acquisition of genes has occurred in nature during evolutionary developments of organisms. By means of different computational methods, the horizontally acquired genes are recognizable. Briefly, these methods are based on parametric (sequence composition) and phylogenetic (evolutionary history) approaches. Compositions of nucleotide sequences in each species are utilized as specific indicator to trace the horizontally transferred gene. In the other words, nucleotide compositions are used as genome signatures (Karlin and Burge 1994), such as GC content or abundance of codons used for synthesis of amino acids (codon usage patterns), which are specific for each species (Sharp and Li 1987, Syvanen 1994).

Parametric analysis is bound to detect the more recent HGTs, while phylogenetic approaches identify more easily a number of ancient HGT events in the genome, using reconcilement of phylogenetic trees with the reference tree for various genes within the genome of a species. By this approach, time and direction of

gene transfer can be identified (Beiko and Ragan 2008). These methods and their application in evolutionary studies are extensively described in Koonin et al. 2001.

Acquisition of gene(s) by horizontal or lateral gene transfer system plays an important role in evolution of organisms, and in particular constitutes an evolutionary mechanism to evolve the prokaryotes (Ochman et al. 2000). This gene transfer route can assemble genes from different origins in a given organism and is not confined inter taxa in nature and in the laboratory (by experimentally gene transfer). For instance, the hypertermophilic species of bacteria could have exchanged genes with archaea (Aravind et al. 1998, Nelson et al 1999). In *Methanosarcina mazei* (a methonogenic archaean) about 30 % of genes have been shown to be of bacterial origin, indicating the phylogenetic roots for the metabolic evolution of this archaean species (Deppenmeier et al. 2002). In bacteria, clustering the protein families reveals at least one horizontal gene transfer event at the species level and two at the phylum level, which are mediated frequently by conjugation (Kloesges et al. 2011). It is shown that about 18 % of the *Escherichia coli* genome was acquired by horizontal gene transfer. Most of these genetic changes are due to transduction by temperent bacteriophages that integrated into the *E. coli* chromosome (Lawrence and Ochman 1998). Bacteria can take up genes horizontally by conjugation, by transduction or transformation and thus are able to adapt faster to changing environments (Nielsen 1998). The most popular example is the acquisition antibiotic resistance genes from other bacteria by transfer of conjugative plasmids (Falkow et al. 1971) or by conjugative transposons (Nielsen 1998).

Horizontal acquisition of genes is not restricted by taxonomic borders even between kingdoms. Increasing evidences show trans-kingdom genetic exchanges between prokaryotes and eukaryotic phyla. For example, the protozoan human parasite, *Trichomonas vaginalis*, is assumed to have obtained its neuraminate lyase genes (participating in amino-sugar metabolism) from a bacterial source, *Haemophilus influenzae* or a closely related ancestor. The amino acid alignments of these lyases showed 80 % identity with its bacterial counterpart (de Koning et al. 2000). In the extremophilic red alga, *Galdieria sulphuraria*, high metabolic

flexibility and adaptation to environmental conditions have been traced back to genetic events that include acquisition of at least 5 % of its protein-coding genes from various bacteria and archaea (Schönknecht et al. 2013). Most of these evolutionary evidences show the transfer flow from prokaryotes, especially bacteria, to eukayotic organisms. In 1989, Heinemann and Sprague found that an F-dependent conjugative system participates in an inter-kingdom DNA-transmission based on experimental genetic modification of the yeast *Saccharomyces cerevisiae* by conjugative plasmids of *Escherichia coli*. A different conjugative system was also effective in other experimental gene transfers. The tumor inducing plasmid (Ti-plasmid) in *Agrobacterium* strains was discovered as the crown gall inducer in plants (Zaenen et al. 1974), thus, a gate was opened to laboratory genetic engineering, based on the observation that these bacteria can transfer part of their genome into other organisms via plasmids (Schell and Montagu 1977).

A different natural way to obtain genes horizontally is by genetic consequences of endosymbiosis. For instance, gene transfer from *Tremblaya princeps*, a bacterial endosymbiont, constitutes with its insect host, *Planococcus citri* (mealybug), a close symbiosis, whilst this bacterium itself obtained endosymbiosis genes from its own bacterial endosymbiont, *Moranella endobi* (Husnik et al. 2013). Similar endosymbiotic gene transfer events (EGT) from previous cyanobacterial symbionts of *Arabidopsis thaliana*, rice, *Oryza sativa, Chlamydomonas reinhardtii*, and a red alga, *Cyanidioschyzon merolae*, four photosynthetic eukaryotes, have been estimated to approximately 14 % of the hosts genomes (Deusch et al. 2008).

Increasing evidences indicate that genetic alterations gradually replace dispensable genes by new genes, acquired from prokaryotes by endosymbiosis or phagotrophy in early eukaryotes (Doolittle 1998). These genes have normally been translocated from organelles, mitochondria and plastids to the nucleus during evolution (Doolittle 1998, Bock and Timmis 2008).

1.2. HGT in Fungi

Although lateral gene transfer, at the first glance, seems to be a common behavior predominantly in the bacterial world, genes can be exchanged also between other organisms including fungi and other eukaryotes. Richards et al. (2006, 2011) presented evidences that oophytes like *Phytophthora* species have obtained genes by transfer from fungi, especially ascomycetes as *Magnaporthe grisea* (rice blast fungus). These transfers seem to have contributed plant parasitic traits within the oophytes. Furthermore, fungi also can have parasexual recombination as a consequence of anastomosis formation. Anastomosis is the fusion between vegetative hyphal branches of compatible fungi. However, gene transfer by this process highly depends on genetical similarity and consequently compatibility between organisms (Glass et al. 2000). Sometimes anastomosis seems to improve plant pathogenic fungi. For instance, in the ascomycetous plant pathogen, *Fusarium oxysporum*, this mechanism affects the efiiciency of root colonization but is not essential for infection of its host (Prados Rosales and Di Pietro 2008). In *Colletotrichum lindemuthianum*, a pathogen of common bean with no sexual state, conidial anastomosis tubes have been shown to form heterokaryotic colonies that are phenotypically different from their incompatible parents (Roca et al. 2003, Ishikawa et al. 2012). Fusion of conidial anastomosis tubes led to asynchronous mitoses and consequently different patterns of nuclear division in fused conidial germlings of *C. lindemuthianum* (Ishikawa et al. 2013). This fusion can presumably increase the genetic diversity in this fungus and may provide it with new pathogenic traits (Ishikawa et al. 2012). In *Rhizoctonia solani*, causing stem canker in many plants, anastomosis groups (AG) distinguish the host range of this plant pathogen (Parmeter and Whitney 1970). These groups also showed variation in sensitivity to fungicides, which may be due to their capacity to metabolize the active ingredients in fungicide preparations (Campion et al. 2003).

Gene transfer associated with parasitism is also a natural phenomenon, in which genetic exchange can occur between host and parasite. Gene transfer processes between two organisms with completely different genetic lineage are often

accomplished either in parasitic or symbiotic relationships (Wijayawardena et al. 2013). Excluding many HGTs with a bacterial donor, there are few cases with a fungal donor. For example one of these HGTs is found in pea aphids with a red-green color polymorphism. It is known that the red aphids biosynthesize the carotenoids, whereas the encoding genes are of fungal origin, obtained from a fungal pathogen or symbiont (Moran and Jarvik 2010).

Mycoparasitism is another way for exchanging genetic material between fungi. Among fungi, few facultative mycoparasites within zygomycetes (Mucorales) don't follow the common processes used by other mycoparasits like *Trichoderma* species such as nutrition competition (Chet 1987) or secretion of antifungal metabolites (Dennis and Webster 1971). These zygomycetous mycoparasies have evolved special parasitism mechanisms to infect their hosts that are zygomycetes, too. This type of parasitism is named biotrophic fusion (Jefrries and Young 1994). Biotrophic fusion starts with growth of parasite mycelia towards host's. The contact occurs between aerial hyphae of the parasite and the host above the substrate. In the contact point, the parasite's hypha swells and a septum forms to separate the swelled area from the rest of hypha. This septated part is named as primary sikyotic cell and is surrounded by few short locally produced hyphae of the host. Subsequently a cytoplasmic bridge or a cytoplasmic continuum is produced between the two fungi by fusion of their mycelia in the infection area. The infection is terminated towards the end of nutrient transfer from the host to the parasite, and a morphological structure named "sikyospore" is developed (Burgeff 1924) which is able to germinate under appropriate conditions (Fig1.1). This type of parasitism which is accompanied by biotrophic fusion and formation of sikyospores have been observed in few mycoparasitic zygomycetes, *Chaetocladium brefeldii, C. jonesii* (Burgeff 1920), *Lentamyces parricida* (formerly *Absidia parricidia*; Renner & Muskat 1958) and *Parasitella parasitica* (syn. *Parasitella simplex*) (Blakeslee 1904, Burgeff 1920, 1924). The latter, *P. parasitica,* was shown to exhibit dependencies between parasitism and the mating system, i.e., a minus mating type of host can exclusively be infected by a plus mating type of parasite and conversely (Burgeff 1924). The relationship between parasitism and sexuality in *P. parasitica* is based

on the communication system between the partners. This fungus follows a common pathway in both developmental pathways, parasitism as well as sexuality, by mediation of trisporoid compounds (Schultze et al. 2005). Also the parasitic interaction results in the transfer of the parasite's genomic elements to the host, during parasitic fusion (Kellner et al. 1993, Burmester et al. 2013). The result of the parasexual infection is the arrival of new fungal descendants, so-called para-recombinants, exhibiting properties of both host and parasite (Kellner et al. 1993).

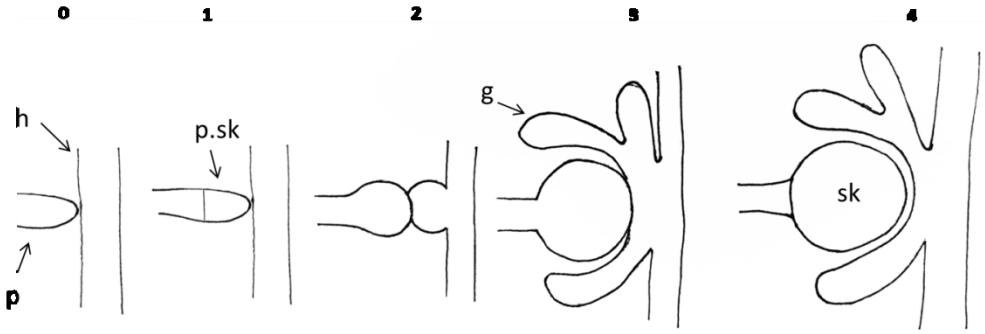


Fig. 1.1. Schematic development of infection structures in biotrophic fusion. The schematic image shows the development of infection structures between the mycoparasite, *P. parasitica* and its host, *A. glauca* (stages 0-4). The hypha in the left side represents the parasite's hypha (p) coming into contact with the host's hypha (h) in the right side, followed by swelling of the contact area. The resulting bulb is separated by a septum at the side of parasite, thus forming the primary sikyotic cell (p.sk; stage 1), surrounded with appendages, so-called galls (g) produced by the host and creates a fusion region (stage 3). Nuclei from both partners enter into the fusion region. Thus, the gall region at the side of the host contains a mixture of both types of nuclei that subsequently will be able to be spread through the host's mycelium. Finally the infection structure develops, at the side of the parasite, the sikyospores, which should, based on the septum situation, contain exclusively the parasite's nuclei hypha (sk; stage 4).

HGT through biotrophic fusion was ambiguous till 1993 when Kellner et al. have proved the gene transfer from *P. parasitica* to *A. glauca* by complementation of auxotrophs of *A. glauca,* defective in methionine or histidine biosynthesis. They obtained prototrophic recombinants from auxotrophs of *A. glauca* following infection by wild type of *P. parasitica* and concluded that the acquired gene(s) from *P. parasitica* complemented the auxotrophy of the *A. glauca* mutants. Subsequently the para-recombinant strains are able to grow prototrophically. At that time, however, it was not possible to confirm the HGT at the molecular level.
P. parasitica infects many but not all fungi in Mucorales but it seems that *A. glauca* is the best candidate to be used as a host strain for para-recombination studies. Many transformation experiments with a variety of different plasmids, revealed that new acquired genes in *A. glauca* are normally established as extrachromosomal autonomously replicating elements. In these studies, various coding regions with gene and promoter function have been utilized to construct the plasmids, including promoters belonging to the genes for actin and the elongation factor EF-Tu (*tef*), and the coding regions of Tn5-derived neomycine resistance gene as well as the green fluorescent protein gene (Wöstemeyer et al. 1987, Burmester et al 1990, Burmester et al 1992, Burmester 1995, Schilde et al 2001, Bartsch et al 2002, Karimi et al. 2012). Other hosts of *P. parasitica*, especially *Mucor circinelloides,* show higher variation in the mode of maintenance for incoming DNA. Transformation experiments with *M. circinelloides* resulted in some cases in integration of acquired DNA into the genome (Arnau et al. 1991, Arnau and Strøman 1993, Wada et al. 1996, Papp et al. 2012b). In other transformants, the transferred plasmids are replicated autonomously (Anaya and Roncero 1991, Benito et al. 1995, Papp et al. 2012a). In addition to free replication of foreign DNA, *A. glauca* has been shown to contain natural extrachromosomal genetic elements besides the chromosomal DNA. Hänfler et al. (1992) found a surface protein encoded exclusively by an extrachromosomal circular DNA without any additional chromosomal copies. Their analysis using electron microscopy showed in addition many small extrachromosomal DNAs of different lengths. Based on the reasoning that *A. glauca* is able to establish

foreign DNA at least as replicating circles, this organism should be ideally suited to propagate DNA of the fusion parasite *P. parasitica*.

Furthermore, this fungus showed the best efficiency for infection with *P. parasitica* compared with other mucoralean. The production of many aerial hyphae that are needed for developing the high number of infection structures as well as the uni-nucleate mitospores make it a good candidate for para-recombination analysis (Kellner et al. 1993, Wöstemeyer et al. 1995, Wöstemeyer et al. 2002). Accordingly, *A. glauca* is presently the most appropriate model organism to follow the fate of introduced DNA into the host cells on the way to understand para-recombination mechanisms in zygomycetes.

1.3. Gene Manipulation in Zygomycetous Fungi

Zygomycetes, particularly syncytial mucoralean fungi with their aseptate mycelia, that produce numerous mitospores in a short life cycle are interesting as model for basic genetic experiments. In addition, most of these fungi are heterothallic and secrete trisporoid pheromones that induce the complementary mating type for sexual reproduction. The spore produced during the sexual process, the zygospore, is hardly able to germinate in most of species, and therefore traditional genetic analysis is hampered considerably. Furthermore, the fusion of gamentangia containing hundreds of nuclei and thus producing multinucleate zygospores complicates genetic analysis. For example, *Phycomyces blakesleeanus,* the zygomycete with the best germination rates between 1 and 5%, zygospore has 50-60 days dormancy before germination. The dormancy time could be shortened to 32 days, but the number of zygospores producing a germ sporangium with viable spores in the third backcross decreased to zero (Sarabia et al. 1988).

Experimental genetic manipulation also has its own difficulties; autonomous replication of the acquired DNA creates mitotically unstable transformants (Suárez and Eslava 1988, Karimi et al. 2012, Anaya & Roncero 1991, Revuelta and Jayaram 1986, Wöstemeyer et al. 1987). Consequently, even if stability of plasmid segregation ranges around that of yeast plasmids per mitotic division, the

stability of transformants is quite low for experimental propagation cycles starting from a single transformed cell to the next unicellular stage, normally the spore progeny of a complete Petri dish (Suárez and Eslava 1988, Anaya and Roncero 1991).

Nevertheless, these fungi in general are easy to handle in gene manipulation experiments in the laboratory. Increasing the number of organisms, amenable to gene manipulation and their application during recent years, encourage the research community to identify, improve and introduce the reliable zygomycetes for gene transfer studies.

Altogether, the aim of these studies is identification of genes and their function, transformation and preparing the best genomic evidences to consider the mechanism of transformation at the molecular level. Reliable mutants that can be complemented by the corresponding wild type genes are the most efficient way for selection in transformation experiments. These mutant strains must ideally be highly stable with very low reversion frequencies.

In many other transformation systems, complementing genes and also dominantly selectable resistance genes to antibiotics and other chemicals are commonly used as marker genes. Nearly always, complementation of auxotrophs proved to be more reliable with respect to avoiding false-positives in transformation experiments.

In addition to selectable marker genes, the gene for the green fluorescent protein coding gene (*GFP*) has often been applied as a reporter gene. This gene enables the transformed fungus to accumulate the green fluorescent protein in fungal structures visible microscopically. After screening of transformed colonies, the activity of the *GFP* gene in transformants can reduce the selection of false positive transformants. The *GFP* gene works perfectly in *A. glauca* (Karimi et al. 2012, Bartsch et al. 2002, Schilde et al. 2001), *Backusella lamprospora* (Nyilasi et al. 2008) and *Rhizopus oryzae* (Mertens et al. 2006).

1.3.1. Marker Genes

1.3.1.1. Resistance Genes

Transformation experiments start by introducing DNA containing appropriate marker genes, suitable for selection or screening. Genes, conferring resistance to antibiotics, are used in the first steps towards a transformation system, in order to select for those individuals that have acquired the additional genetic information and express it successfully.

Most antibiotic resistance genes come from bacterial sources. The antibiotic resistance markers used in zygomycetes transformation experiments including: the resistance gene against neomycin, an aminoglycoside antibiotic resistance gene (*nptII*) derived from an *E. coli* strain, harbouring the transposon Tn5. The same gene confers resistance to several other aminoglycoside antibiotics; geniticin or G-418, and gentamycine are well-known examples of antibiotics that are of use in fungal transformation experiments. This gene has been used in transformation of *Phycomyces blakesleeanus* (Arnau et al. 1988, Revelta and Jayaram 1986, Suárez and Eslava 1988), *M. circinelloides*, *M. rouxii* and *Rhizomucor pusillus* (Appel et al. 2004). Resistance to neomycin also has been applied in transformation studies of *Absidia glauca* (Karimi et al. 2012, Burmester et al. 1992, Burmester et al. 1990, Wöstemeyer et al. 1987, Bartsch et al 2002) and *Parasitella parasitica* (*P.simplex*; Burmester 1992). The hygromycin B resistance gene, *hph*, is another marker gene which has been utilized for transformation of *Mortierella alpina* (Mackenzie et al. 2000), *Backusella lamprospora* (Nyilasi et al. 2008) and *Mucor circinelloides* (Nyilasi et al. 2005). *Mortierella alpine* has been transformed using a zeocin resistance gene (Takeno et al. 2005).

Furthermore, some compound resistance genes were used as marker genes originating from non-bacterial sources like the carboxine resistance, *cbxR*, from the basidiomycete fungus, *Ustilago maydis* introduced into *Mucor circinelloides* (Ortiz-Alvarado et al. 2006), the acetamide resistance gene from *Aspergillus nidulans* in *Rhizopus oryzae* (Michielse et al. 2004), the statins compounds resistance, *hmgR*, encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase,

isolated from *Rhizomucor miehei* and transferred into *M. circinelloides* (Lukács et al. 2009). In all cases, the results demonstrate that irrespective of the gene source, these zygomycetes are capable to express the exogenous genes.
Due to sensitivity of some antibiotics to environmental factors like temperature and light, these marker genes are not always reliable. In addition, many fungi adapt to antibiotics, possibly by the action of binding proteins at the surface. Therefore, transformation based on resistance genes usually results in high numbers of false-positive colonies (Wöstemeyer et al. 1987). Instead, usage of biosynthetic markers for complementation of auxotrophs, renders the selection of transformants almost unmistakable.

1.3.1.2. Complementing Genes

The first transformation system for zygomycetes was based on complementation of an auxotrophic mutant by the corresponding, homologous gene (van Heeswijck and Roncero 1984). The authors isolated an auxotroph deficient in leucine biosynthesis of *Mucor circinelloides* without having background information on the corresponding gene. Thus, they prepared a genomic library of the wild type and transferred the DNA of the library to protoplasts of mutant. By selecting for growing colonies on leucine-free medium, the corresponding gene was identified. Subsequently, this auxotroph of *M. circinelloides* became a popular model organism for studying molecular genetics in mucoralean fungi. Later on, auxotrophs of additional zygomycetes have been introduced, especially *LeuA* and *PyrG/PyrF* from the biosynthetic pathways for leucine and uracil. *LeuA* codes for isopropylmalate isomerase, *pyrG* and *PyrF* respectively, encode orothidine-5-monophosphate decarboxylase and oratate phosphoribosyl transferase. These genes are responsible for the conversion of orotic acid to uridine monophosphate during the biosynthesis of pyrimidine ribonucleotides. The *LeuA*$^-$ auxotrophic mutants can only be identified by screening for lack of growth on minimal medium and the ability to be supplemented by addition of leucine (Roncero et al. 1989), whereas *PyrG*$^-$ or *PyrF*$^-$ auxotrophs can be selected for in some but not all zygomycetes according to their resistance to 5-fluoroorotic acid added to the

medium (Benito et al. 1995, Yamazaki et al. 1999) after mutagenesis. If 5-fluoroorotic acid is added to the medium, the active *Pyr* genes in prototrophic cells will convert it to the toxic compound, 5-fluorouracil, causing death in the cells containing the wild type *Pyr* genes. In contrast, that lack of the *Pyr* genes leads to *Pyr*$^-$ cells that are not affected by 5-fluoroorotic acid, but depend on addition of uracil or uridine to the medium (Santi 1981).

Complementation studies based on these genes have been performed with the *LeuA* gene for *M. circinelloides* (Roncero et al. 1989), *P. blakesleeanus* (Iturriaga et al. 1992), *Rhizomucor pusillus* (Wada et al. 1996) and *R. niveus* (Takaya et al. 1996). The first transformations using the *pyrG* gene have been accomplished for *M. circinelloides* (Benito et al. 1992), *Rhizopus oryzae* (Skory 2005) and *Mortierella alpina* (Takeno et al. 2004). The vectors harboring the marker genes *PyrG* or *LeuA* are well-studied to trace the transformation efficiency of these mucoralean fungi. A methionine deficient derivative of *M. circinelloides* has also been used for transformation based on the corresponding wild type gene, but the gene was not characterized at the sequence level (Anaya and Roncero 1991). For *A. glauca*, a methionine auxotroph, defective in the *Met2-1* gene has been transformed with a plasmid harboring the corresponding wild type gene. The defect in this gene disabled the fungal strain to produce homoserine O-acetyltransferase, an intermediated enzyme in methionine biosynthesis (Karimi et al. 2012).

1.3.2. Promoter Efficacy

In addition to the coding region, each gene has a control region that is essential for initiating transcription of the gene and for its temporal and spacial regulation. The DNA fragments with promoting function for expression of the transferred gene play very important roles in transformation assays. Basically, marker genes may be preceded by two types of promoters, constitutive or inducible ones. Constitutive promoters facilitate gene expression in all cells of the organism under every circumstance, regardless of developmental stage and the

surrounding environment. In contrast, inducible promoters' activity depends on exogenous conditions, either biotic or abiotic factors (Struhl 1986).

In many transformation systems the homologous promoters for transferred genes are preferred. If necessary, however, other promoters controlling various coding regions even of heterologous origin may also be useful. For instance, the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd1p*) in transformation of *Mucor circinelloides* could successfully control the expression of the homologous *crgA* gene, encoding a regulator of carotene biosynthesis, and the heterologous gene *gox1* encoding glucose oxidase from *Aspergillus niger* (Wolff and Arnau 2002).

Afterwards, it was figured out that only a short part of the control fragment is sufficient to express the transferred gene (Larsen et al. 2004). This promoter was used for transformation of *R. pusillus* and *M. circinelloides* after fusing it to the neomycin resistance gene (Appel et al. 2004). The *gpd1* promoter could also regulate the expression of the gene for malic enzyme from *Mortierella alpina* and from *M. circinelloides* in transformation experiments with *M. circinelloides* (Zhang et al. 2007). This region of the *M. circinelloides gpd1* gene promoted the expression of hygromycin B resistance, *hph* and *GFP* genes in *Bakusella lamprospora* transformants (Nyilasi et al. 2008).

Other examples are, the constitutive promoter of tryptophan biosynthesis (*trpC*) originated from *Aspergillus nidulans* for expression of hygromycin phosphotransferase (*hph*) in *M. circinelloides* (Nyilasi et al. 2005) and the histone H4 promoter fragment which has been applied to introduce the hygromycine B resistance gene in *Mortierella alpina* (Mackenzie et al. 2000).

Mertens et al. (2006) evaluated the effect of three different promoters, two inducible promoters, i.e. glucoamylase A (*amyA*) that is expressed in presence of starch or cellobiose and pyruvate decarboxylase A (*pdcA*) that needs the presence of glucose for expression in addition to a constitutively expressed promoter from *R. oryzae,* phosphoglycerate kinase (*pgk1*), as regulatory regions for expression of the *GFP* gene. They compared transcription and protein accumulation of the green fluorescent protein for each vector. The results showed

a direct correlation between expression and the promoter. *PdcA* provided the highest protein accumulation, followed by *amyA* and *pgk1*.

Transformation of *Parasitella simplex (syn. P. parasitica*) was accompolished with the neomycin resistance gene, controlled by the constitutive promoter of the translation elongation factor gene (*tef*) from *A. glauca* (Burmester 1992). The same cassette could also be expressed in *A. glauca* (Burmester 1995). In addition, *A. glauca* has been transformed with several other genes; neomycin resistance (Wöstemeyer et al. 1987, Burmester et al. 1990), *GFP* (Schilde et al. 2001, Karimi et al. 2012) and methionine biosynthesis genes (Karimi et al. 2012) under the control of the constitutive *actin* promoter. Therefore, the *actin* promoter originated from *A. glauca* proved to be especially suitable for transformation of this fungus and could promote the expression of different genes of various origins, like *GFP* gene from jellyfish and neomycin resistance gene from *E. coli* (Wöstemeyer et al. 1987, Burmester et al. 1990, Burmester et al. 1992, Kellner et al. 1993, Burmester, 1995, Schilde et al. 2001, Bartsch et al. 2002).

1.4. Experimental DNA Uptake Techniques for Zygomycetes

DNA uptake in zygomycetes has been achieved by four different techniques.

1.4.1. Polyethylene Glycol (PEG)

Polymeric ethylene glycol is used as adhesive compound in transformation studies to enhance DNA binding to the surface of protoplasts and as a fusogenic agent (Kawai et al. 2009, Chen et al. 2008). Nevertheless this compound is toxic for the cells in high concentration (Peberdy 1979). PEG- mediated transformation is used as standard technique or *Aspergillus* species (Balance et al. 1983, Koukaki et al. 2003, Ahuja et al. 2008, Dave et al. 2012) and *Saccharomyces cerevisiae* (Kawai et al. 2010). This method is abundantly applied for transformation of zygomycetes, too (Wöstemeyer et al. 1987, Yamazaki et al. 1999, Papp et al. 2006, Gutiérrez et al 2011, Wada et al. 1996, Papp et al. 2012). Furthermore, PEG is utilized to attach different protoplasts in cell fusion experiments (Wöstemeyer et al. 1990).

1.4.2. *Agrobacterium tumefaciens*

The plant pathogenic bacterium *A. tumefaciens* is able to transfer part of its DNA (T-DNA) including two direct repeats, the left and right borders, to its host (Gelvin 2000). T-DNA is located on a tumor-inducing (Ti) plasmid, influences the plant growth regulatory system and leads to uncontrolled plant cell growth (Zhu et al. 2000). By applying appropriate small signal molecules, *A. tumefaciens* can undergo conjugation with many different cells that are completely unrelated to the natural hosts, i.e. dicotyledonous plant cells. To use this DNA-transferring ability in experimental transformations, a binary vector system is designed, i.e. the donor *A. tumefaciens* strain contains two plasmids: the disarmed Ti plasmid, containing a virulence region (*vir*) and only two borders of its natural T-DNA and a binary vector, containing the T-DNA. In transformation experiments, the disarmed Ti plasmid is accompanied by another plasmid containing an additional DNA fragment, which has been inserted between two borders of T-DNA. The virulence region on the Ti-plasmid encodes the T-DNA transfer machinery (Hooykaas and Beijersbergen 1994, de Groot et al. 1998).
This technique to introduce the DNA into zygomycetes, avoides the necessity for protoplast formation and may offer better possibilities for integration of introduced DNA into the host genome (Papp et al. 2012, Michielse et al. 2004, Niylasi et al. 2005). This method is somewhat more complicated due to using various antibiotics in starter cultures and the difficulty in removing the bacterium to obtain pure fungal transformants. On the other hand, the results of Michielse et al. (2004) reported that *pyrG* marker gene was truncated in *R. niveus* during chromosomal integration.

1.4.3. Micro-Projectile Bombardment

In this technique the plasmid DNA to be introduced is abound to the surface of small metals beads, made of gold or tungsten, which subsequently are shot on fungal protoplasts or ideally on germlings under slight vacuum conditions (Sanford et al. 1993). Only few reports exist for mucoraleans in which this

technique has been used. These are *M. circinelloides,* transformed by the homologous *leuA* marker gene (Gonzalez-Hernandez et al. 1997), *A. glauca,* transformed the plasmids containing the *GFP* and the neomycine resistance conferring gene, *nptII* (Bartsch et al. 2002), *Mortierella alpina* transformed with the *PyrG* marker gene (Takeno et al. 2004, Takeno et al. 2005), and *R. oryzae* also transformed with *pyrG* containing plasmids (Skory 2002, 2004).

1.4.4. Electroporation

Fungal protoplasts and the DNA to be introduced are given to a plastic cuvette equipped with two metal electrodes. A high tension direct electrical pulse is applied by a capacitor in a pulser machine. By this treatment, the membranes are depolarized, and the DNA is able to enter the cytoplasm. Before treatment, fungal protoplasts can also be treated with salts such as calcium chloride (Van Heeswijck and Roncero 1984, Burmester et al. 1987) in order to enhance the permeability of membrane for DNA entrance. The salt concentration correlates inversely with the voltage of the pulses. In case of salt application, the use of high voltage is not possible due to increased conductivity and this heat stress of the protoplasts. Electroporation has been used for transformation of *M. circinelloides* using *leuA* and *pyrG* marker genes (Gutiérrez et al 2011). Transformation experiments in *Absidia glauca* have frequently been performed by means of this technique (Burmester et al. 1990, 1992, 1987, Burmester 1995).

1.5. Aim of the Project

The need for reliable marker genes for the model organism, *Absidia glauca*, encouraged us to use auxotrophic mutants of this fungus. Starting with a stable methionine auxotrophic strain, we identified the corresponding gene, in order to introduce it as a selectable marker in transformation experiments. This gene was also meant to trace the fate of introduced DNA via the parasitic interaction between *A. glauca* and its fusion parasite *P. parasitica.* In this respect, the project

represents a first step on the way to understanding the formation of para-recombinants at the mechanistic level.

The experiments allowed to conceive the methionine biosynthesis and subsequently to complement a methionine auxotroph of *A. glauca* as an important host for *P. parasitica*. For these purposes, it was necessary to identify the deficiency in the mutant at the biochemical level.

Based on a model for methionine biosynthesis in *Neurospora crassa* and *Aspergillus nidulans,* methionine can be produced from cysteine and (or) homoserine via O-acetyl homoserine by means of several subsequently acting enzymes (Fig. 1.2). Two enzyme activities, homoserine O-acetyltransferase and γ-cystathionine synthase, are essential to convert homoserine to cystathionine (Wiebers and Garner, 1967, Yamagata 1989, Marzluf 1997, Brzywczy and Paszewski 1993; Hacham et al. 2003).

Knowledge of the pathways in ascomycetes should considerably facilitate the identification of the gen that is affected in the methionine-deficient *A. glauca* mutant.

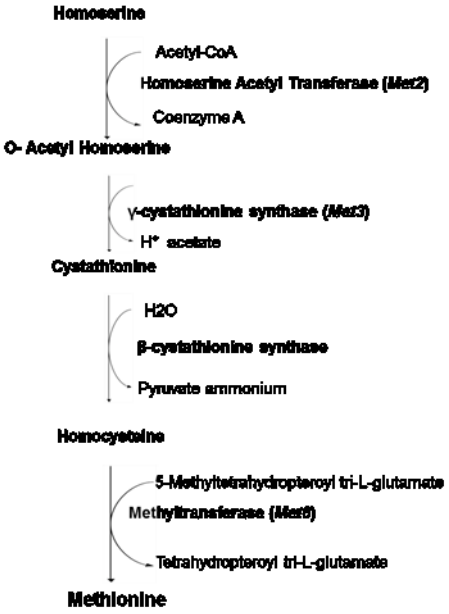


Fig. 1.2. Methionine biosynthesis pathway. Presumptive pathway for methionine biosynthesis in *A. glauca* based on described pathways for *Aspergillus nidulans* and *Neurospora crassa* (Marzluf 1997).

2. Materials and Methods

2.1. Strains

Wild type strains of *Absidia glauca* plus mating type CBS 100.48 and the corresponding minus mating type CBS 101.48 have been utilized. These strains were already employed in the experimental work of Hans Burgeff (Burgeff 1924). They were isolated from the same location and have been deposited by Burgeff in the CBS collection in 1948. The mutant strain of *A. glauca,* RVII-324 *met*$^-$ (Wöstemeyer *et al.*, 1990), was obtained from the CBS 101.48 minus mating type by X-ray mutagenesis (Wöstemeyer and Brockhausen-Rohdemann 1987). It has been used for infection with *Parasitella parasitica* in para-recombinant co-cultures and also for transformation experiments.
The *Parasitella parasitica* mutant strain NGI-315 *ade*$^-$, a derivative of CBS 412.66 plus mating type obtained by nitrosoguanidine mutagenesis, was used for infection studies (Burmester et al. 2013).
The *Escherichia coli* strain XL1-Blue (*endA1, gyrA96* (nal^R), *thi-1, recA1, relA1, supE44, hsdR17* (r_K^- m_K^+), *lac glnV44 F'[proAB*$^+$ *lacI*^{q} *Δ(lacZ)M15::Tn10* (Tet^R)*]*) (Bullock et al., 1987) was used for cloning and transformation experiments.

2.2. Media

Fungal strains were grown on complete (sup; Wöstemeyer, 1985) or minimal medium (MM; Burmester et al., 1990) supplemented with different amino acids, or their intermediates (Sigma-Aldrich and Reanal) at a final concentration of 250 µM to identify the deficiencies. Para-recombination experiments have been performed on malt extract agar (MEX) and zygomycetes induction medium (Schimek et al. 2005). *Escherichia coli* was grown on Luria Bertani (LB) medium (Miller 1972). The media and their contents are listed in table 2.1.

Table 2.1. Media and their contents.

Medium*	Contents/ litre	Reference
Minimal Medium (MM)	10g glucose monohydrate, 1g NH_4Cl, 4g KH_2PO_4, 0.9g K_2HPO_4, 0.25g $MgSO_4.7H_2O$.	Burmester *et al.*, 1990
Supplemented minimal medium (sup)	MM + 5g yeast extract (Roth)	Wöstemeyer, 1985
Malt extract medium (MEX)	50 g malt extract	
Zygomycetes induction medium (IM)	20g maltose, 10g KNO_3, 5g KH_2PO_4, 2.5g $MgSO_4.7H_2O$, 1g yeast extract	Schimek et al. 2005
Luria Bertani (LB)	10 gtryptone, 5 g yeast extract, 10 g NaCl	Miller, 1972
* For solid media 12 g agar (Roth) was added.		

2.3. Cultivations

2.3.1. Feeding Experiments

Feeding experiments have been done to determine the defects of mutants on solid minimal medium supplemented with the diverse amino acids (Reanal) or their intermediates including homoserine, O-succinylhomoserine, cystathionine and homocysteine (Sigma–Aldrich) at 250 µM final concentrations.

2.3.2. Para-recombination Cultures

Para-recombination experiments have been performed by co-cultivation of *P. parasitica* and *A. glauca* mutant strains in 1cm distance on complete medium, induction medium (IM) and malt extract medium with or without using sterile cellophane sheet between the plates' surface and the mycelia. The formation of sikyospores was followed with a dissecting microscope. Sporangiospores were collected from the plates by cutting the cellophane sheet in the infection zone or by washing the plates with sterile water. Dilutions were spread on minimal medium as well as on complete medium. The colonies which were able to grow were counted. Single spore isolates were transferred on fresh minimal medium plates and grown till sporulation. Single spore cultures were isolated from several

subsequent sporulation cycles to determine the mitotic stability of transferred gene(s).

2.4. Transformation

A spore suspension containing 10^6-10^9 spores of the *A. glauca met*$^-$ mutant strain, counted in a Thoma chamber, was grown in liquid complete medium for 8 hours. After filtration using 0.45 µm membrane filters in a sterile filter unit under weak vacuum in order to concentrate the suspension, protoplasting was done using streptozyme (Wöstemeyer and Brockhausen-Rohdemann 1987) in 0.7 M sorbitol, 3 mM $CaCl_2$ and 10 mM MOPS/KOH, pH 6.3 (sorbitol solution), for 45-60 minutes. Streptozyme is a lytic enzyme mixture secreted by *Streptomyces* No. 6 and includes chitinase and chitosinase as well as several other activities, which is very helpful to prepare protoplasts in zygomycetes (Wöstemeyer and Brockhausen-Rohdemann 1987). The enzyme solution was removed by centrifugation at 1,500 rpm for 10 minutes. The protoplast pellet was resuspended in sorbitol solution and centrifuged twice at 1,500 rpm for 10 minutes (Wöstemeyer et al. 1990). The germinated spores were suspended in 1 ml sorbitol solution and transferred into an electroporation cuvette with 0.2 or 0.4 cm distance between the electrodes, together with 5µg of plasmid vector. Electroporation was performed with a GenePulser device (BioRad, München) set at: 400 V, 25 µF and without an additional shunt ($\Omega=\infty$). For each electroporation, the time constant values were noted. Typical pulse times between 5 and 10 milliseconds were obtained for 0.2 and 0.4 cm cuvettes respectively.

Before and after electroporation, the spore solution was kept on ice for 10 minutes followed by plating on minimal medium agar containing 0.7 M sorbitol (Burmester et al. 1992). 3-5 days later, the colonies were transferred as agar blocks to new minimal medium plates and were allowed to sporulate. After collecting the spores by washing the plates with sterile water, they were plated on minimal medium. Single spore colonies from the minimal medium were isolated and used for analysis in subsequent sporulation cycles. The asexual spores

which are produced by a colony originated from one single spore or one germinated spore from a single sporulation cycle.

2.5. Nucleic Acids Preparation

2.5.1. Growth Condition for DNA and RNA Isolation

For DNA and RNA isolation from fungal strains, 250 ml Erlenmeyer flasks containing 100 ml minimal medium for wild type and transformants strains and sup medium or minimal medium supplemented with methionine for RVII-324 *met*$^-$, mutant strain of *A. glauca,* and adenosine for NGI-315 *ade*$^-$ mutant strain of *P. parasitica* were inoculated with 10^5-10^7 spores. The cultures were incubated for 3-6 days on 120 rpm shaker under room conditions (temperature and light; Wöstemeyer and Brockhausen-Rohdemann 1987).

2.5.2. DNA Isolation

The mycelia were harvested on filter papers on a Buchner funnel and homogenized by grinding with a pestle in a chilled mortar with liquid nitrogen.

2.5.2.1. Cesium Chloride Gradient Centrifugation

The mycelium powder obtained from 100 ml medium was mixed with 20 ml of buffer, containing 50 mM EDTA, 200 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1 % SDS in Greiner centrifuge tubes and incubated for 15 minutes at 70 °C. Subsequently, 7 ml of 4 M sodium chloride were added and mixed gently. The tubes were cooled on ice for 30 minutes. To remove insoluble cell residues, the suspension was centrifuged at 10,000 g and 4 °C for 10 minutes (Sorvall RC6, rotor: SS-34). To precipitate the DNA, the supernatant was transferred to a new tube and solid polyethylene glycol 6000 (Serva) was added to a final concentration of 1 g/10 ml. The mixture was shaken gently at room temperature to dissolve the PEG. After incubating the tubes on ice for one hour, the

preparations were centrifuged at 10,000 g and 4 °C for 20 minutes (Sorvall RC6, rotor SS-34). After dissolving the pellet in 4 ml 25 mM Tris-Cl pH 8.0, 5 mM EDTA, 10 mM sodium chloride and 1 % Triton X–100, 1 g cesium chloride and 20 µl of 10 mg/ml ethidium bromide were added per ml of solution, followed by ultracentrifugation for 24 hours at 44,000 rpm at 25 °C (Sorvall UltraPro 80, rotor: TV865B). The genomic DNA band was removed with a syringe under UV light (Schilde et al. 2001, modified).

The DNA was extracted three times with 2-propanol saturated with water and sodium chloride to remove the ethidium bromide. To remove the cesium chloride, the DNA was dialyzed three times, 4 hours each, against 1.5 l of 10 mM Tris-Cl, pH 8.0, 1 mM EDTA.

2.5.2.2. Trichloromethane (Chloroform) Extraction

0.1 g mycelial powder was gently mixed with 0.5 ml of buffer, containing 50 mM EDTA, 30 mM Tris-Cl pH 8.0, 100 mM sodium chloride, and 0.5 % SDS in 1.5 ml tubes and incubated at 55 °C for 10 minutes. 0.4 ml chloroform was added and the samples were centrifuged for 10 minutes at 10,000 rpm at 4° C. The upper phase was carefully transferred into a new tube. 20 µl of heat treated RNaseA (1 mg/ml) was added and incubated at room temperature for 15 minutes. Then, 0.4 ml of chloroform was added, mixed gently, and centrifuged at 12,000 rpm for one minute. The upper phase was transferred to a new tube and 2 volumes of ethanol were added, stored at –20 °C for 20 minutes and then centrifuged for 10 minutes at 12,000 rpm. The resulting pellet was washed with 70 % ethanol twice by centrifugation at 12,000 rpm for 5 minutes. After drying, the DNA was dissolved in 30 µl sterile water (Sambrook and Russell 2001, modified). The DNA amount and quality was checked by spectrophotometry and agarose gel electrophoresis, respectively.

2.5.3. Gel Extraction

In order to isolate specific fragments of DNA after loading on the agarose gel and visualizing by ethidium bromide, the target fragments were cut out from the agarose gel and 1 ml of 6 M NaI was added in a 1.5 ml tube. Incubation at 55 °C melted the gel completely. 5 µl of glass milk were added, the suspension was mixed and incubated again at 55°C for 10 minutes with shaking every 2 minutes. After incubation on ice for 5 minutes, the DNA attached to glass particles was precipitated by centrifugation at 12,000 rpm for 2 minutes. To remove the high salt solution, the glass particles were washed twice with 1 ml of a solution containing 20 mM Tris-Cl, pH. 7.2, 200 mM NaCl, 2 mM EDTA, pH. 7.2, and 51% ethanol. The sample was centrifuged at 12,000 rpm for 2 minutes followed by discarding the supernatant. The glass particles' pellet was dissolved in 10-20 µl deionized distilled water and incubated at 55 °C for 30 minutes. The glass particles were settled down by centrifuging at 12,000 for 2 minutes, and the DNA solution was transferred to a new tube.

2.5.4. RNA Isolation

Total RNA was purified form the fungal mycelium powder using TRIzol® reagent containing guanidinium thiocyanate and phenol (Invitrogen). About 100 mg of mycelium powder were suspended in 1 ml of preheated Trizol, shaken and incubated for 5 min at 60 °C. The samples were centrifuged for 30 min at 4 °C and 10,000 rpm. The supernatant was mixed with 200 µl chloroform, vigorously shaken for 15 seconds and incubated for 3 min at room temperature, then centrifuged for 20 min at 4°C and 10000 rpm. The upper phase was removed and mixed with 0.5 volumes of ice-cold isopropanol and 0.5 volumes of a solution containing 0.8 M sodium citrate and 1.2 M NaCl. After 10 min incubation at room temperature, the RNA was precipitated by centrifugation for 30 min at 4 °C and 12,000 rpm. The pellet was washed with 1 ml of 70 % ethanol, centrifuged for 10 min at 4 °C and 10,000 rpm and finally dissolved in 50 µl of RNase-free water treated with 0.1 % DEPC (Diethylpyrocarbonate, Sigma-Aldrich).

2.5.5. Complementary DNA (cDNA)

2 µg of RNA was used in the reverse transcription (RT) reaction in the presence of 2 µl of 10x RT buffer (containing 500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30mM MgCl2 and 50 mM DTT), 2µl dNTP mix (0.5 mM each dNTP), 1 µM oligo-dT primer, 1 µl (10 units) RNase inhibitor, 1 µl omniscript revese transcriptase (4 units) at the final concentration of 20 µl. Except RNase inhibitor from Fermentas, the rest of material in the reaction prepared from Qiagen (Omniscript RT Kit, Qiagen, Hilden, Germany). To recognize contamination of cDNAs with residual chromosomal DNA, the cDNAs were checked with primers for the actin gene as reference gene. Actin gene is a housekeeping gene and expressed in all cells to maintain the basic cellular functions (Berepiki et al. 2011). The fragments amplified using primer set for *actin* gene in cDNAs don't consist the intron part within this gene, therefore the amplified fragments for cDNAs are shorter than chromosomal DNA.

2.6. Amplification

2.6.1. PCR

To amplify fragments in order to compare wild type and recombinants with mutant strains, and also to prepare the labeled probes for Southern blot analysis, polymerase chain reactions were performed using a MWG-Biotech thermocycler with the following temperature profile: 5 min at 94 °C, 30 cycles with: 45 seconds at annealing temperature 55-60 °C, 60 seconds for extension at 72 °C, and 45 seconds for denaturation of DNA at 94 °C. For each reaction, 25 ng chromosomal DNA or 1 ng plasmid DNA were mixed with 2 µl of 10 x PCR buffer (containing 100 mM Tris-Cl, pH 8.8, 500 mM KCl and 0.8 % Nonidet P40), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer (forward and reverse) and 1 unit Taq DNA polymerase (Fermentas) at the final volume of 20 µl.

2.6.2. Inverse PCR

2.6.2.1. Recognition of the *Met2-1* Gene in *A. glauca*

To detect, trace and amplify the DNA coding regions, degenerate primers were designed and utilized based on conserved amino acid motifs of the related zygomycetes, *Rhizopus oryzae*, *Mucor circinelloides* and *Phycomyces blakesleeanus* using the BioEdit Sequence Alignment editor software. The amplified fragments were used for hybridization of genomic DNA, digested with various restriction enzymes, in Southern blot experiments. On the basis of Southern analysis results, eligible enzymes were selected for DNA digestion. The Southern patterns for selected enzymes were expected to show one or two fragments with lengths of up to 5-6 kb.

2.6.2.2. Ligation of *A. glauca*'s Chromosomal DNA

Digested DNA fragments were re-ligated under these conditions: 1 µg digested DNA in restriction assays, in which the enzyme was deactivated by heat treatment (10 min at 70 °C) was mixed with 2.5 µl T4 DNA ligase (Fermentas) and 5 µl 10 x concentrated ligase buffer at the final volume of 50 µl. 10 x ligase buffer contains 400 mM Tris-Cl, pH. 7.8, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT) and 5 mM ATP, the latter is added as the last ingredient when using the buffer (Sambrook and Russell 2001). Ligation was performed at 12 °C for 16 hours in a cooled water bath. The DNA from ligation assays were ethanol-precipitated, washed by ethanol, and used as templates for inverse PCR.

2.6.2.3. Amplification by Inverse PCR

Genomic DNA of *A. glauca* CBS 101.48 minus wild type strain was digested with *Hind*III, ligated and amplified with primer pairs A, A1, B and C for the *Met2-1* gene (Table 2.2). The sequences of the resulting fragments were exploited for designing specific primers. All primers used to amplify parts of the putative genes

for *Met2-1* and *Met2-2* genes, encoding homoserine O-acetyltransferase, and *Met3*, encoding γ-cystathionine synthase are listed in Table 2.2.

The amplification by PCR with Taq polymerase was performed as described in 2.6.1.

Table 2.2- list of primers used for amplifications. Restriction sites are underlined (Karimi et al. 2012, Burmester et al. 2013).

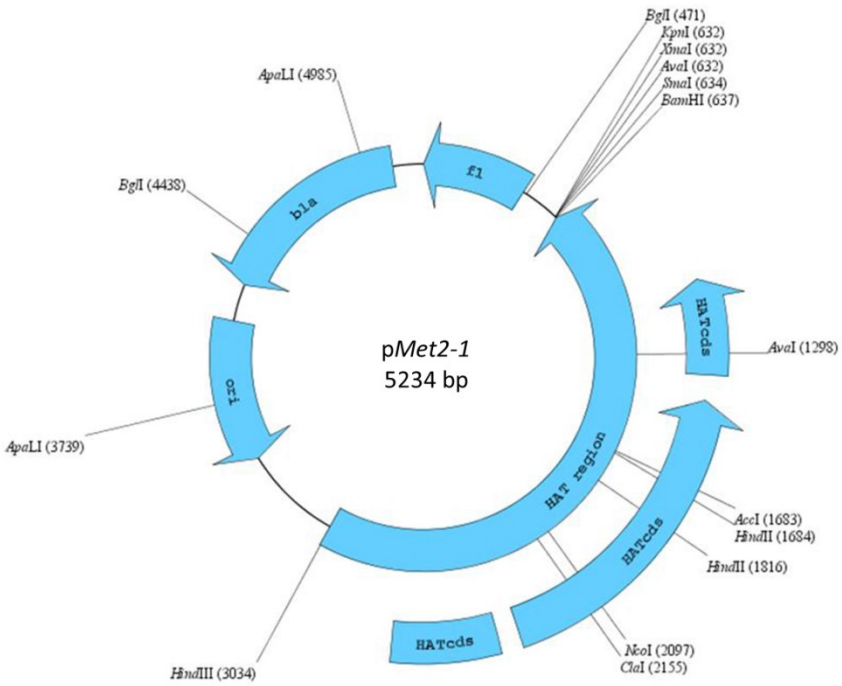
Pair	Direction	Nucleotide sequence	Used for
A	For Rev	5′-GGGGATCCGCNGAYGTNGARGAYTGG-3′ 5′- GGGTCGACTARCARTTNGCRTTCRAA-3′	*Met2-1*
A1	For	5′-GGGGATCCATGGGNGGNATGCA-3′	*Met2-1*, *2-2*
B	For Rev	5′-GGGGATCCGGNTGGTGGGARGAYTT-3′ 5′-GGGTCGACATYTCYTTYTGYTGCCA-3′	*Met2-2*
C	For Rev	5′-GGGGATCCAARCARTTYTGGCARCA-3′ 5′-GGGTCGACGGRAANCCRAARCANAC-3′	*Met3*
D	For Rev	5′-GGGTCGACGTTGGTCTCTCAGCTGCAAG-3′ 5′-CCGGATCCAAGACTTGCATACCACCCATGG-3′	*Met2-1*, *A.g.* iPCR
D1	For Rev	5′-ATGTCGACGCCAACTGTTACATTGC-3′ 5′-CCCTGCAGCACCATTGCGTAATGTCCACC-3′	
E	For Rev	5′-GGAAGCTTTGACAGCTGTTTCATTGATG-3′ 5′-TCGGATCCCTTGAAGCCAGGTACGATAATGTC-3′	*Met2-1*, *A.g.*
F	For Rev	5′-CTGTACACAATTGAACAAGATGG-3′ 5′-CTCTAGAAAGAACTCGTCAAG-3′	NeoR
G	For Rev	5′-TGTTGACCGAAGCTCCTTTGA-3′ 5′-GCCAAGATGGAACCACCATC-3′	*A.g. actin*
H	For Rev	5′-CCACCATGGTGAGCAAGGGC-3′ 5′-CTTGTACAGCTCGTCCATGCC-3′	*GFP*
I	For Rev	5′-GGGTCGACTTGGACTGTCGGCGGCTCGC-3′ 5′-CCGGATCCAGCACTTGCATGCCGCCCATTG-3′	*P.p. Met2-1*, iPCR
J	For Rev	5′-CTTGAATTCGACCAGATCAATCG-3′ 5′-CCAAAGCTTGACTGGATCACAATCATC-3′	*A.g. Met2-1*, iPCR
K	For Rev	5′-CAAGGTCGACAGGTTCGACCCCTGT-3′ 5′-GGCAAGAAGGAATGCAATGCAAGCTT-3′	*A.g. Met2-1*, iPCR
L	For Rev	5′-GTCAGCTGGATCGAGACAACTGGGACC-3′ 5′-GTTCTGATGGCGGATCCTAATTGG-3′	*A.g. Met2-2*, iPCR
M	For Rev	5′-CCATCCACAAAGAAACAGGCGAAC-3′ 5′-CCCTGCAGCACCATTGCGTAATGTCCACC-3′	*A.g. Met2-1,* label
N	For Rev	5′-GACAAGGSWGACAACTGTATGGT-3′ 5′-CCATTGAGTGATGTCTACTTCTGC-3′	*A.g. P.p. Met2-1*, label

2.7. Plasmid Construction

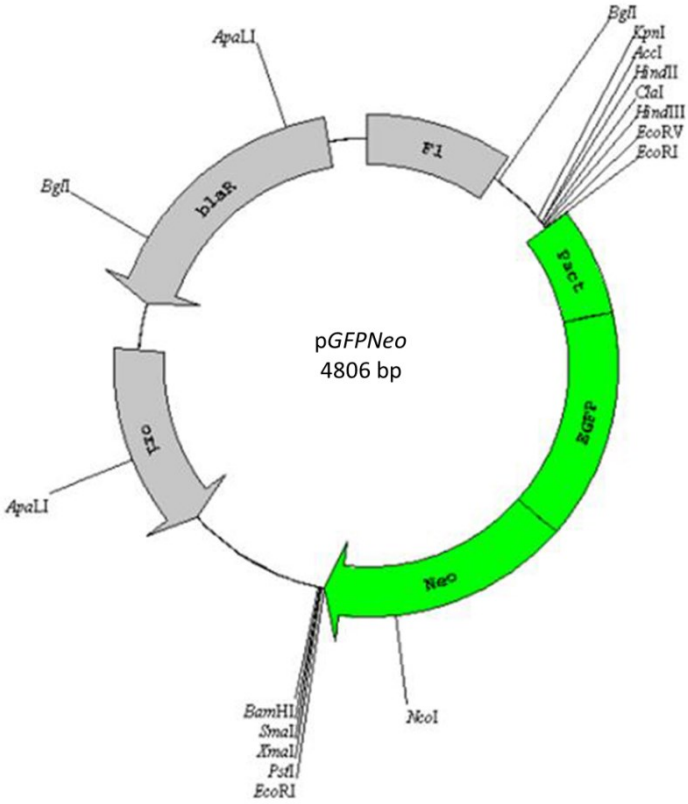
The complete *A. glauca Met2-1* gene was amplified with primer pair E, digested with the restriction enzymes *Bam*HI and *Hin*dIII and cloned into the corresponding restriction sites of pTZ19R creating p*Met2-1* (Fig 2.1.A). The actin promoter of *A. glauca* was amplified and digested with *Hin*dIII and *Bsp*HI, and inserted between the *Hin*dIII*/Nco*I sites of p*EGFP* (Clontech, Palo Alto, USA), creating pa*EGFP*. The neomycin-phosphotransferase gene was amplified by primer pair F with p*EBFP* (Clontech, Palo Alto, USA) as template. The fragment was digested with *Bsr*GI and *Xba*I and cloned into the appropriate sites of pa*EGFP*. The complete cassette was cut out with *Eco*RI and subcloned in pBluescriptIISK (Stratagene, La Jolla, USA) creating p*GFPNeo* (Fig 2.1.B). The cassette was cut out with *Bam*HI and *Hin*dIII, inserted between the *Hin*dIII and the partially restricted *Bgl*II sites of p*Met2-1*, creating p*Met2GFPNeo* (Fig 2.1.C).

The *A. glauca* actin promoter was amplified with primer pair G, digested with *Bam*HI and *Hin*dIII and cloned in the corresponding sites of YesNTC (Invitrogen, Carlsbad, California, USA) harbouring already the coding sequence of the *Mucor mucedo sexM* transcription factor fused to *EGFP* (this group, unpublished), a vector constructed for transformation of *Saccharomyces cerevisiae*. The cassette was cut out with *Hin*dIII and *Xba*I and inserted in *Hin*dIII-digested p*Met2-1*. Remaining sticky ends were filled in using Klenow DNA polymerase. The fragments were ligated for a second time prior to transformation of *E. coli*. The resulting vector was named p*Met2SexMGFP* (Fig 2.1.D, Karimi et al. 2012).

A


BglI (471)
KpnI (632)
XmaI (632)
AvaI (632)
SmaI (634)
BamHI (637)
ApaLI (4985)
BglI (4438)
f1
bla
HATcds
AvaI (1298)
pMet2-1
5234 bp
ori
ApaLI (3739)
HAT region
AccI (1683)
HindII (1684)
HindII (1816)
HATcds
HATcds
NcoI (2097)
ClaI (2155)
HindIII (3034)

B


BglI
KpnI
AccI
HindII
ClaI
HindIII
EcoRV
EcoRI
ApaLI
f1
BglI
blaR
pact
pGFPNeo
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EGFP
ori
ApaLI
Neo
NcoI
BamHI
SmaI
XmaI
PstI
EcoRI

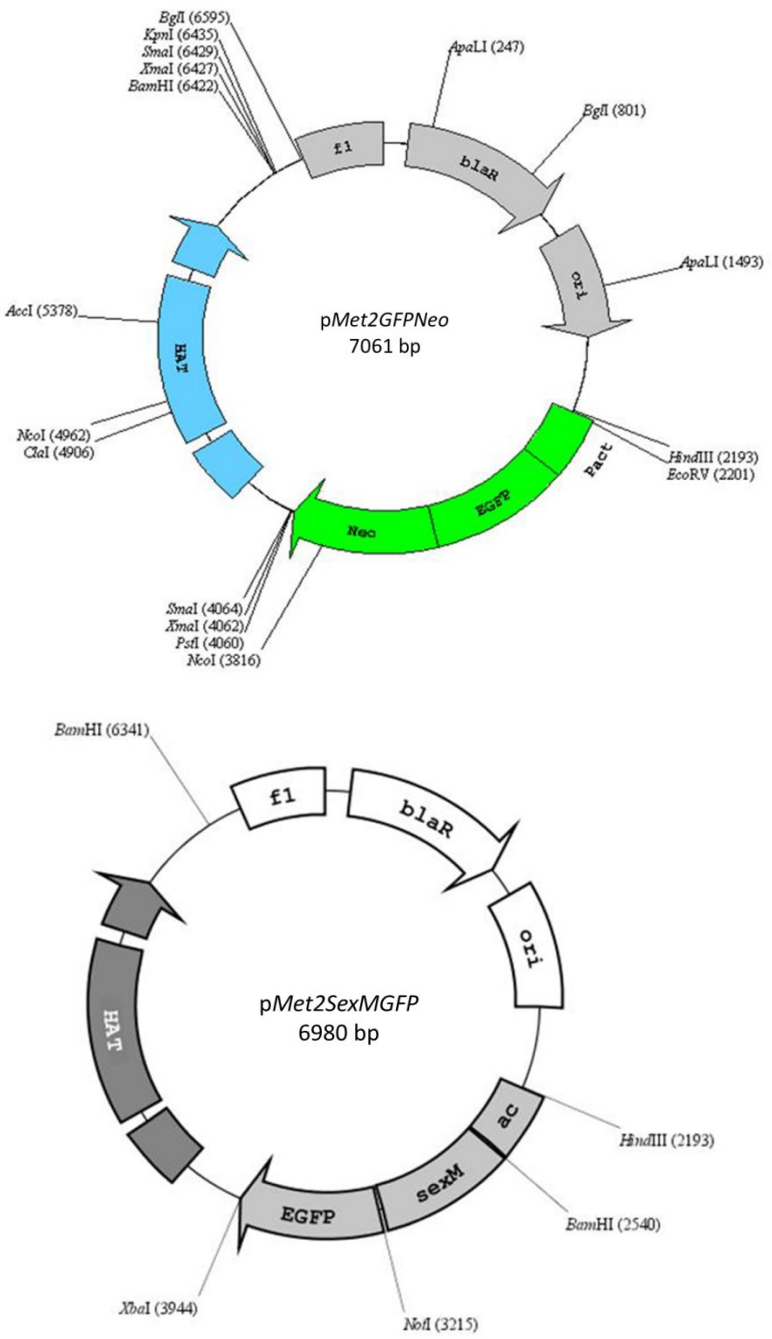

Fig.2.1. **Map of transformation vectors.** (A) p*Met2-1* consists of pTZ19R carrying the *Met2-1* gene, (B) p*GFPNeo* carries the neomycin-phosphotransferase gene (*Neo*) and the gene for enhanced green fluorescent protein (*EGFP*) under the control of the *A. glauca* actin promoter (ac). (C) p*Met2GFPNeo* contains the *Met2-1* gene along with the *Neo* and *EGFP* genes under the control of the actin promoter. (D) p*Met2SexMGFP* harboring the open-reading frame for the *sexM* transcription factor gene (*sexM*) of *Mucor mucedo*.

2.8. Southern Blot Analysis

Blotting was performed based on the procedure described by Southern (1975) with modifications. Genomic DNA of transformed, wild type and mutant strains was digested in amount of 3 µg and of 10 ng of vector plasmids, using restriction enzymes purchased from Fermentas and NEB companies at 37 °C. Digested DNAs were loaded on 1 % agarose gels along with undigested DNAs. The nucleic acids were denatured by washing the agarose gel (in size of 7.5 x 8.5 cm or 14.3 x 13.7 cm) in a solution containin g 0.5 M NaOH and 1.5 M NaCl (150 ml solution for small gels and 300 ml for big gels) for 20 minutes. The gels were neutralized in 0.5 M Tris-Cl, pH 7.0, and 3 M NaCl wash solution twice for 20 minutes each. By placing a nylon membrane over the gel and a pile of dry absorptive paper, the DNA was transferred to the membrane in the presence of 20 x SSC (3M NaCl, 0.3 M Na-citrate, pH 7.0) overnight. In order to fix the DNA to membrane, the membrane was dried in a vacuum oven at 80 °C for 3 hours.
Hybridization was carried out at 42 °C in two steps; pre-hybridization and second hybridization. To prevent non-specific binding to the membrane, pre-hybridisation was performed for 4 hours using a buffer containing 50 % formamide, 5 x SSC, 1 x Denhardt solution (1% bovine serum albumin, 1 % Ficoll and 1 % polyvinylpyrrolidone), 0.1 % SDS, 50 mM sodium phosphate buffer, pH 7.0, and 0.5 mg/ml denatured DNA from salmon fish's sperm in the final volume of 20 ml.
Hybridization was done overnight in 20 ml buffer containing 50 % formamide, 5 x SSC, and 1 x Denhardt solution, 0.1 % SDS, 20 mM sodium phosphate buffer, pH 7.0, 0.1 g/ml salmon sperm denatured DNA and the digoxigenin-labeled probe.
To prepare the digoxigenin-labeled probes, 100 ng of 1kb/ 1kb Plus DNA ladders (Fermentas) and plasmid vectors were used. The DNAs were denatured at 95 °C for 10 minutes in a waterbath and cooled immediately on ice. Subsequently, 1µl dNTP labelling mix, 1 µl hexanucleotide primer mix, and 0.5 units of the Klenow fragment of DNA polymerase I (Roche) were added at the final volume of 10 µl (DIG DNA Labelling Kit, Roche). The mixture was incubated at 37 °C for 1 hour.

Every 2µl of labelled probes were cleand by passing through a column filled with Sephadex G50 in 0.4 ml 10 mM Tris-Cl pH 8.0, 1 mM EDTA, pH 8.0 and 0.1 % SDS. The column was washed with 0.4 ml portions of cleaning buffer. The second fraction contained the digoxigenin-labelled DNA probe.

For genes, corresponding to homoserine acetyl transferase and green fluorescent protein, the labelled fragments were obtained by PCR amplification using dNTP labelling mix.

After hybridisation, to remove excess probe and prepare the membrane for detection, it was washed according to the following regime: 1) two washes in 2 x SSC and 0.1 % SDS for 20 minutes at 42 °C, 2) two washes in 2 x SSC and 0.1 % SDS at 50 °C for 20 minutes, 3) two washes in 0.2 x SSC and 0.1 % SDS at 50 °C for 10 minutes, 4) one wash in 100 mM maleic acid, pH 7.5, 150 mM NaCl and 0.1 % TritonX-100 for 5 minutes. The latter solution plus 1 % skimmed milk powder was used to block non-specific binding sites for 30 minutes in a plastic cover. The membrane was incubated in fresh block buffer consisting anti-digoxigenin-alkaline phosphatase conjugate (Roche) in 1:10,000 dilution, sealed into a plastic bag for another 30 minutes, followed by two washing steps in solution 4 for 15 minutes each. Finally, the detection buffer containing 100 mM Tris-Cl, pH 9.5, and 100 mM NaCl was used for 5 minutes. The membrane was sealed into a plastic bag, while 250 µM CSPD (Bronstein et al. 1991) in 1 ml detection buffer were distributed over the membrane. After 15 minutes incubation at 37 °C, an X-ray film (Kodak XAR-5) was placed on top of the membrane. Depending on the signal strength, after 45 minutes to 2 hours, the film was developed and fixed in darkness.

2.9. Microscopic Investigations

Microscopic investigations were performed with a Zeiss Axiophot microscope. Differential interference contrast (DIC) was used for light microscopy. In order to investigate the expression of GFP gene in transformants, the following Zeiss filter set was used: BP 485 excitation, FT 510 dichroic and 515–585 emission filters.

3. Results

3.1. Natural Transformation during Parasitism

The host-parasite system *Absidia glauca–Parasitella parasitica* represents an excellent example of biotrophic fusion parasitism in the fungal world. In fusion parasitism, the hyphae of both partners necessarily undergo fusion during infection (Jeffries and Young 1994). There are several reports claiming that *P. parasitica* transfers its genomic content to its invaded host. At the microscopic level, Burgeff had described this fusion process in 1924. According to his microscopic observations, the infection process is accompanied by mixing the cytoplasm of both organisms as a consequence of fusion. He also showed that this mycoparasitism depends on the mating types of the partners. In the experiments with the fungal isolates he used, parasitic structures are exclusively produced between complementary mating types of host and parasite. In the 1952, Lederberg predicted that the *Parasitella* interaction could be the most promising experimental system for proving horizontal gene transfer between non-identical species in the eukaryotic world. Later on Kellner et al. (1993) provided the evidence, for horizontal gene transfer in this system. In their studies, two auxotrophic mutants of *A. glauca* were used for infection with *P.parasitica*. After parasitic fusion, prototrophic recombinants were found, indistinguishable in morphology from to *A. glauca.* They concluded that these new strains, which were named para-recombinants, obtained the prototrophic growth ability from *P. parasitica*. It was also proven that the hormonal recognition in mating- dependent parasitism follows the same pathway as in the sexual system in *P. parasitica* (Schultze et al. 2005). Nevertheless, the horizontal gene transfer in this mycoparasitic system, so far, has, at least for chromosomally located genes, not been proven at the molecular level. This is the first study which corroborates this claim by means of two auxotrophic mutants of *P. parasitica* and *A. glauca* in order to analyze prototrophic descendants at the DNA level.

3.1.1. Screening for Prototrophic Para-recombinants

The genetic consequences of mycoparasitism interaction between *A. glauca* and *P. parasitica* were analyzed using two auxotrophic strains from both partners.

As mentioned, two auxotrophic mutants of *A. glauca* have been utilized by Kellner and her co-workers (1993) to follow the fusion parasitism by *P. parasitica*. One of these strains was RVII-324 *met*$^-$ mutant strain, a derivative of *A. glauca* CBS101.48 wild type strain, which has deficiency in methionine biosynthesis. Furthermore, this methionine auxotroph has been checked for reversion frequency by Wöstemeyer et al. (1990) and showed reversion ratio of $\leq 10^{-7}$ (no revertanta among 2 x 10^7 spores found). Therefore, RVII-324 *met*$^-$ mutant strain, with respect to its reasonably good maximal reversion rate, is chosen as the host for parasitism investigations for this thesis.

For *P. parasitica*, an adenine auxotrophic strain, NGI-315 *ade*$^-$, has been used (Burmester et al. 2013). This mutant grows exclusively on minimal medium in the presence of adenine. Phenotypically this mutant gives rise to mycelia with a red-orange color. This pigment accumulation is observed in other fungal *ade1* or *ade2* mutants, too (Roman 1956, Fischer 1969). These mutants are defective either in the gene for N-succinyl- 5-aminoimidazole-4 carboxamide ribotide synthase (*ade1*), or phosphoribosyl-aminoimidazole carboxylase (*ade2*). Neither this mutant nor the *A. glauca* mutant can grow prototrophically on minimal medium. Exclusively recombinants (so-called para-recombinants), obtained after co-cultivation of both partners, can grow on minimal medium. Co-cultivation of *A. glauca* as the host and *P. parasitica* as the parasite, with complementary mating types, causes the parasite to grow towards the host, mediated by the sex pheromonal system (Schultz et al. 2005). After infection, fusion of mycelia from both partners is terminated by producing special spore-like structures (so-called sikyospores; Burgeff 1924). These spores are seperated from the producer mycelia by a septum (Fig. 1.1).

To achieve these structures, co-cultivation of the partners has been performed on complete medium. After appearance of infection structures (sikyospores) on complete medium (Fig.3.1), sporangiospore progeny was plated on minimal

medium. Those colonies which were able to grow prototrophically on minimal medium were transferred to fresh minimal medium plates. The mitotic sporangiospres of these primary recombinant colonies were checked for the stability of prototrophic growth on minimal medium during several sporulation cycles. The mitotic stability of para-recombinants showed high fluctuation in different sporulation cycles, but in general it was unstable (Fig 3.2). This instability was also previously observed for other mutants of *A. glauca,* RVII382 his^{-}*,* a histidine auxotrophic mutant strain of *A. glauca,* which was used as the host for infection by *P. parasitica* in previous experiments (Kellner et al., 1993).

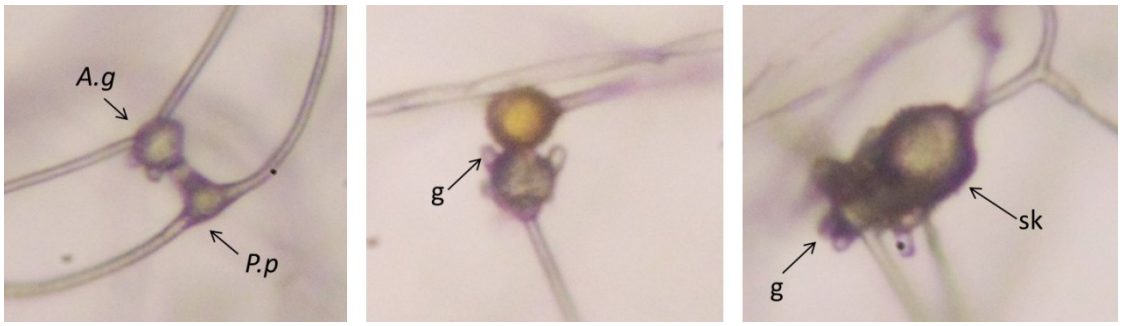

Fig. 3.1. Microscopic observations of interactions between *P. parasitica* and *A. glauca*. The development of sikyotic cell in parasitic interaction between *P. parasitica* (*P.p*) and *A. glauca* (*A.g*); gall (g), sikyospore (sk).

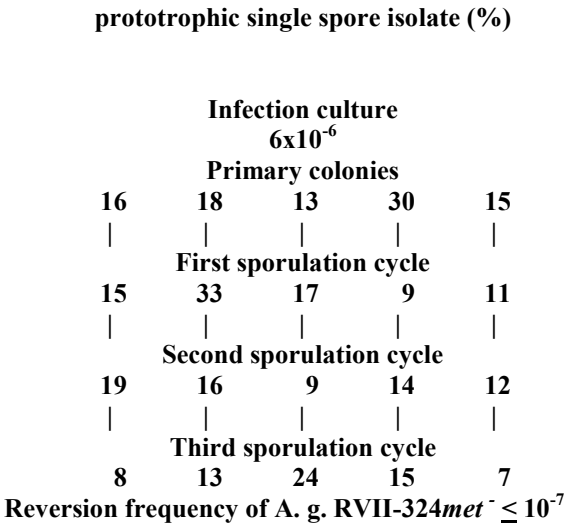

Fig. 3.2. Mitotic stability of para-recombinants. Co-coltivation of *P. parasitica ade*$^-$ and *A. glauca met*$^-$ strains has been performed on complete medium. After sikyospores development, mitotic sporangiospores were harvested, diluted with sterile water and plated on minimal medium, where only para-recombinants could grow. The number of total spores was determinated using a Thoma counting chamber. Single spore isolates of para-recombinants were obtained by plating on minimal medium, and the ration of prototrophic colonies was calculated for several sporulation cycles.

Para-recombinants appeared phenotypically similar to the methionine deficient mutant of *A. glauca,* and the only difference was their ability to grow on minimal medium prototrophically. Based on the phenotype similarity of para-recombinants with recipient mutant of *A. glauca*, it is assumed that para-recombinant strains have essentially the genetic background of *A. glauca* RVII-324 with only few additional genomic elements from *P. parasitica,* especially and maybe exclusively the selective marker gene that enables prototrophic growth. Therefore, characterization of the genetically defective gene in the *A. glauca* recipient is a prerequisite for molecular analysis of these para-recombinants.

3.2. Analysis of the Methionine-deficient *A. glauca* Mutant

Feeding experiments on minimal medium supplemented with essential intermediates in methionine biosynthesis showed that the RVII-324 *met*$^-$ mutant of *A. glauca* grows in the presence of cystathionine and homocysteine, but not with homoserine or O-succinyl homoserine.

In the methionine biosynthesis pathways of different organisms, homoserine is acylated to either O-acetyl homoserine (Marzluf 1997) or converted to O-succinyl homoserine (Hacham et al. 2003) by the enzyme homoserine O-acetyltransferase. These intermediates are converted to cystathionine by another enzyme, γ-cyctathionine synthase (According to the methionine-biosynthesis pathway shown in Fig 1.2).

The inability of *A. glauca* RVII-324 *met*$^-$ to convert homoserine to cystathionine could be caused by genetic defects either in the gene for homoserine O-acetyltransferase or the gene for γ-cystathionine synthase, two involved enzymes in this conversion. Therefore, the putative genes, i.e. *Met2* for homoserine O-acetyltransferase and *Met3* for γ-cystathionine synthase were identified in the three sequenced genome among mucoralean fungi, *Mucor circinelloides, Phycomyces blakesleeanus* and *Rhizopus oryzae* in order to get access to the corresponding genes in *A. glauca*. Based on conserved amino acid motifs of these fungi, two putative genes *Met2 (Met2-1* and *Met2-2)* and one putative *Met3* gene were determined in three genomes. By degenerated primers, the putative genes from *A. glauca* were partially amplified and sequenced. The entire coding areas for these genes were characterized after amplification by inverse PCR using specific primers and sequencing. In the next steps, molecular investigations for wild type and methionine auxotrophic strain of *A. glauca* have been performed by comparing the PCR patterns and Southern blot signals as well as expression in both strains. In addition, complementation of the mutant strain by introducing the cloned wild type allele was achieved in transformation experiments.

PCR patterns using specific primers for each of the three genes showed no differences in the *Met2-2* and *Met3* genes between wild type and mutant strain of *A. glauca*, while the gene fragment for *Met2-1,* obtained for the *A. glauca* wild

type, could not be found for the auxotrophic mutant (Fig. 3.3.A). The wild type fragment for *Met2-1* was labeled with dNTP labeling mix and used in Southern blot analysis to trace this gene in the wild type and the mutant. The results also revealed different patterns in wild type and mutant strain and displayed a rearrangement in the chromosomal DNA at the *Met2-1* locus of the RVII-324 mutant. Using a labelled wild type *Met2-1* probe for hybridization with *Hind*III digested genomic DNA from wild type and mutant strains, two signals were observed for the mutant strain with lengths of 1.7 kb and 6.5 kb, whereas in the wild type, there was only one signal at 2.4 kb (Fig. 3.3.C). The fragments for DNA, digested with *BamH*I, in the mutant strain are 1.1 kb and >10 kb, while the wild type displays only one fragment with lengh of > 10 kb. These signal patterns point towards a division of the *Met2-1* gene into two distal parts with one restriction site for each restriction enzyme, *Bam*HI and *Hind*III, inside the coding region of the *Met2-1* gene in the mutant. These divided parts could correspond to the coding region of the N and the C-terminal parts of the protein, respectively. The sequencing results of the N-terminal part, adjacent to the *Hind*III fragment, indicate a large insertion in the RVII-324 mutant (GenBank accession nos JX448412 and JX448413) in comparison with *A. glauca* wild type (GenBank accession no. JQ898110). Accordingly, the schematic genetic maps in the mutant and wild-type *A. glauca* are shown in Fig. 3.3.D.

In order to figure out the probable effects of this rearrangement for the *Met2-1* gene function, the complementary DNA (cDNA) of both strains have been prepared and amplified by RT-PCR. These analyses revealed that the *Met2-1* gene is not transcribed in the mutant strain. Using primer pair D1 (Table 2.2), where the primers bind to the template in the distal part of *Met2-1* in the mutant and the wild type, amplification can be initiated in the chromosomal DNA of both strains, while amplification was not possible in the mutant strain with complementary DNA (obtained by RT-PCR) as template (Fig. 3.3.B). This part of the gene is shown as the short underlined part in the *Met2-1* schematic map in Fig. 3.3.D. Unlike *A. glauca* wild type, the *Met2*-1 gene in the mutant could not be transcribed in the distal part of the gene, which is located now on in a new genomic position. The large insertion in the *Met2-1* gene, which caused the

rearrangement in the RVII-324 mutant, explains the high stability of the mutant phenotype. Large insertions or transpositions exclude the possibility for reversion events.

In addition, the cloned *Met2-2* region of wild-type and the methionine-auxotrophic mutant didn't show any difference in hybridization analysis and also in nucleotide sequences (GenBank accession no. JX448411, Fig. 3.4). Furthermore, transformation assays using the *Met2-2* gene could not complement the mutant. Consequently, *Met2-2* is unable to accomplish the *Met2-1* function; *in vivo,* these genes have different functions. *Met2-2* has an unclear role in *A. glauca*, otherwise the mutant phenotype would not have been recognized even during the initial mutant screenings.

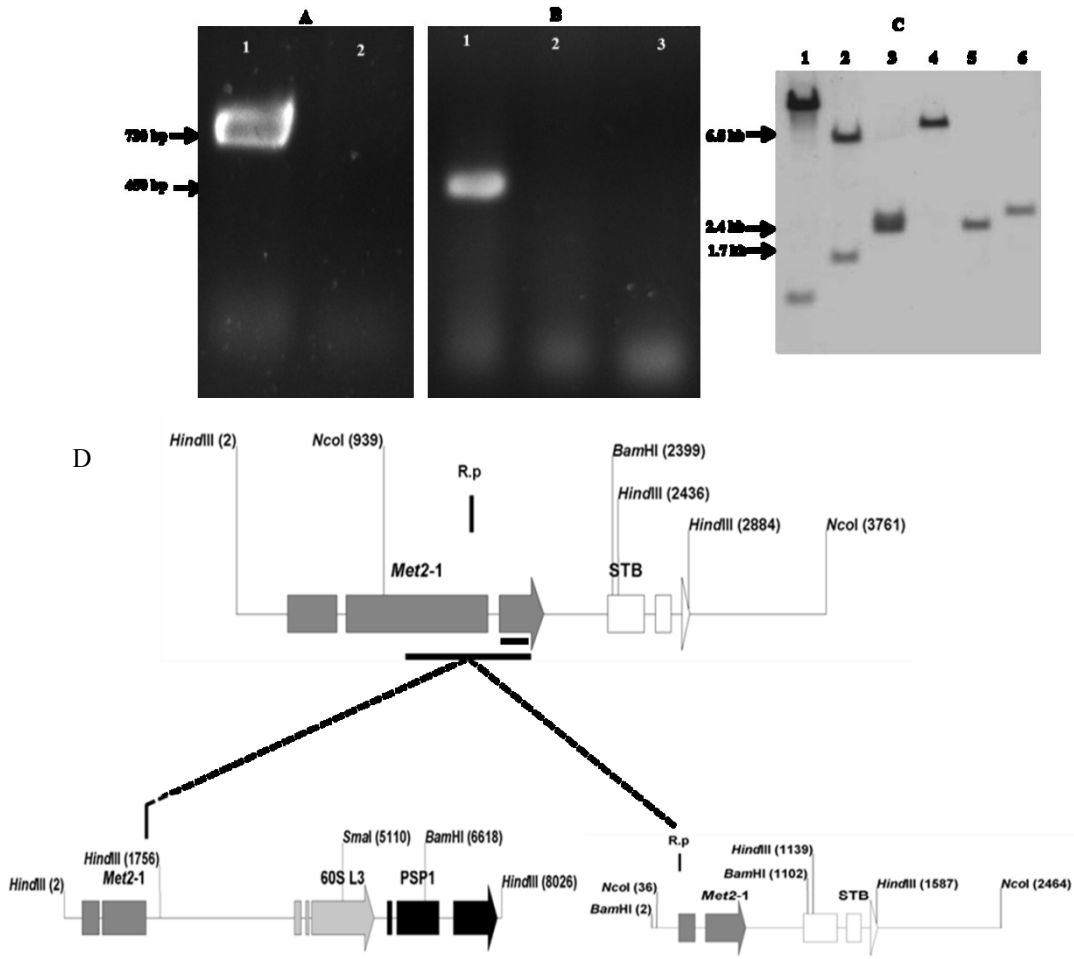

Fig. 3.3. Molecular characterization of *Met2-1* gene in the *A. glauca met*$^-$ mutant in comparison with the parental wild type strain. (A) PCR with genomic DNAs of wild

type (lane1) and mutant strain (lane2). (B) RT- PCR with primer set in undamaged part of *Met2-1*, wild type (lane1) and mutant strain (lane2), without DNA as control (lane3). (C) Hybridization of RVII-324 genomic DNA (lanes 1–3) and CBS 101.48 DNA (lanes 4–6), DNAs were restricted with *BamH*I (lanes 1, 4) *Hind*III (lanes 2, 5) and *Nco*I (lanes 3, 6). (D) Map of the sequenced *A. glauca Met2-1* wild type gene compared with mutant *Met2-1* gene. The long underlined part shows the positions of recombination point disrupting the open reading frame in the mutant (R.p). There are two additional restriction sites for *Bam*HI and *Hind*III in this inserted fragment, and restriction with each enzyme caused two signals in Southern blot analysis in comparison with the wild type strain without restriction sites for these enzymes inside the coding region. The short underlined part shows the fragment with the recognision sites for primer set D1 used in RT-PCR experiments.

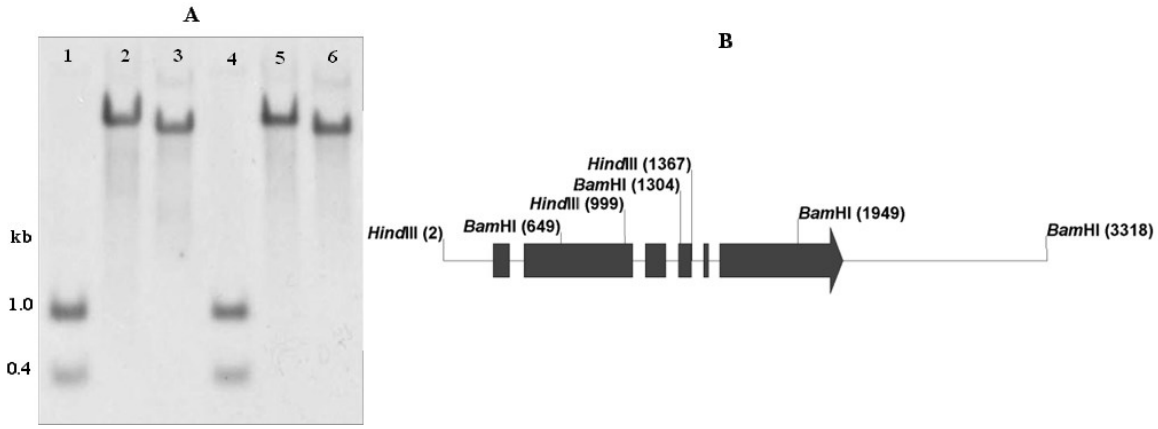

Fig. 3.4. Characterization of *Met2-2* gene in *A. glauca.* *A. glauca Met2-2* loci are identical in wild type and mutant. (A) Genomic DNA of the wild type (lane 1-3) and the *met*$^-$ mutant (lane 4-6) were restricted with *Hind*III (lanes 1,4), *Xho*1 (lanes 2,5), *Kpn*I (lanes 3,6) and hybridized with the *Met2-2* fragment (B) Restriction map of the *A. glauca Met2-2* region.

3.2.1. Transformation of the Methionine-deficient Mutant by the *Met2*-1 Wild Type Allele

Transformation experiments have been performed to support the function of the *Met2-1* gene in methionine biosynthesis of *A. glauca.* The *Met2-1* gene of *A.*

glauca wild type strain was utilized for complementation experiments with the methionine auxotrophic mutant of this fungus, RVII-324 *met*$^-$, by introducing vector plasmids harbouring the *Met2-1* gene into the mutant strain.

3.2.1.1. Vectors, Transformation Efficiency and Stability

Several vectors harbouring the *Met2-1* gene from *A. glauca* wild type were used for transformation experiments. p*Met2-1* was employed for selection of transformants on minimal medium without methionine. For constructing this vector, the *Met2-1* gene on a fragment of 2.4 kb from *A. glauca* wild type was restricted with *Hind*III and *Bam*HI restriction enzymes and inserted into the corresponding restriction sites of pTZ19R. The other vectors, p*Met2GFPNeo* and p*Met2sexMGFP* contained additional genes.

p*Met2GFPNeo* harbours two additional genes, i.e. the green fluorescent protein encoding gene as a reporter gene (*GFP*; Schilde et al. 2001), fused to the neomycin phosphotransferase gene as an additional selective marker gene under the control of the *A. glauca* actin promoter (Wöstemeyer et al. 1987), inserted into the pBluescriptIIsk plasmid (Stratagene). Each transformant who has received this vector, can grow on minimal medium containing 400 µg/ml neomycin and shows the fluorescent activity.

p*MET2sexMGFP*, carries the *sexM* transcription factor gene from *Mucor mucedo* (Wetzel et al. 2012), fused to *EGFP* and controlled by the *A. glauca* actin promoter, inserted into the yeast plasmid pYes2NTC (Invitrogen). In transformation studies of *Saccharomyces cerevisiae,* the resulting *sexM* protein co-transported the *GFP* fluorescence into nuclei. It also showed the expression of the *GFP* gene at high levels (Wetzel et al. 2012). Therefore, transformation experiments with the *A. glauca* methionine auoxotrophic mutant, *sexM* was used to check for the possibility of nuclear delivery of plasmid-encoded gene products.

The results of these transformation experiments reveal that for 10^5-10^6 protoplasts, between 1-10 methionine-independently growing transformants could be achieved per 5-10 µg of plasmid DNA. There are no significant differences in transformation efficiency between these three vectors. However, the

transformants obtained with p*Met2sexMGFP* showed higher mitotic stability than with p*Met2-1* and p*Met2GFPNeo* after several sporulation cycles, independent of selective or non-selective growth conditions (Table 3.1). Therefore, according to microscopic observations for green fluorescent protein expression, two transformants obtained with p*Met2sexMGFP* were chosen for characterization by Southern type hybridization experiments.

Sporulation cycle	Total number of plated spores	Number of prototrophic colonies	Ratio of prototrophic colonies
p*Met2sexMGFP*			
Primary colony	8×10^{6}	37	4.6×10^{-4}
1	1.2×10^{5}	86	7.2×10^{-2}
2	2.4×10^{5}	93	3.9×10^{-2}
p*Met2-1*			
Primary colony	1.4×10^{7}	42	3×10^{-4}
1	7.3×10^{5}	34	4.6×10^{-3}
2	6×10^{5}	21	3.5×10^{-3}
p*Met2GFPNeo*			
Primary colony	7.2×10^{6}	39	5.4×10^{-4}
1	3.5×10^{5}	31	8.8×10^{-3}
2	1.6×10^{5}	26	1.6×10^{-2}

Table 3.1. Statistical estimation of transformation stability. Stability of the prototrophic phenotype of single spore isolates from typical transformants using three vector plasmids harbouring the *A. glauca Met2-1* gene, i.e. p*Met2sexMGFP*, p*Met2-1* and p*Met2GFPNeo*. The primary colony starts from a multi-nucleated protoplast containing an unknown number of nuclei. At least one of them is transformed. Sporulation cycles 1 and 2 are started from a single uni-nucleated spore. Aliquots of the total spore progeny were plated on complete medium for counting total progeny and on minimal medium, in order to determine the number of prototrophs after these sporulation cycles.

3.2.1.2. Expression of Transferred Genes

3.2.1.2.1. *GFP* Expression

As mentioned, the *GFP* gene was used as reporter gene to distinguish the transformant unequivocally from those derivatives that might have regained their growth ability on minimal medium without externally added methionine by reversion events. For this mutant, however, the reversion rate is very low (Wöstemeyer et al. 1990), and the high degree of chromosomal rearrangement underlying this mutant renders the probability for reversion very low anyhow. Additionally, this gene was used to trace expression of the introduced DNA in the transformant mycelia. The expression of *GFP* was more obvious in fusions with the transcription factor *sexM* of *M. mucedo* in p*Met2sexMGFP* (Fig. 3.5,D-F) as has already been observed in *Saccharomyces cereviseae* with the same translational fusion (Wetzel et al. 2012) in comparison with p*Met2GFPNeo* (Fig. 3.5,A-C).

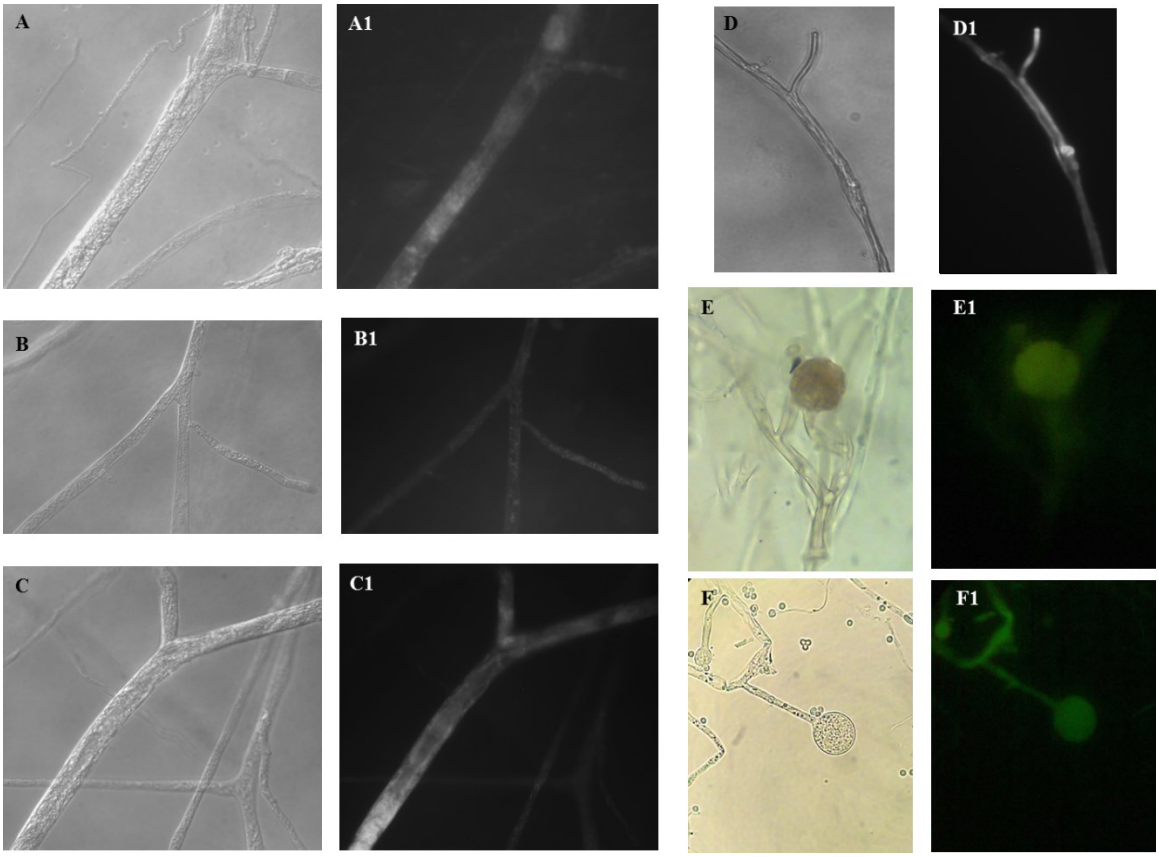

Fig. 3.5. *GFP* expression in *A. glauca* transformants. (A1, B1 and C1) Transformants' mycelia express the *GFPNeo* cassette. (D1, E1 and F1) Mycelium of a *SexMGFP* expressing transformant. The following filter combination excitation was used: 485 ± 20 nm, emission: 515–585 nm. A,B,C,D,E and F were obtained by differential interference contrast.

3.2.1.2.2. *Met 2-1* Transcription in RT-PCR Experiments

In addition to prototrophic growth and visible green fluorescent protein in transformants, the expression of introduced *Met2-1* gene in the transformed strains of RVII-324 *met*$^-$ was also investigated at the transcriptional level. The cDNAs were obtained from mRNA of transformants as well as from mutant and wild type strains by RT-PCR. Using primers for actin as a reference gene (Fig. 3.6.B) assured that the cDNA is not contaminated with genomic DNA, due to the known length differences between amplicons of actin cDNA and the

corresponding, intron-containing chromosomal DNA (Fig. 3.6.A,C). The primers used for the amplification of the undamaged part of the *Met2-1* (primer pair D1; Table 2.2) could initiate the amplification when the chromosomal DNA of the mutant used as the template, whereas, this amplified band was not achieved with its complementary DNA, meaning that *Met2-1* gene is not transcribed in the mutant because of the occurred rearrangement in this gene due to mutagenesis. This part of *Met2-1* gene is shown as a short underlined part in the schematic map in Fig. 3.3.D. In transformants, this primer set allows the cDNAs to be amplified as well as in the wild type strain. These results also confirm the role of introduced DNA for surviving in the absence of methionine.

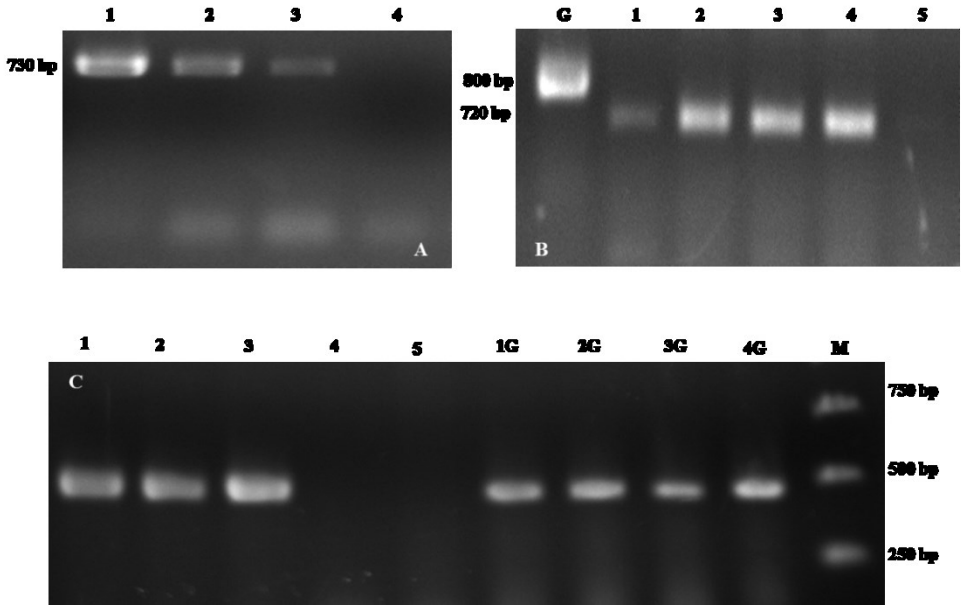

Fig. 3.6. *Met2-1* and actin genes' expression. Expression of the genes for *Met2-1* (A) and actin (B) in transformants (lanes 1,2), wild type (lane 3) and mutant (lane 4); lane 5 doesn't contain DNA as control. The genomic template for actin primers contains an intron (B: lane G). (C) Comparison between amplification patterns in complementary DNA (lanes 1,2 for transformants, 3 for wild type, 4 for mutant strains and 5 without DNA as control) and genomic DNA (lanes 1G, 2G for transformants, 3G for wild type, 4G for mutant strains) using the primer pair D1 (Table 2.2) for the undamaged part of the *Met2-1* gene. These results show the disability of *met*$^-$ mutant strain in expression of *Met2-1* gene.

3.2.2. Southern Blot Analysis of Transformants

DNAs from transformants and the mutant were digested with *Hin*dIII and after hybridization, compared with the hybridization signals of the vector and the wild type. Hybridization was performed with a probe for *EGFP* (Fig. 3.7.A,B) as well as with the *Met2-1* probe of *A. glauca* (Fig. 3.7.C). The signal patterns were identical in digested DNA or in bulk DNA with the plasmid vectors, when hybridized with labeled *EGFP* and the initial plasmid vector probes. These results indicate that the plasmid replicates autonomously and does not integrate into the chromosomal DNA of transformants. The plasmid vector could also be rescued from transformants' bulk DNA and re-transformed competent *E. coli* cells. As additional evidence, the *Met2-1* patterns in transformants were the same as in the parental mutant RVII-324 (Fig. 3.7.D).

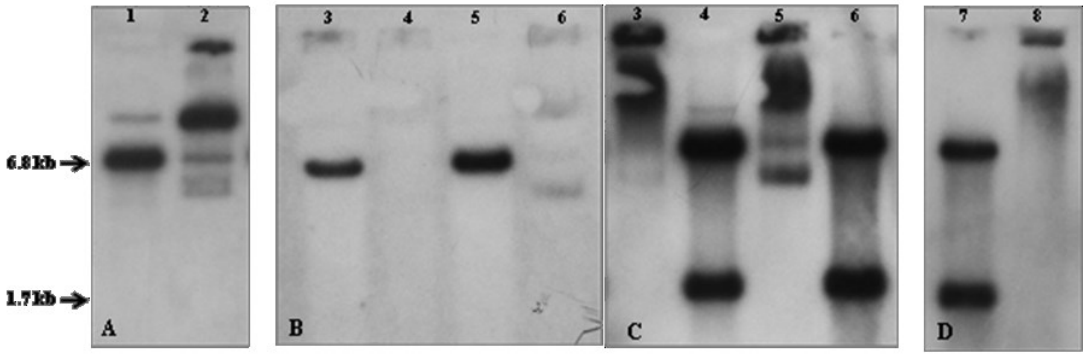

Fig. 3.7. Hybridization analysis of *A. glauca*, transformed with p*Met2sexMGFP*. DNAs were restricted with *Hin*dIII (lanes 1,3,5 and 7) or loaded without digestion (lanes 2,4,6 and 8). (A) Vector DNA and (B) genomic DNAs of transformants hybridized with a labeled *GFP* probe. Alternatively, the *Met2-1* gene fragment (C) genomic DNAs of transformants and (D) control DNA of the untransformed RVII-324 mutant were hybridized with a *Met2-1* probe.

The stability of the introduced *Met2-1* gene into transformant strains was checked by allowing them to grow and sporulate on minimal medium. The spores were collected by washing the agar plates with sterile water and plating to fresh minimal medium in suitable ditutions. After 2-3 days the single colonies obtained

from single spores were transferred to new plates. This was done for several times after sporulation of colonies, and DNA from each sporulation cycle was checked by PCR and hybridization patterns.

After several sporulation cycles, two hybridization signals were observed in one transformant obtained with p*Met2sexMGFP*. One signal matched with the linearized vector in hybridization with the *GFP* probe, but the second signal had a shorter size in comparison with the vector (Fig. 3.8.A). Re-cloning in *E.coli* competent cells showed a smaller plasmid beside the original one as well. The new plasmid was linearized with *Hind*III and *Bam*HI restriction enzymes and compared with the original linearized p*Met2sexMGFP* for restriction patterns. The linearized new plasmid showed a shorter size with *Hind*III in comparison with the original vector plasmid, and thus pointed towards a missing part in the new plasmid (Fig. 3.8.B). Plasmids, digested with *Bam*HI, also split the original p*Met2sexMGFP* in two fragments, but only one fragment for the truncated one (Fig. 3.8.C). There are two restriction sites for *Bam*HI in the plasmid vector p*Met2sexMGFP* (Fig. 2.1.D) resulting two fragments for this plasmid when digested with *Bam*HI, while only one band obtained from the *Bam*HI-digested of the truncated new plasmid (Fig. 3.8.C). This pointed towards the missed part in the new plasmid, contained one of the *Bam*HI restriction sites, and therefore this site has been eliminated by a deletion event. Nevertheless, PCR could still amplify both, *GFP* and *Met2-1* genes, and yields fragments with the same size in both plasmids (Fig. 3.8.F). This means that the truncation does not reside in these parts of inserted gene cassette.

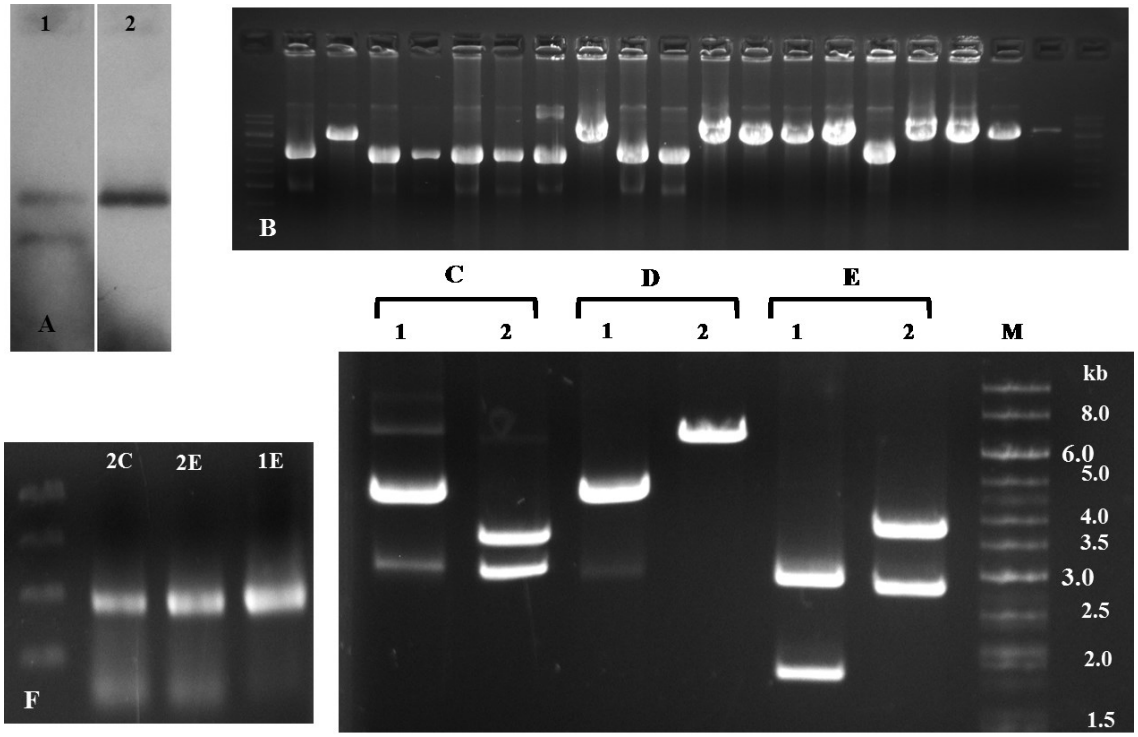

Fig. 3.8. Rearrangment in an introduced plasmid. In one transformant rearrangement in the acquired plasmid DNA was observed after several sporulation cycles. Beside the original transferred plasmid, an additional shorter plasmid was observed too. (A) Hybridization signal patterns obtained with the *GFP* probe were compared (lane 1) with p*Met2sexMGFP* (lane 2), digested with *Hind*III. (B) Two different plasmid sizes obtaind after re-cloning in *E. coli*. Three restriction patterns in new re-arranged shorter plasmid (lane 1) in comparison with p*Met2sexMGFP* (lane 2) using *Bam*HI (C), *Hind*III (D) and *Hind*III plus *Bam*HI (E) restriction enzymes, M indicate the DNA size marker. (F) PCR amplification of the fragments from digested plasmids showed in C and E using *Met2-1* primers.

3.3. Molecular Analysis of Para-recombinants

The genetic and physiological background of the methionine auxotrophy has been elucidated unequivocally by characterization of the gene defect in the *A. glauca* mutant, RVII-324 *met*$^-$, and by complementation of the corresponding gene in transformation experiments. The information on this methionine mutant of

*A. glauc*a in addition to sequence data of the *Met2-1* and *Met2-2* genes of *P. parasitica* (GenBank accession numbers JX448409 and JX448410 respectively, Fig. 3.9), helped to make use of complementation assays for prototrophic growth of para-recombinants after infection of *A. glauca* with *P. parasitica*.

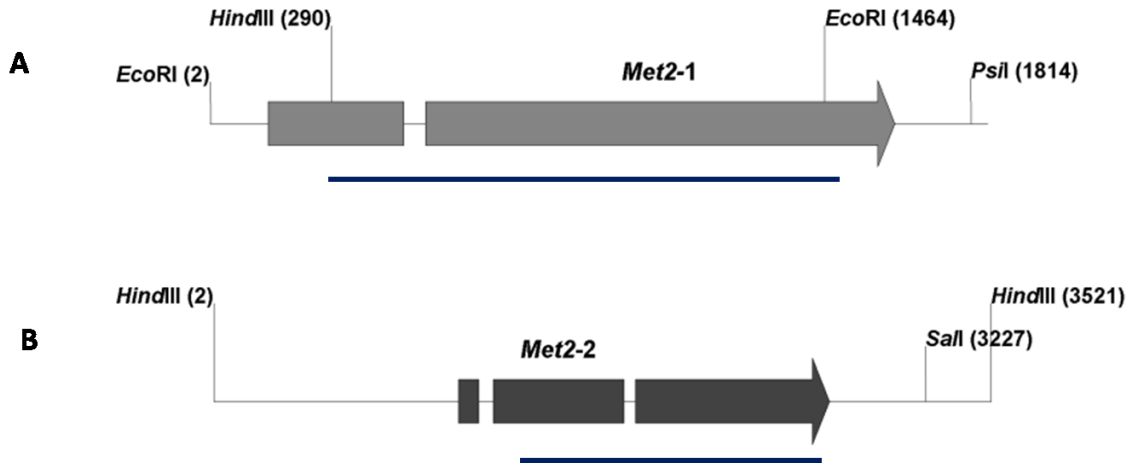

Fig. 3.9. Restriction maps of *Met2* putative genes of *P. parasitica*. *Met2-1* (A) and *Met2-2* (B) genes. The underlined fragments were used for hybridization.

For analyzing the para-recombinants at the molecular level, genomic DNAs of para-recombinant strains were prepared from colonies, obtained via single spore isolation. These DNAs and the DNA from the *P. parasitica* adenine auxotrophic strain, NGI-315 *ade*$^-$, and the wild type strain of *A. glauca*, CBS 101.48 and its methionine mutant, RVII324 *met*$^-$, were digested with *Hind*III and used in Southern blot hybridization. Labelled probes were prepared for *Met2-1* and *Met2-2* genes of *P. parasitica* (from underlined parts in Fig. 3.9) and wild type strain of *A. glauca*. *Hind*III digested genomic DNA from para-recombinants showed an extra signal of 3 kb length in comparison with their parental auxotrophic strains and with the wild type strain of *A. glauca,* when hybridized with the *Met2-1* probe

of *P. parasitica,* while cross-hybridization did not occur for digested DNA of *A. glauca* wild-type and the methionine auxotrophic mutant (Fig. 3.10.A). After hybridization with the *Met2-1* probe of *A. glauca,* the 3kb signal was observed as well, and cross-hybridization did not occur for digested DNA of *P. parasitica* (Fig. 3.10.B). The same signal of 3kb length was found after hybridization with a labelled *Met2-2* probe of *A. glauca* (Fig. 3.10.C) but not with the *P. parasitica Met2-2* probe.

The hybridization patterns of these para-recombinants for the *Met2-1* gene were identical with the *A. glauca* mutant pattern. This indicates that no integration at homologous loci has occurred. On the other hand, the same additional 3 kb signal as in the para-recombinant has been observed using differently laballed probes from *P. parasitica, Met2-1* as well as *A. glauca Met2-1* and *Met2-2.* Accordingly, this 3 kb fragment bears some similarity with *Met2-1* of *P. parasitica* and with *Met 2-1* and *Met2-2* of *A. glauca* (Fig. 3.10).

The acquired 3 kb genetic element in para-recombinant strains, which achieved in their restricted genomic DNA with *Hind*III, can be explained by assuming duplication in an internal part of the *A. glauca Met2-2* gene, because the chromosomally located copy of *Met2-2* in *A. glauca* contains two *Hind*III restriction sites in the coding region (Fig. 3.4). This can also be explained by eventual uptak of a part of the *P. parasitica Met2-1* gene, by which the methionine auxotrophy of *A. glauca* could be complemented.

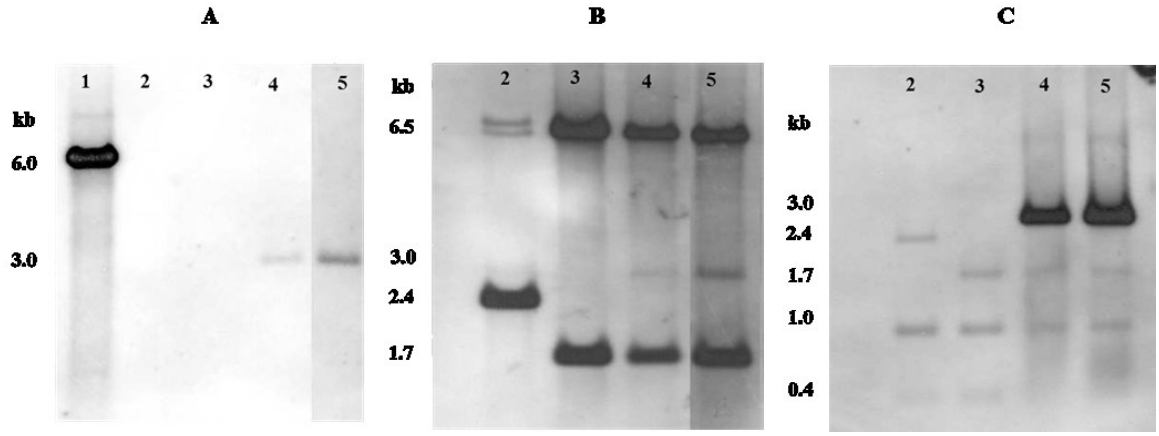

Fig. 3.10. Southern blot analysis of para-recombinant strains. Interaction between *P. parasitica* and *A. glauca* auxotrophic strains leads to methionine prototrophic strains. Apparently, this event occurred by taking up part of the *Met2-1* gene of *P. parasitica*. The DNAs of *P. parasitica* (lane 1), *A. glauca* wild type (lane 2), *A. glauca* mutant (lane 3) and para-recombinant strains (lanes 4,5) were digested with *Hind*III and hybridized with (A) *P. parasitica Met2-1*, (B) *A. glauca Met2-1* and (C) *A. glauca Met2-2* labelled probes. Hybridization with labelled probe of *Met2-1* gene of *A. glauca* showed similar signals in methionine deficient and para-recombinant strains (B), while an additional signal of approximately 3 kb length could be seen in para-recombinants when hybridized with labelled probes of *P. parasitica*'s *Met2-1* gene (A) and *A. glauca*'s *Met2-2* gene (C).

The genetic elements obtained by infection and complementing the mutant, appeared to be maintained extra-chromosomally in para-recombinants. Digestion of genomic DNA of a para-recombinant strain with different restriction enzymes showed an additional signal of approximately 3 kb length, when hybridized with a labelled fragment of *A. glauca*'*s Met2-2* gene (Fig. 3.11). This indicates that the modified *A. glauca*'s *Met2-2* gene in para-recombinants is not chromosomally located, otherwise the hybridization patterns should vary between enzymes. The similarity in size of signal patterns (Fig. 3.11) can only be explained if this part of the *Met2-2* gene resides on a plasmid-like DNA in para-recombinants. These extrachromosomal elements are linearized by the restriction enzymes. According to the hybridization of digested para-recombinant genomic DNA with the labelled

Met2-1 gene of *P. parasitica* (Fig. 3.10), the acquired part of the *Met2-1* gene of *P. parasitica* by para-recombination, accompanies the duplicated fragment of *A. glauca*'s *Met2-2*.

The results of the transformation experiments with the *A. glauca* methionine mutant also proved the extra chromosomal establishment of acquired DNA. This information and similar reports on the existence of extra chromosomal DNA in *A. glauca,* natural elements (Hänfler et al. 1992) as well as artificial ones (Burmester et al. 1992), strongly supports the assumption that the acquired genetic elements from the parasite generally propagated through autonomous replication, and are only rarely integrated into the chromosomal DNA of para-recombinants.

Overall, the methionine auxotrophy in *A. glauca* RVII-324 *met*$^-$, has been changed into methionine prototrophy in para-recombinants as a consequence of infection, and the *Met2-1* DNA of *P. parasitica* is assumed to be responsible for complementation of the genetic defect in the mutant recipient strain.

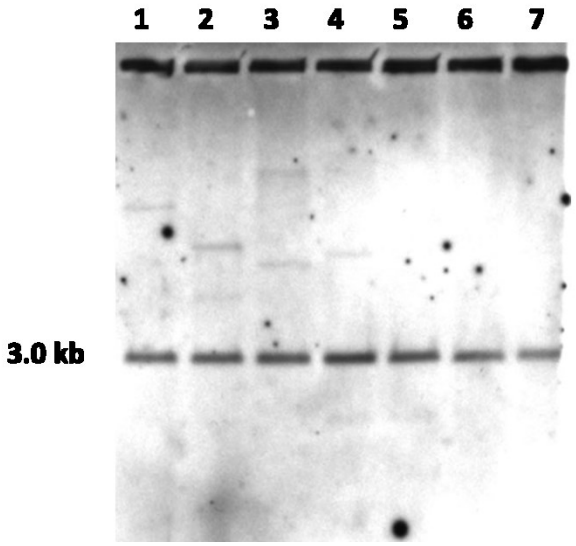

Fig. 3.11. Identical hybridization patterns of para-recombinants' digested DNA by different enzymes. Genomic DNA of the para-recombinant strain was digested with *Bam*HI (lane1), *Eco*RI (lane 2), *Xba*I (lane 3), *Hind*III (lane 4), double digestion with *Hind*III and *Bam*HI (lane 5), *Hind*III and *Eco*RI (lane 6), *Hind*III and *Xba*I (lane 7). The blot was hybridized with a labelled probe of the *A. glauca Met2-2* gene.

4. Discussion

4.1. Para-recombination

Parasitella parasitica (syn. *P. simplex*; Bainier 1903) is a facultative mycoparasite for several zygomycetous fungi including *Absidia glauca.* The characteristic feature of this interaction is the formation of a cytoplasmic continuum between the mycelia of the host and the parasite, during infection (Burgeff 1924). This type of parasitism also has been found in few other mycoparasites of zygomycetes, *Chaetocladium brefeldii*, *Chaetocladium jonesii* (Brefeld 1872, 1881) and *Absidia* (*Lentamyces*) *parricida* (Renner and Muskat 1958) and was termed biotrophic fusion parasitism (Jeffries and Young, 1994). For the interaction between *P. parasitica* and *A. glauca*, the parasitic fusion is formed between complementary mating types of parasite and host (Burgeff, 1924).

The infection experiments with *P. parasitica* and *A. glauca* showed that the parasexual relationship is associated with gene transfer from the parasite to its host after formation of the cytoplasmic continuum between the partners following infection (Kellner et al. 1993; Wöstemeyer et al., 1995, 2002). Kellner et al. (1993) demonstrated the para-recombination event and obtained prototrophic strains following infection of two different *A. glauca* auxotrophic strains by wild type *P. parasitica.* At that time, however, the transfer of chromosomally located genes from *P. parasitica* to *A. glauca* could not be studied at the molecular level. Congruent combinations between auxotrophic recipient mutants and the corresponding genes were not available, and consequently it was not possible to analyze the genetic inheritance of recombinants at the DNA level. This research provides this possibility to trace the acquired genetic traits in para-recombinants formed in parasexual relationship between *P. parasitica* and *A. glauca* using auxotrophic mutants of both partners. This system offered the opportunity to isolate the prototrophic para-recombinants by selection for growth on minimal medium. The *A. glauca* mutant RVII-324 *met*$^-$ is unable to grow on minimal medium without adding methionine or its precursors, cystathionine or homocysteine. This mutant has been used as the host strain in co-cultivation with

an adenine-deficient mutant of *P. parasitica*, NGI-315 *ade*$^-$, as the parasite strain, defective in one of the genes, needed for adenine biosynthesis.
Due to the inability of both auxotrophic strains to grow prototrophically on minimal medium, only para-recombinant spore progeny generated during the infection cycle are able to develop viable mycelia on minimal medium. This prototrophic growth on minimal medium has already been seen in earlier experiments (Kellner et al. 1993). These colonies carry genetic information from both partners of the parasitic interaction. The phenotypes always correspond to that of *A. glauca*. Accordingly, the hypothesis has been established that para-recombinants essentially contain *A. glauca*'s genomic complement and only very little additional information, obtained from *P. parasitica*. It is reasonable to assume, that in most cases this is not much more than the DNA that is experimentally selected for in the complementation experiments, here essentially the *Met2-1* gene. Hybridization analysis with *Met2-1* gene probes, obtained from both partner strains, showed that the para-recombinants contain an identical copy of the *Met2-1* gene of *A. glauca,* in addition to an extra fragment similar to the *Met2-1* gene of *P. parasitica*. Additionally, transformation experiments with the *Met2-1* gene from *A. glauca* revealed that this mutant strain is perfectly complemented by a functional *Met2-1* gene. Hence, the acquired functional *Met2-1* gene of *P. parasitica* is assumed to complement the *A. glauca* genetic defect background in para-recombinant strains (Burmester et al. 2013).

4.2. *A. glauca* RVII324 *met*$^-$ Deficiency

The methionine-auxotrophic *A. glauca* derivative, RVII324 *met*$^-$, is not able to convert homoserine into O-acetyl homoserine. The *Met2* gene codes for homoserine O-acetyltransferase in methionine biosynthesis in *Saccharomyces cerevisiae* and has the same function in filamentous fungi (Baroni et al. 1986, Goyon et al. 1988). According to the information obtained from other zygomycetes, two putative *Met2* genes, *Met2-1* and *Met2-2,* were identified in *A. glauca* too. The sequenced *Met2-2* from *A. glauca* wild type showed is identical with *Met2-2* in the methionine-deficient mutant. Transformation experiments with

the cloned *Met2-2* gene of *A. glauca* wild type couldn't produce prototrophic strains from RVII-324 *met*$^-$. Therefore, the role of the *Met2-2* gene in *A. glauca* remains unclear. In any case, this gene can not fulfill the function of the *Met2-1* gene in transformation experiments. In addition, the isolation of the mutant in the original mutagenesis experiments would not have been possible, if *Met2-2* could acts in the same way as *Met2-1*. We may assume that the *A. glauca Met2-2* gene is not more than a pseudogene that is not functionally expressed at the protein level. Splicing might be impaired at one or more of the introns, or some nucleotide sequence differences between the *Met2-1* and *Met2-2* alleles might lead to a protein with different function or no enzymatic function at all. Alternatively, the *Met2-2* protein, if there is any, might occur in different compartments within the cell. Computational analyses point into this direction: the N-terminal part of the *Met2-2* coding region shows similarity to signal peptides, necessary for mitochondrial import in *A. glauca*, *Phycomyces blakesleeanus*, *M. circinelloides* and *R. oryzae*.

Comparing the sequence data of *Met2-1* gene from wild type and methionine mutant shows a large insertion in this gene. The rearrangement in the *Met2-1* gene could be a consequence of repair processes at DNA breakage point after the X-ray treatment, applied for the initial induction of mutants. X-rays are known to lead to considerable rearrangements via cellular repair mechanisms. In our mutant, one large fragment has been inserted into the *Met2-1* coding region. This rearrangement explains the observed low reversion frequency for this mutant. Furthermore, complementation of the methionine auxotroph by the wild type *Met2-1* gene in transformation experiments shows clearly that no additional mutations affecting the biochemical pathway towards methionine have been accrued. This feature offers the chance to pursue the genetic changes in para-recombinants by analyzing different hybridization patterns in comparison between wild type and mutant strains of *A. glauca*. This is an advantage of *A. glauca* RVII324 *met*$^-$ to be used as the recipient strain whether in para-recombination studies or transformation experiments. In contrast, other type of mutations, point mutations or frameshifts, do not normally give rise to differential hybridization patterns of restriction fragments.

An additional major experimental achievement becomes possible after having access to the combination of the characterized *Met2-1* gene in *A. glauca* and the corresponding auxotrophic mutant. This combination provides the basis for a reliable transformation system, based on complementation of the mutation. Especially in zygomycetes with their known difficulties in artificial gene manipulation, this may be regarded as a considerable advancement (Karimi et al. 2012). To date, only few characterized mutants are available in those model zygomycetes that are amenable to transformation. These complementation-based systems are highly selective and avoid the well-known problems with those based on antibiotic resistance. *Absidia glauca* bears the additional advantages that primary transformation rates for *A. glauca* are higher than for the other mucoralean zygomycetes and that *GFP*-expressing vectors as well as those classical ones, based on dominant selection via a gene conferring neomycin resistance are also available in many different plasmids.

4.3. The Fate of Acquired DNA

Experimental evidence shows that, for the majority of events, *A. glauca* establishes incoming DNA extrachromosomally. The results of transformation experiments with artificial constructs reveal that incoming plasmids are replicated autonomously without integration into the chromosomal DNA (Wöstemeyer et al. 1987, Burmester et al. 1992, Karimi et al. 2012). It is also proven that *A. galuca* harbours many different small plasmids, at least one of which codes for a cell wall-associated protein. The gene for this protein occurs exclusively extrachromosomally and has no additional copy on the chromosomal DNA (Hänfler et al. 1992). Hence, it may be regarded as highly probable that newly acquired genetic elements from the parasite could be propagated by autonomous replicating rather than by integration into chromosomal DNA.

So far, permanent homologous integration in transformation of *A. glauca* has only been reported under special circumstances, mediated by highly repetitive elements where integration into the genomic DNA after transformation with a neomycin resistance gene as selective marker has been observed (Burmester et

al. 1990). This general feature of autonomous replication as major mechanism for establishment of novel DNA holds also true for our new vectors, harbouring the *Met2-1* gene as selective marker.

In para-recombinants we cannot always be sure about the mode of establishment of the incoming *Parasitella* DNA. Nearly always, however, para-recombinants show some mitotic instability, which is more convincingly compatible with extra-chromosomal establishment of foreign DNA than with integrative recombination events.

One reasonable hypothesis is to assume a general defense mechanism of *A. glauca* in confrontation with foreign DNA that starts to eliminate the incoming DNA as far as this elimination doesn't affect fitness, here methionine independency. In transformation experiments, part of the vector was truncated after some generations, but the inserted genes (*Met2-1* and *GFP*) remained. This observation is conclusive for the *Met2-1* gene, because it is selected for during growth on minimal medium, but less convincing for the *GFP* marker, because selection is not to be assumed for this gene. From previous experiments we know that interactions between DNA on a plasmid and the homologous copies in the chromosomes actually occur. Mutations at chromosomal loci for the translational elongation factor, *TEF*1α were observed after transformation of *A. glauca* with a plasmid harbouring part of the *TEF*1a gene (Burmester, 1995). Similar mutational events including deletions could be assumed for acquired *Parasitella* DNA in para-recombinants, based on the hybridization results with *Parasitella*'s *Met2-1* probe. Here, a shorter signal band was observed for para-recombinats (3 kb) in comparison with *P. parasitica* wild type (6 kb) or with respect to the failure to amplify the expected larger fragment of the *Met2-1* gene in PCR experiments using the primer set for *Parasitella,* and also regarding the partial duplication at *A. glauca Met2-2*.

On the other hand, co-hybridization of para-recombinants with *A. glauca Met2-2* and *P. parasitica Met2-1* labeled probes showed the 3 kb band after restriction with *Hind*III. This length is similar to hybridization with only the *P. parasitica met2-1* probe. This can only be explained by assuming a duplication at a small internal

part of the *A. glauca Met2-2* gene, accompanied by the taking up part of the *Met2-1* gene of *P. parasitica* on an extra chromosomal element.

Similar rearrangements and deletions were also observed in other mucoralean fungi. In transformation experiments of *Phycomyces blakesleeanus* with a plasmid containing a geneticin resistance gene, rearrangement and deletion was observed not only in the incoming DNA, but also lead to increased mutation and nuclear as well as cellular death in the fungus (Obraztsova et al. 2004). In *Mucor miehei* transformants, the vector plasmid was extensively modified by deletions occurring in the fungus (Monfort et al. 2003).

In transformation experiments of *Rhizopus oryzae*, the incoming DNA is propagated extrachromosomally and sometimes forms tandem multiple copies of vector in the transformants (Skory 2002, 2004, Michielse et al. 2004).

Artificial integration of introduced DNA is regarded as difficult and at best as rare events in all zygomycetes. For *M. circinelloides*, there are different publications reporting integration of exogenous DNA into the recipient's chromosome (Arnau et al. 1991, Arnau and Strøman 1993, Nyilasi et al. 2005, Papp et al. 2012). Nevertheless, the localization of introduced DNA varies among the transformant derivatives. After transformation of a *leuA* mutant of *M. circinelloides,* defective in leucine biosynthesis, integrative transformations has been achieved by homologous recombination at the *leuA* locus (Arnau et al. 1991, Arnau and Strøman 1993), as well as by gene replacement and ectopic integration (Arnau and Strøman 1993). The situation is of course much more complicated for the analysis of para-recombinants than for transformants. Due to variation in placement of incoming DNA and due to the impossibility to predict length and nature of the incoming DNA, it is only possible to provide data on fate and location of the marker gene that has been selected for following infection, and not on adjacent elemets or on additional DNA fragments that might have been co-introduced.

4.4. Recombination Efficiency and Stability

Complementation of the *A. glauca* methionine auxotroph, either by transformation or via para-recombination always showed non-integrative and thus mitotically unstable events. Nevertheless, the low reversion frequency renders this auxotrophic mutant best candidate for using it in genetic manipulation experiments. Whereas there is no general possibility to follow the establishment pathway of incoming *Parasitella* DNA in para-recombinants, the mitotic instability clearly favours extrachromosomal replication.

In most transformation reports for zygomycetes, the transferred DNA was shown to replicate autonomously (Burmester 1992, 1995, Obraztsova et al. 2004, Appel et al 2004, Skory 2005). It seems that *A. glauca* follows this general rule and doesn't integrate exogenous DNA to its chromosomes (Burmester et al. 1992, Schilde et al. 2001). Different hypotheses have been discussed to explain this phenomenon. Especially in the *A. glauca* genome, nucleotide sequences, capable to increase transformation frequency and maintenance of introduced DNA have been found. A DNA element, which was named *SEG1* (for segregation element) was shown to increase stability of transformants considerably, if inserted into vector plasmids. This fragment was assumed to contain structural elements involved in partitioning and stable segregation of plasmids (Burmester et al. 1992). In *Mucor miehei,* autonomously replicating sequences (ARS) from *Phycomyces blakesleeanus* provided plasmid constructs with significantly higher transformation frequency under selective conditions in comparison to the constructs without these ARS elements (Monfort et al. 2003). These sequences also influence transformation frequency. Nonetheless, the exact location and function of these sequences in the genome is still unknown. Therefore, it seems probable that such ARS element(s), whether from *A. glauca* or *P. parasitica,* helped *Parasitella*'s DNA to be established and subsequently expressed in para-recombinants.

As already denoted, instability of markers acquired by transformation, might be a consequence of a defense mechanism against exogenous DNA (Monfort et al. 2003, Michielse et al. 2004). The nature of the coenocytic mycelia, a general

feature of mucoralean zygomycetes, might influence the instability of transformants by elimination of foreign DNA (Monfort et al. 2003). It is comprehensible that transferred plasmid are eliminated by degradation due to cell defense mechanism as foreign DNA. In nature, these systems are probably directed especially against viruses and transposable elements. Selective conditions, however, favour the stability of transformants. Under natural conditions, selective conditions are certainly less pronounced than in laboratory experiments. Thus, new autonomously replicating DNA, acquired by any mechanism, is expected to be maintained only for a limited number of mitotic divisions.

Defense systems against foreign DNA, accompanied by endogenous repair systems, maintain the genome integrity of the cell. In the case of double strand breaks, the breakage may be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ), two different recombination pathways in eukaryotes. In the NHEJ system, the presence of homologous sites is not essential for repair of broken DNA, whereas HR depends on undamaged homologous sequences that serve as template to repair the double strand breaks (Paques and Haber 1999, Lieber 1999, Lewis and Resnick 2000).

In NHEJ the broken DNA ends, by an ATP-independent pathway, bind to Ku80-Ku70 heterodimers. Subsequently, a complex is formed together with a DNA-dependent protein kinase (DNA-PK) that enables DNA ligase IV together with its cofactor XRCC4 to ligate the broken ends. For *R. oryzae*, it has been hypothesised that integration could arise by homologous or non-homologous recombination via intermolecular end-to-end joining repair (Skory 2002, 2004). Different tandem integration events have been observed that are assumed to be a consequence of a primary double strand break, followed by 5'-to-3' resection, strand invasion annealing, and cleavage of the resulting Holliday junctions (Skory 2002, 2005). According to results achieved by Skory (2004), gene conversion in the target gene could be the reason for complementing a *pyrG*$^{-}$ mutant of *R. oryzae* following transformation with a mutated vector plasmid via these proposed repair events. In his study, the mutated *pyrG* gene on the plasmid and at its chromosomal location led to duplication of the *pyrG* copy only on the plasmid,

one non-functional copy carrying both mutations, from the plasmid as well as from the chromosome, and one functional copy without mutations. This experiment result strongly resembles classical gene conversion events, in this case on the plasmid.

M. circinelloides is probably the best-studied mucoralean funus with respect to specific mechanisms for the detection and elimination of foreign DNA. One of the recognized mechanisms in this fungus is silencing by interfering RNA (siRNA), RNA strands, which are able to regulate the gene function (Nicolas et al. 2003). By silencing the interfering RNA that regulates *carB* gene expression, a gene involved in carotenoid biosynthesis, a high number of transformed strains were obtained by introducing a plasmid carrying *carB* into an albino mutant. These transformants contained high copy numbers of that plasmid (Nicolas et al. 2009). In addition, the silencing effect was helpful to create a knockdown tool for manipulation of the *carRP* gene in *M. circinelloides* (Nicolas et al. 2008).

Overall, the recognized mechanisms, involved in establishment and integration of foreign DNA, are not sufficient to obtain a clear picture for zygomycetes. In contrast to studies with artificial plasmids, the *Parasitella* model, described in this thesis, offers the additional advantage to study consequences of natural gene transfer in these fungi.

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Declaration

I declare in accordance with the conferral of the degree of doctor from the faculty of biology and pharmacy of the Friedrich Schiller University, Jena that the submitted thesis is written only with the assistance and literature cited in the text. This thesis has not been previously submitted either to the Friedrich Schiller University Jena or to any other university.

Jena (March 2014) ----------

(Sedigheh Karimi Dorcheh)