2 *Sordaria macrospora*, a Model System for Fungal Development

Ulrich Kück[1], Stefanie Pöggeler[2], Minou Nowrousian[1], Nicole Nolting[2,*], Ines Engh[1]

CONTENTS

I. Introduction

The application of forward or reverse genetic approaches in model organisms has made great contributions to our fundamental biological knowledge. Associated with these studies is the technical progress in understanding and manipulating the genomes of the relevant organisms. Basic biological studies with model organisms have led, for example, to the identification of signal transduction pathways, transcription control circuits, and cell cycle checkpoints. Moreover, studies using model systems continue to uncover the details of genetic interactions controlling developmental plasticity. The acquired knowledge usually can be applied to organisms of higher order having more complex morphologies that are difficult to manipulate experimentally. A further advantage of model systems compared to complex highly developed organisms (e.g. humans) is their potential for a rapid and inexpensive genetic analysis.

In this review, we focus on the filamentous fungus *Sordaria macrospora*, which for long has been used as a model for conventional genetic analysis, fruiting body development, and the analysis of meiosis (Esser and Straub 1958; Heslot 1958; Zickler 1977; van Heemst et al. 1999; Pöggeler et al. 2006a). Since the first combination of classical tetrad analysis and modern molecular genetics, this fungus has become an important eukaryotic model system to study developmental biology.

While this review was in preparation, the genome of *S. macrospora* was sequenced, using next generation sequencing techniques. De novo assembly of Illumina/Solexa paired end reads and subsequent comparative assembly with genome data from *Neurospora* species yielded a first draft version with about 10 000 predicted genes. This genome sequence will greatly enhance the value of *S. macrospora* as a eukaryotic model system (Nowrousian M, Kamerewerd J, Engh I, Pöggeler S, Stajich J, Read N, Kück U, Freitag M, personal communication).

A. Fungal Organisms as Model Systems for Developmental Biology

So far about 74 000 fungal species have been identified and classified, but even conservative estimates suspect that about 1.5×10^6 species exist in nature

[1]Lehrstuhl für Allgemeine und Molekulare Botanik, Fakultät für Biologie, Ruhr-Universität Bochum, Universitätsstrasse 150, 44780 Bochum Germany; e-mail: ulrich.kueck@ruhr-uni-bochum.de
[2]Abteilung Genetik eukaryotischer Mikroorganismen, Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen, Grisebachstrasse 8, 37077 Göttingen, Germany; e-mail: spoegge@gwdg.de
[*]Present address: Heinrich Petk Institute for Experimental Virology and Immunology at the University of Hambury, Germany

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(Hawksworth 2001). Fungi are true eukaryotes, with a nucleus and (in most cases) mitochondria. Exceptions are those species having mitosomes or mitochondria-related hydrogenosomes instead of mitochondria. However, recent evolutionary studies indicate that both mitosomes and hydrogenosomes might be derived from mitochondrial ancestors (Embley and Martin 2006). Fungi can be either uni- or multicellular. Irrespective of their taxonomy, unicellular fungi are named yeasts, while the terms filamentous or mycelial fungi indicate multicelluar fungi with complex, multicellular propagation structures. Depending on their environmental growth conditions, some fungi are able to undergo a transition from uni- to multicellular growth and vice versa. This dimorphic development is a characteristic of certain species that are considered to be either yeasts or filamentous fungi.

Baker's yeast, *Saccharomyces cerevisiae*, is the most prominent fungal model system and has contributed enormously to the basic understanding for example of eukaryotic signalling pathways or the biogenesis of eukaryotic organelles (e.g. mitochondria, peroxisomes, endoplasmic reticulum). Thus, yeast is the main model system for eukaryotic cell biology (Botstein and Fink 1988).

However, multicellular models are essential since many genes play different roles in defined cell types at different developmental stages. These complex cell and tissue interactions cannot be mimicked in unicellular organisms like yeasts. Therefore other model organisms, such as filamentous fungi, have proven their value in specialized research areas.

Filamentous fungi are characterized by the extreme polar growth of their hyphae, which is reminiscent of the growth behaviour in neurons or plant pollen tubes. This polarized growth in fungi is characterized by vesicle transport towards the apex. In contrast to yeasts, filamentous fungi can undergo major morphogenetic changes that are accompanied by a switch from polarized to isotropic growth (Harris and Momany 2004; Fischer et al. 2008). These changes in cellular development occur regularly during asexual or sexual propagation.

During asexual propagation, the typical formation of conidiospores is observed (e.g. *Aspergillus* spp., *Penicillium* spp.). Another, often overlooked, asexual differentiation process is the fragmentation of hyphae into arthrospores. Both these processes are dependent on environmental conditions, such as light.

Similarly, sexual propagation structures occur in true filamentous fungi due to environmental changes such as nutrient starvation. In almost all filamentous fungi, fruiting body formation is the major developmental step during the sexual reproduction cycle. Fruiting bodies vary in size between 200 μm and several centimetres as found for example in the morel. Their major function is the protection and further distribution of the sexual spores that are generated after the meiotic divisions that follow karyogamy (Pöggeler et al. 2006a).

B. Why Choose *Sordaria macrospora*?

Sordaria macrospora is a coprophilic homothallic pyrenomycete first described in 1866 (Auerswald 1866), which is closely related to *Podospora anserina* and *Neurospora crassa*. In contrast to these heterothallic species, single strains of *S. macrospora* produce fruiting bodies (perithecia) without the presence of a mating partner. As a consequence of self-fertility, fruiting body development is an apandrous process, allowing the direct testing of recessive mutations that lead to defects in fruiting body formation. The life cycle of *S. macrospora*, as depicted in Fig. 2.1, can be completed in the laboratory within seven days. During vegetative growth, chemical and physical stimuli such as biotin or light induce branching of hyphal tips, which is followed by adhesion of several hyphae to each other. This entry into the sexual phase results in the formation of a network of interconnecting hyphae, leading to the first of two consecutive morphological stages: first, the development of primordia (protoperithecia), and second, the transition of protoperithecia into mature fruiting bodies, called perithecia. In the homothallic fungus *S. macrospora*, transition between these two stages takes 72 h. In heterothallic fungi such as *N. crassa*, this transition requires crossing between two strains of opposite mating types.

Ascus development, as detailed in the next section, starts with the formation of female gametangia called ascogonia. The ascogenous cells are enveloped by sterile hyphae to form fruiting body precursors. Subsequent tissue differentiation gives rise to an outer pigmented peridial tissue and, following karyogamy, inner ascus initials embedded in sterile paraphyses are formed. Mature fruiting bodies from *S. macrospora* harbor 50–300 asci, which after meiosis and postmeiotic

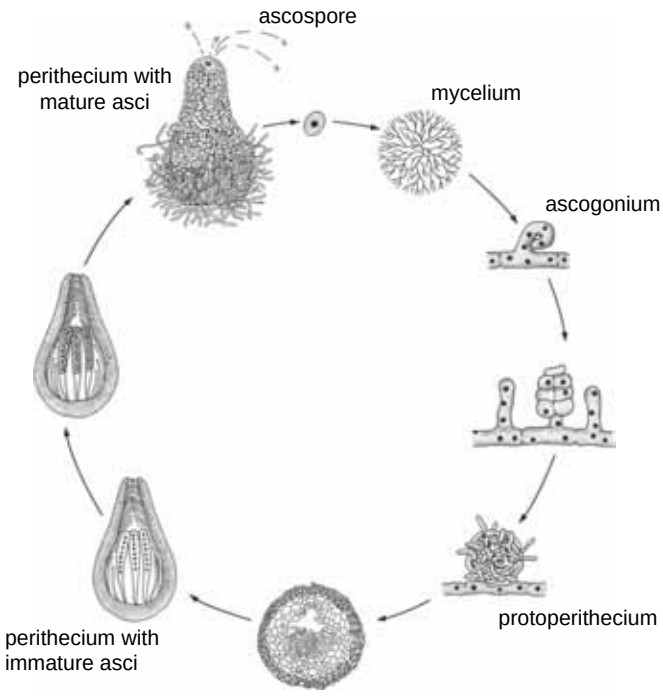

Fig. 2.1. Life cycle of *Sordaria macrospora*: The cycle starts with a germinating ascospore and is completed in the laboratory within seven days by the formation of mature fruiting bodies (perithecia) that discharge the mature and melanized ascospores

divisions contain eight ascospores each. Mature black ascospores are discharged through an apical pore (ostiole) at the neck of the fruiting body. In conclusion, fruiting body development requires the differentiation of the mycelia into several specialized tissues; and regulation of these morphological and physiological changes requires a number of different genes (Esser and Straub 1958).

There is another developmental feature that makes *S. macrospora* a favourable object for fruiting body development. *S. macrospora* produces only meiotically derived ascospores, whereas asexual spores, such as conidia, are absent. Thus, there is no interference between two different developmental programmes, which makes it easier, for example, to analyse differentially expressed genes involved in ascocarp development.

II. Biology

In order to manipulate the development of a fungal model system, detailed knowledge of its life cycle is an essential prerequisite for any experimental study. The sexual system of fungi can be either heterothallic (self-incompatible), pseudo-homothallic (secondary homothallic, pseudo-compatible), or homothallic (compatible) (Lin and Heitman 2007).

A. Life Cycle

Similar to heterothallic filamentous ascomycetes, the homothallic *S. macrospora* has a dikaryophase in its life cycle. During this phase, pairs of nuclei synchronously and repeatedly divide inside the ascogonium, thereby producing a great number of nuclei. The nuclei then migrate in pairs to the developing dikaryotic ascogenous hyphae emerging from the ascogonium. The ascogenous hyphal tips develop a U-shaped crozier containing two nuclei. These nuclei divide mitotically in synchrony and, subsequently, two septa appear which divide the hook cell into three sections: a lateral and a basal cell with one nucleus each, and an apical cell with two nuclei. The two nuclei in the apical cell then fuse. Immediately after karyogamy within the young ascus, the diploid nucleus undergoes meiosis, which is followed by a post-meiotic mitosis, resulting in the formation of eight nuclei. Each of the eight nuclei is incorporated into its own ascospore (Esser and Straub 1958; Esser 1982). The self-fertilization mode of *S. macrospora* is termed autogamous fertilization. This means

that a pair-wise fusion of nuclei present within the ascus initial occurs, without cell fusion having taken place before (Esser and Straub 1958).

B. Homothallism

In homothallic species, like *S. macrospora*, a mycelium derived from a uninucleate ascospore is self-fertile and able to perform all steps of meiosis. In contrast, heterothallic species, like *N. crassa*, require another compatible individual for sexual reproduction. The mating partners of heterothallic fungi are morphologically identical and are only distinguished by their mating type (*MAT*) loci. In *N. crassa*, these are termed *MAT1-1* (*matA*) and *MAT1-2* (*mata*). The alternative versions of the mating-type locus on homologous chromosomes of the mating partners are called idiomorphs because they are completely dissimilar in the genes they carry (Metzenberg and Glass 1990). The *MAT1-1* idiomorph of *N. crassa* contains three genes: *MAT1-1-1* (*mat A-1*), *MAT1-1-2* (*mat A-2*), and *MAT1-1-3* (*mat A-3*; Glass et al. 1990a; Ferreira et al. 1996, 1998; Fig. 2.2). The MAT1-1 polypeptide has a DNA-binding motif that shows similarity to the *S. cerevisiae* MATα1p mating-type protein. *MAT1-1-2* encodes a protein without a characteristic DNA-binding motif, but with a conserved region encompassing 25 amino acids with three invariant *h*istidine, *p*roline, and *g*lycine residues (the HPG domain; Debuchy and Turgeon 2006). The *MAT1-1-3* gene encodes a protein with a *h*igh *m*obility *g*roup (HMG) DNA-binding motif (Ferreira et al. 1996). The *N. crassa MAT1-2* idiomorph contains two genes, *MAT1-2-1* (*mat a-1*) and MAT1-2-3 (*mat a-2*). While the *MAT1-2-1* gene encodes a HMG-domain protein that is the major regulator of mating in *MAT1-2* strains, the function of the *MAT1-2-2* encoded protein is unknown (Staben and Yanofsky 1990; Pöggeler and Kück 2000).

Pseudo-homothallic members of the Sordariaceae (e.g. *Neurospora tetrasperma*) or the Lasiosphaeriaceae (e.g. *P. anserina*) develop four-spored asci in which most ascospores carry two nuclei, one of each mating type. Another form of pseudo-homothallism is accomplished by mating-type switching, but this has not been identified in members of the Sordariales (Lin and Heitman 2007).

There are three ways fungi can be homothallic: (1) they can harbour a fused *MAT* locus of both idiomorphs, (2) they can harbour both *MAT* alleles at different loci in the genome, and (3) they can sexually reproduce but carry only one *MAT* locus (Lin and Heitman 2007). In Sordariales all three forms of homothallism can be found in the same order.

S. macrospora as well as *Neurospora pannonica* and *Neurospora terricola* belong to the first group and contain sequences similar to both the *MAT1-1* and the *MAT1-2* idiomorphs. In the homothallic *Chaetomium globosum* in addition to a *MAT1-1* locus, a *MAT1-2* locus was identified at another genomic locus (Glass et al. 1990a; Pöggeler et al. 1997a; Debuchy and Turgeon 2006). However, according to data from Southern hybridization, the homothallic Neurospora species *N. africana*, *N. dodgei*, *N. galapagonensis*, and *N. lineolata* possess only the *MAT1-1* locus (Glass et al. 1990a; Pöggeler 1999). The fused mating-type-loci of *S. macrospora, N. pannonica*, and *N. terricola* appear to be derived from a heterothallic ancestor. The molecular mechanisms responsible for the fusion of *MAT* regions are

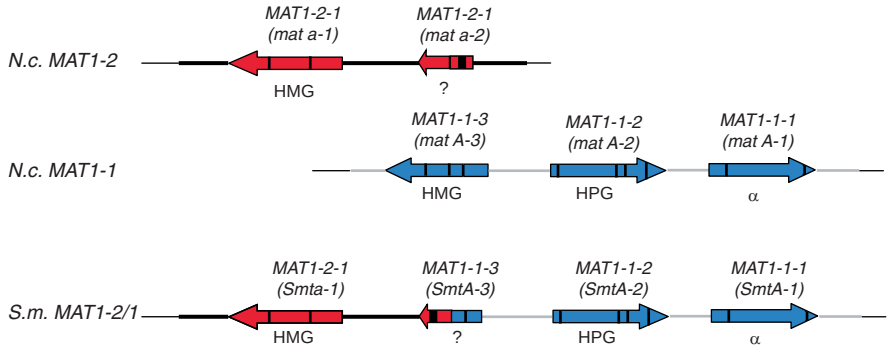

Fig. 2.2. Comparative maps of mating type loci from the two *N. crassa* (*N. c.*) mating type idiomorphs and from the *S. macrospora* (*S. m.*) mating type locus. For further details of designations see text

so far unknown, but it is likely that a recombination event led to the close linkage of *MAT1-1*- and *MAT1-2*-specific sequences on a single chromosome.

Cloning and sequencing of the entire mating-type locus of *S. macrospora* revealed that it encodes four different ORFs: *MAT1-2-1* (*Smt a-1*), *MAT1-1-3* (*Smt A-3*), *MAT1-1-2* (*Smt A-2*), and *MAT1-1-1* (*SmtA-1*). Except for *MAT1-1-3*, proteins encoded by the *S. macrospora* mating type genes show strong sequence similarities to the corresponding *N. crassa* mating-type ORFs. The *S. macrospora MAT1-1-3* gene has a chimeric character and exhibits sequence similarity to the *MAT1-1-3* and the *MAT1-2-2* ORF of *N. crassa*. In contrast to the *N. crassa* MAT1-1-3, the *S. macrospora* MAT1-1-3 protein lacks an HMG domain (Fig. 2.2).

Sequence analysis of the cDNA from the *S. macrospora MAT1-2-1*, *MAT1-1-3*, *MAT1-1-2*, and *MAT1-1-1* revealed that introns in each gene are spliced, indicating that all of the mating-type genes identified in the mating-type locus of *S. macrospora* are transcriptionally active (Pöggeler et al. 1997b). Surprisingly, RT-PCR experiments demonstrated co-transcription of the *MAT1-1-3* gene and *MAT1-2-1* as well as optional splicing of two introns within the *MAT1-1-2* gene (Pöggeler and Kück 2000).

To investigate the functional conservation of mating-type genes of *S. macrospora*, cosmid clones containing the entire mating-type locus from *S. macrospora* were transformed into both *P. anserina MAT1-1* and *MAT1-2* strains. Similar to *N. crassa MAT1-1* strains, the *P. anserina MAT1-1* contains three genes (*FMR1* = *MAT1-1-1*, *FMR2* = *MAT1-1-2*, *SMR3* = *Mat1-1-3*), while the *P. anserina MAT1-2* locus contains only a single gene (*FPR1* = *MAT1-2-1*) encoding an HMG-domain protein (Debuchy and Coppin 1992; Debuchy et al. 1993). After introduction of the *S. macrospora MAT* locus into the homokaryotic strains of *P. anserina*, transformants were capable of forming fruiting bodies without crossing with a mating partner of the opposite mating type. However, instead of asci with four ascospores, the perithecia of the transformants contain only a gelatinous mass and have no structures such as hook cells, croziers, asci or spores (Pöggeler et al. 1997b). Interestingly, transformation of the entire *S. macrospora MAT* locus into a *P. anserina* Δ*MAT* strain without an endogenous *MAT* locus led to fertile perithecia containing rosettes of asci (Pöggeler et al. 1997b). Thus, the mating-type genes from *S. macrospora* confer self-fertility to the heterothallic *P. anserina* when they do not interfere with a resident mating-type locus. This result suggests that the *S. macrospora* mating-type genes are functional and most probably are involved in fruiting body development and ascosporogenesis.

Due to the presence of *MAT1-1*-type homothallic species in the genus *Neurospora* (Glass et al. 1990b; Metzenberg and Glass 1990) and due to the fact that *MAT1-1* strains of the heterothallic *S. brevicollis* were shown to produce apandrous fruiting bodies with ascospores under certain culture conditions (Robertson et al. 1998), it was unclear whether *MAT1-2* specific genes are essential for sexual reproduction in homothallic members of the family Sordariaceae. Deletion of *MAT1-2-1* converts the self-fertile *S. macrospora* to a self-sterile fungus, no longer able to produce fruiting bodies and ascospores. Thus, the Δ*MAT1-2-1* mutant phenotypically resembles the *S. macrospora pro* mutants (see Sect. IV) which also do not produce any perithecia. However, no irregularities in vegetative growth and mycelial morphology were observed when the mutant strain was compared to *S. macrospora* wild type This finding demonstrates that, at least in the homothallic *S. macrospora*, the *MAT1-2-1* gene is required for fruiting-body development and sexual reproduction (Pöggeler et al. 2006b).

Cross-species microarray technology (see Sect. V.D) showed that the *S. macrospora* MAT1-2-1 affects the expression of at least 107 genes, including a common set of ten putative developmental genes deregulated in sterile *pro* mutants. At least 74 and 33 genes are two-fold up- or down-regulated in the *S. macrospora* Δ*MAT1-2-1* mutant strain. Of these 107 genes, 80 have homologues with known or putative function and were sorted into ten putative functional categories (Pöggeler et al. 2006b).

Transcription of cell type-specific genes in the ascomycetous yeast *Saccharomyces cerevisiae* has been shown to rely on the interaction of mating-type proteins (Herskowitz 1989). A two-hybrid approach in conjunction with protein cross-linking analysis demonstrated that in *Sordaria macrospora* the MAT1-1-1 and MAT1-2-1 proteins, homologous to polypeptides encoded by opposite mating partners of the heterothallic *N. crassa*, are able to form a heterodimer (Jacobsen et al. 2002).

III. Phylogeny

S. macrospora belongs to the family Sordariaceae (Sordariales, Ascomycetes) which comprises taxa characterized by dark, ostiolate fruiting bodies, and unitunicate, cylindrical asci (Kirk et al. 2001). The Sordariaceae family presently contains 7–10 genera (Kirk et al. 2001, Eriksson et al. 2004) and is represented by well known genera such as Gelasinospora, Neurospora, and Sordaria. It is also morphologically related to the Lasiosphaeriaceae, another family within the Sordariales, which contains the important genus *Podospora* (Huhndorf et al. 2004). Phylogenetic studies have revealed that species of the genus *Sordaria* are closely related to the genus *Neurospora* (Pöggeler 1999; Cai et al. 2006) which includes the fungal model organism *N. crassa* (Davis and Perkins 2002; Galagan et al. 2003; Borkovich et al. 2004; Fig. 2.3).

Phylogenetic trees based on sequences of the conserved glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) and on gene fragments of *MAT1-1-1* and *MAT1-2-1* have shown that *Neurospora* and *Sordaria* are monophyletic units (Pöggeler 1999). Moreover, there is a strict separation of heterothallic and homothallic species within both genera and a separation of homothallic strains with fused mating-type loci and *MAT1-1*-type homothallic species within *Neurospora*. The phylogenetic analyses suggest that changes in the reproductive strategy may represent a single event in each genus (Pöggeler 1999).

The *MAT* genes of members of the Sordariaceae appear to evolve more quickly than other regions of the genome; a phenomenon also observed in other ascomycetes (Turgeon 1998; Pöggeler 1999; Voigt et al. 2005). Interestingly, analysis of non-synonymous and synonymous substitution rates within *MAT* genes of the genus *Neurospora* revealed that the rapid divergence of *MAT* genes is due to an adaptive evolution within heterothallic members of this genus, whereas it is due to the lack of selective constraints within homothallic members of *Neurospora* (Wik et al. 2008).

IV. Mutants and Morphology

A major step in the molecular genetic analysis of components controlling cellular development is the functional analysis of mutants having a defect or block in defined morphogenetic steps. Such morphological mutants can be generated either by conventional mutagenesis or by the application of homologous recombination procedures (see Sect. V.B). The detailed molecular and cellular analysis of mutants will decipher molecular determinants involved in cellular differentiation processes. Using a conventional genetic approach, more than hundred developmental mutants with defects in fruiting body formation were generated. Mutants were obtained after UV mutagenesis of a protoplast suspension derived from the wild-type strain. Protoplasts were exposed to UV light (254 nm) for 15 min, with a survival rate of 0.1%. The protoplasts were regenerated on complete medium, supplemented with 10.8% sucrose (Masloff et al. 1999). After 24 h, individual clones were transferred to corn-meal medium to investigate clones with phenotypic variations in fruiting body development. In total, more than 23 000 clones were screened in this way. Presumptive developmental mutants were further tested for mitotic stability, before single-spore isolates were generated for a detailed molecular analysis. The mutants were grouped into four different phenotypic classes (see Fig. 2.4A):

1. Asc mutants. These mutants show a very early developmental block in the life cycle. They form ascogonia that sometimes have a non-wild-type structure. In more than 100 developmental mutants, we detected only five having this phenotype. They are sterile and never form protoperithecia.
2. Pro-mutants (Fig. 2.4B). Some 45 mutants have been isolated and they show a developmental block just after protoperithecia formation. Therefore, all pro-mutants are sterile. Protoperithecia have a round-shaped structure and a diameter of 30–55 m. The average size can vary in the different mutants but has been found to be constant in a defined mutant when grown on corn meal agar. As shown in Sect. VI. E, these mutants have contributed greatly to our current understanding of the molecular genetic network controlling fruiting body development. The first mutant used for a complementation analysis was pro1, where the *pro1* gene encodes a development-specific GAL4-like C_2H_6 transcription factor (Masloff et al. 1999).
3. Per-mutants. The 44 mutants of this type are characterized by the presence of a more or less

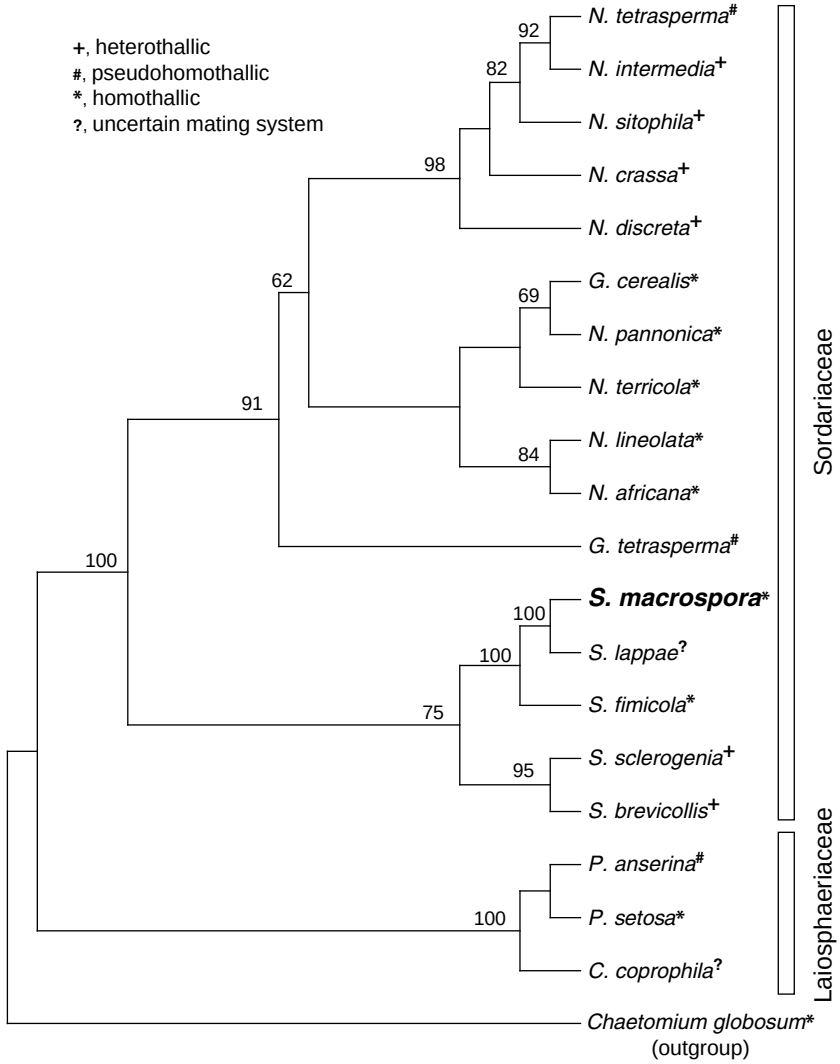

Fig. 2.3. Phylogenetic tree of partial *gpd* sequences generated by neighbor-joining analysis. 436 nucleotide positions were included in the analysis. The tree shown is based on a consensus tree calculated with the Neighbor program (Phylip). The *numbers* (percentages) indicate the bootstrap support based on 1000 replications. Abbreviations and accession numbers are as follows: *N. tetrasperma* (*Neurospora tetrasperma,* AJ133018.1); *N. intermedia* (*Neurospora intermedia*, AJ133019.1); *N. sitophila* (*Neurospora sitophila*, AJ133020.1); *N. crassa* (*Neurospora crassa*, U67457.1); *N. discreta* (*Neurospora discreta,* AJ133021.1); *G. cerealis* (*Gelasinospora cerealis*, AF388934.1); *G. tetrasperma* (*Gelasinospora tetrasperma*, AF388935.1); *N. pannonica-*. (*Neurospora pannonica*, AJ133016.1); *N. terricola* (*Neurospora terricola*, AJ133017.1); *N. lineolata* (*Neurospora lineolata*, AJ133015.1); *N. africana* (*Neurospora africana*, AJ133012.1); *S. macrospora* (*Sordaria macrospora*, AJ133007.1); *S. lappae* (*Sordaria lappae*, EF197111.1); *S. fimicola* (*Sordaria fimicola*, AJ133009.1); *S. sclerogenia* (*Sordaria sclerogenia*, AJ133011.1); *S. brevicollis* (*Sordaria brevicollis*, AJ133010.1); *P. anserina* (*Podospora anserina,* EF197096.1); *P. setosa* (*Podospora setosa,* EF197110.1); *C. coprophila* (*Cercophora coprophila*, EF197091.1). *Chaetomium globosum-* (*CHGG_07980.1*; http://www.broad.mit.edu/annotation/genome/chaetomium_globosum) was used as the outgroup

developed perithecium, but otherwise they are sterile. This is due to the fact that they are unable to generate mature ascospores, although in some cases, they are able to form immature asci. A good example is mutant per5 (see Sect. VI.E), which was one of two mutants that provided the first example for the applicability of the *S. macrospora* system for a molecular genetic analysis (Nowrousian et al. 1999).

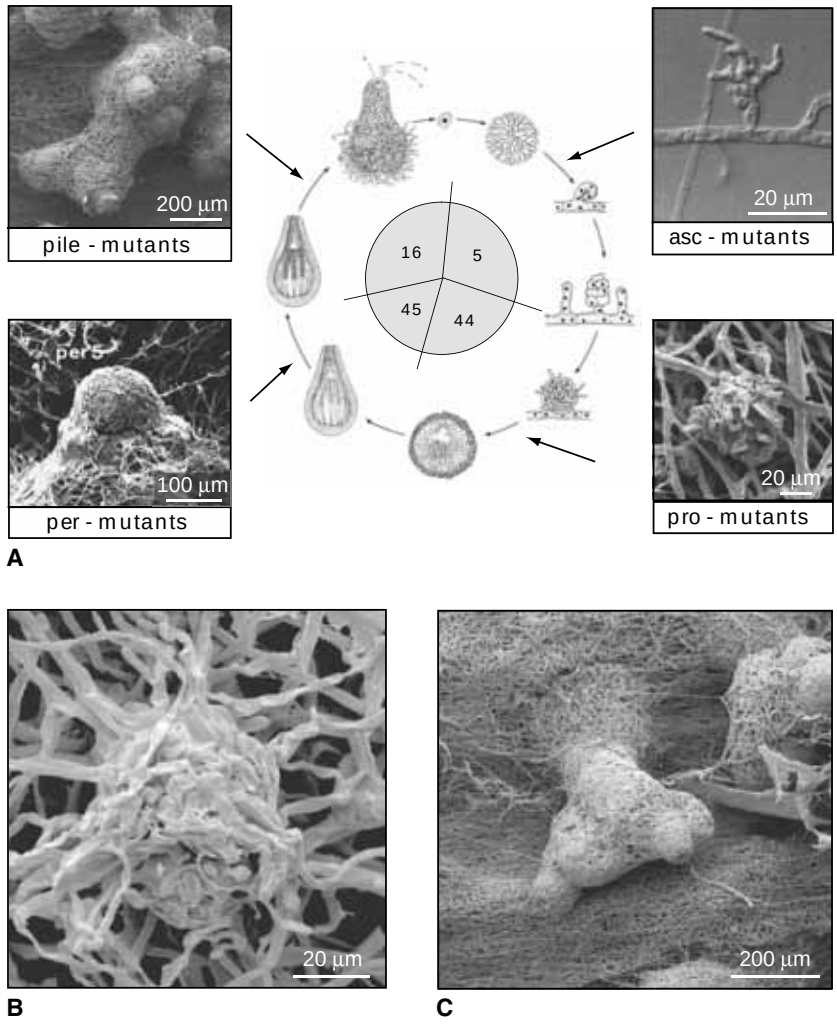

Fig. 2.4. Phenotypes of developmental mutants from *Sordaria macrospora*. **A** Life cycle and number of mutants that can be grouped in four different morphological classes having distinct developmental blocks. **B**, **C** Scanning electron micrographs of a pro (**B**) and pile mutant (**C**)

4. Pile mutants (Fig. 2.4C). These mutants show a rather complex phenotype. The spatial order of perithecium formation is disturbed. One perithecium can sit on top of another, thus forming a pile of many perithecia. Initially, we obtained 16 isolates of this mutant type which seem to be genetically instable. Pile mutants are often fertile, but show a defect in the formation of the pigment melanin (Engh et al. 2007a).

As described in the sections to come, different developmental mutants were used for the further molecular investigation of the developmental process. Restoration of a fertile phenotype in these sterile mutants allowed isolation of a wild-type gene copy, which is defective in the recipient strains.

V. Molecular and Genetic Tools

Development of an organism as a genetic model system is greatly dependent on the application of modern molecular genetic techniques to a hitherto only conventionally manipulated genetic system. In *S. macrospora* the use of molecular genetics started with DNA-mediated transformations and recently applied techniques such as microarray hybridization and novel cellular markers for fluorescent

microscopic investigations (Nowrousian et al. 2005; Engh et al. 2007b; Nowrousian et al. 2007a).

A. Tetrad Analysis

Tetrad analysis investigates the four products that are generated from a diploid cell during two meiotic divisions. In fungi, meiosis occurs during sexual spore formation, resulting in each of the spores in the meiosporangium (the ascus in the case of ascomycetes) resembling one product of meiosis. In ascomycetes ordered tetrads as can be found in *S. macrospora* can be distinguished from non-ordered tetrads present in yeast. If alleles are segregated during the first meiotic division, this leads to ascus halves in ordered tetrads with spores of the same genotype. However, cross-over may occur during prophase of the first meiotic division between the centromere and the gene of interest. Different alleles are then present on sister chromatids and do not segregate until the second meiotic division. This leads to ascus halves with spores of different genotypes. For further analysis, spores can be isolated and germinated on agar medium.

S. macrospora is an excellent organism for tetrad analysis for several reasons (Esser and Straub 1958; Esser 1982), some of which are outlined here.

The tetrad is ordered and the ascospores are linearly arranged. A post-meiotic mitosis leads to eight instead of four spores. Sister products remain adjacent to one another; thus, the position of each ascospore reflects the preceding nuclear division.

The ascospores of *S. macrospora* are about 28 µm long, allowing for easy manual isolation.

Several spore colour mutants exist that can be used in crosses. This is important, because *S. macrospora* is self-fertile and one cannot distinguish selfed and non-selfed fruiting bodies. However, when using a strain with a spore colour defect in sexual crosses, recombinant perithecia are identified by the segregation of spore colour alleles within the asci (Fig. 2.5).

Experiments have shown that not only spore colour alleles but also heterologous marker genes like *egfp* segregate in a Mendelian manner (Pöggeler et al. 2003).

S. macrospora has a very short sexual cycle that can be completed within seven days under laboratory conditions.

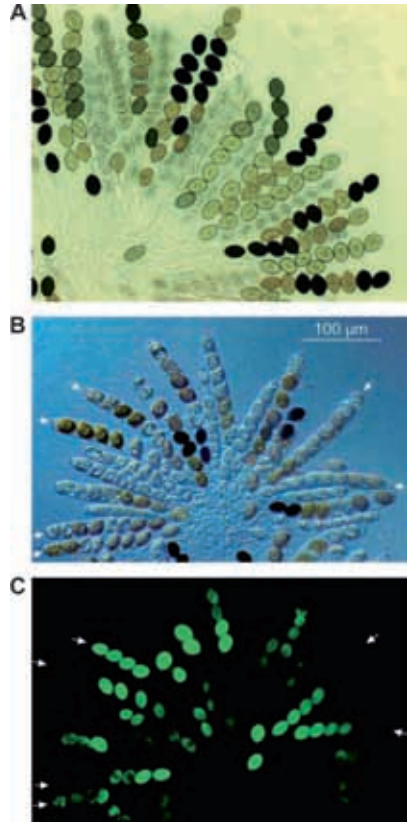

Fig. 2.5. Ascus rosettes from a cross between two different *S. macrospora* strains. **A** Tetrad analysis from a cross between a wild-type strain having black ascospores and a mutant strain with yellow ascospores. Light (**B**) and fluorescence (**C**) micrographs from ascus rosettes that were derived from a cross between a strain carrying two different ascospore colour mutations and a transformant carrying the gene for the green fluorescence protein (*gfp*). *Arrows* indicate asci in which the alleles for the ascospore colours or green fluorescent protein were separated by first and second division segregation, respectively

What is the advantage of a tetrad analysis? Traditionally, it is used to examine gene distribution and gene–centromere distances by analysing the frequency of second division segregation. This was made mostly obsolete by genome sequencing. However, tetrad analysis is still a powerful tool for genetic analysis. For example, recently a Δ*ku70* mutant was generated that facilitates the generation of knockout strains (Pöggeler and Kück 2006), which still carry the *ku70* deletion (see next chapter). In order to obtain a knockout strain in the wild-type background, crosses against a spore colour mutant are performed for further tetrad analysis. This is a fast and easy method leading to homokaryotic mutant strains

(Pöggeler and Kück 2006). Another important advantage of a tetrad analysis is the possibility to generate double mutants by crossing the two mutants carrying distinct single mutations. The double mutant can be isolated even if: (1) both single mutants were generated using the same selection marker, or (2) the double mutant has the same phenotype as one of the single mutants. This is feasible, because segregation into parental and recombinant spores is taken into account and the ordered tetrad allows for the identification of both. Using this method, numerous *S. macrospora* double mutants have been generated (Mayrhofer et al. 2006; Kamerewerd et al. 2008). As a consequence, conventional genetic analysis strongly increases the potential of *S. macrospora* as a model organism for studying diverse biological phenomena.

B. DNA-Mediated Transformations and Gene Libraries

A variety of options recently became available to introduce and control genetic elements, as well as to help the construction of different molecular libraries, displaying the importance of *S. macrospora* as model organism for basic research (Kück and Pöggeler 2005).

At the beginning, the development of a reproducible DNA-mediated transformation protocol was of vital importance. Transformation of *S. macrospora* was first reported by Le Chevanton and co-workers. They used an auxotrophic *ura5* strain as the recipient. Appropriate selection was performed by complementation of the uracil auxotrophy, by transforming with a homologous *ura5* gene (Le Chevanton et al. 1989).

Selection of *S. macrospora* transformants with a dominant marker became feasible using the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) under control of fungal promoter and terminator elements (Walz and Kück 1995). The advantage of such a dominant selectable marker is that basically any recipient strain can be used in transformation experiments. Expression of the *hph* gene was initially directed by the upstream region of the isopenicillin N synthetase gene (*pcbC*) from the cephalosporin C producing fungus *Acremonium chrysogenum*. Recently, the strong constitutive promoters of the *A. nidulans gpd* and *trpC* genes were shown to be useful for driving *hph* expression (Walz and Kück 1995; Pöggeler et al. 2003), with transformation frequencies of about 5–15 transformants per 10 μg of DNA (Walz and Kück 1995).

In order to generate an alternative dominant marker system that does not exhibit cross-resistance to hygromycin B, the *nat1* gene conferring resistance to nourseothricin was used to transform *S. macrospora* (Hoff and Kück 2006). The *nat1* gene product is the nourseothricin acetyltransferase from *Streptomyces noursei* (Krügel et al. 1993) and was expressed under the control of the *A. nidulans trpC* promoter. Transformation frequencies of 10–40 transformants per 10 μg of plasmid DNA were obtained with this dominant selection system (Hoff and Kück 2006).

Alternative selection systems were developed based on novel auxotrophic recipient strains. Using conventional mutagenesis, a leucine auxotrophic strain was generated, which lacks the complete wild-type copy of the *leu1* gene, encoding β-isopropylmalate dehydrogenase (Kück 2005). A uracil auxotrophic mutant strain was constructed by isolating the *ura3* (syn. *pyr4*) gene, encoding orotidine-5′-phosphate decarboxylase from the *S. macrospora* genomic DNA (Nowrousian and Kück 1998) and subsequently disrupting the *ura3* open reading frame in vitro by insertion of a *hph* resistance cassette. The resulting recombinant plasmid was used in *S. macrospora* transformations to isolate a fungal derivative, in which the wild-type *ura3* copy was substituted by the chimeric *ura3-hph* gene through site specific homologous recombination (Kück and Pöggeler 2004). The *leu1* and *ura3* strains can be transformed with the corresponding wild-type genes from *S. macrospora*; and the transformation frequency is comparable to that described for the *hph* gene.

Transformed DNA was shown to be ectopically integrated into the genomic DNA of *S. macrospora*. The copy number varied between transformants. Interestingly, the analysis of *S. macrospora* transformants by pulse field gelelectrophoresis revealed that all vector molecules usually integrate into the same chromosome (Walz and Kück 1995).

Targeted gene replacement via homologous recombination is a routinely used approach to elucidate the function of unknown genes. Integration of exogenous DNA in the genomic DNA requires the action of double-strand repair mechanisms. However, similar to most filamentous fungi, plants, and animals, transformed DNA is

predominately ectopically integrated into the genome of *S. macrospora*.

Most probably this occurs by non-homologous end joining (NHEJ), a mechanism that involves the binding of the Ku heterodimer (Ku70/Ku80) at the ends of a DNA double-strand break (DSB; Pastwa and Blasiak 2003).

Hence, the low rate of homologous recombination exhibited by the *S. macrospora* wild-type has hindered highly efficient gene knockout protocols.

In *N. crassa*, Ninomiya and co-workers demonstrated that deletion of the genes *ku70* and *ku80* results in a dramatic increase in the frequency of homologous recombination resulting in 100% of transformants exhibiting integration only at homologous sites (Ninomiya et al. 2004). This strategy for targeted gene integration was extended to *S. macrospora* and a ku70 deletion mutant was generated by replacing the *ku70* gene with a *nat1* resistance cassette. No phenotypic defects regarding vegetative or sexual development were observed in the ku70 strain, making it an attractive recipient strain for transformation and a valuable tool for gene disruption of developmental genes. Compared to the wild type, which shows homologous integration efficiency between 0.1% and 5.0%, a Δ*ku70* strain showed up to 100% of transformants with homologous integration of exogenous DNA (Pöggeler and Kück 2006). As described in the previous section, the Δ*ku70*::nat1 mutation can easily be eliminated from a disruption strain by crossing to a spore color mutant with an otherwise wild-type genetic background. Tetrad analyses allow the isolation of ascospore isolates that lack the Δ*ku70* mutation, but carry the substituted gene copy.

Based on procedures described for *N. crassa* (Colot et al. 2006), the *S. macrospora* transformation procedure was expanded by substituting conventional cloning with recombinational cloning in yeast (Oldenburg et al. 1997; Raymond et al. 1999). Using this method, traditional restriction digestion and ligation for the generation of knockout fragments are unnecessary and components of the final construct are synthesized individually by PCR. Amplified fragments contain short overlapping sequences and are cotransformed into yeast for assembly by the recombination machinery. This "two-step" transformation procedure including homologous recombination in yeast and transformation of the knockout fragment into *S. macrospora* Δ*ku70* strain presents a valuable tool for gene disruption and a crucial improvement for the molecular research on *S. macrospora* (Mayrhofer et al. 2006; Nolting and Pöggeler 2006a).

Genetic transformation protocols contribute greatly to the molecular dissection of the *S. macrospora* sexual development. Another important development for genetic engineering of *S. macrospora* comes from the construction of various gene libraries of *S. macrospora* DNA. An indexed cosmid library and a cDNA library, which is suitable for yeast two-hybrid screens, were used to identify important components involved in sexual reproduction and fruiting-body development (Pöggeler et al. 1997a; Mayrhofer and Pöggeler 2005). Clones of indexed cosmid libraries are kept individually in microtitre dishes to ensure equal representation of each clone. Several methods for the rapid screening of pooled cosmid DNA have been published, such as isolation of conserved fungal genes by means of colony filter hybridization of cosmid clones or rapid screening by means of PCR using pooled cosmid DNA (Pöggeler et al. 1997a). In other approaches, this library was used to clone novel developmental genes by restoring the fertile wild-type phenotypes in otherwise sterile mutants of *S. macrospora* (see Sects. IV, VI.E; Masloff et al. 1999; Nowrousian et al. 1999, 2007a; Pöggeler and Kück 2004; Engh et al. 2007b). The second genomic library was generated for yeast two-hybrid studies and consists of yeast vectors containing *S. macrospora* cDNA (Nolting and Pöggeler 2006a). This library facilitated the screening of interaction partners of developmental proteins and was shown to be useful for the identification of transcriptional activators and other developmental genes (Nolting and Pöggeler 2006a).

C. Tools for Fluorescence Microscopy

Fluorescence microscopy has become a powerful tool for studying the biology of filamentous fungi in detail, e.g. gene regulation, signal transduction, and protein localization. It relies on the fluorescent labelling of proteins or cellular components. This can be achieved by different methods, i.e. fluorescent dyes, fluorescent proteins or a combination of specific primary antibodies with conjugated secondary antibodies. While fluorescent dyes are mostly used to label cell organelles or

membranes, fluorescent proteins and antibodies are often employed to visualize proteins. All three applications were conducted with *S. macrospora* as an experimental system.

Using immunofluorescence, *S. macrospora* was employed as a model organism for cytoskeleton interactions during ascus development (Thompson-Coffe and Zickler 1992, 1993), using antibodies against actin and tubulins in combination with secondary antibodies conjugated with fluorescent probes. In addition, antibodies against meiotic proteins like e.g. RAD-51 were generated for the analysis of meiosis (Tessé et al. 2003; Storlazzi et al. 2008). A similar approach was used to determine the subcellular localization of the developmental protein ACL1 (Nowrousian et al. 1999).

The advantage of fluorescent proteins over immunofluorescence is that no antibodies are necessary and in vivo time-lapse imaging is possible. For studying the localization of developmental proteins, a number of plasmids with genes for different fluorescent proteins were generated (Table 2.1, Fig. 2.6). These plasmids encode N- or C-terminal fusion proteins between the gene of interest and the fluorescent protein. These fluorescent markers segregate during meiotic division (Pöggeler et al. 2003). Using EGFP and DsRed, the localization of the developmental proteins PRO40, PRO41, and MCM1 were identified as Woronin bodies, the ER, and the nucleus, respectively (Nolting and Pöggeler 2006b; Engh et al. 2007b; Nowrousian et al. 2007a). For PPG1, the pheromone precursor protein, and PRO41, a novel component of the ER, putative signal sequences for translational insertion into the ER were verified by Western analysis with an anti-EGFP-antibody that detected the secreted PPG1-EGFP and PRO41-EGFP fusion proteins (Mayrhofer and Pöggeler 2005; Nowrousian et al. 2007a).

However, fluorescent proteins are not only powerful tools for characterizing protein localization. In fungi, they have been used to study the dynamics of organelles (Mouriño-Pérez et al. 2006) and to screen for targeting mutants (Ohneda et al. 2005). For *S. macrospora*, several plasmids encoding organelle-targeted fluorescent proteins were constructed (Table 2.1, Fig. 6A, C, E–H) by fusing targeting signals to genes encoding fluorescent proteins. One example is plasmid pDsRed-SKL, encoding the *DsRed* gene fused to a short sequence, encoding the SKL motif. This C-terminal peroxisomal targeting sequence (PTS1) targets the fusion protein to the peroxisome (Elleuche and Pöggeler 2008). Likewise, ER markers pEGFP-KDEL and pDsRed-KDEL were constructed using EGFP and DsRed together with the C-terminal retention signal KDEL and the N-terminal secretion signal sequence of the *ppg1* pheromone precursor gene (Mayrhofer and Pöggeler 2005; Nowrousian et al. 2007a). The ER marker proteins were successfully used to identify the localization of fluorescent signals from labelled developmental proteins (Engh et al. 2007b, Nowrousian et al. 2007a). Using organelle-targeted GFP proteins, we have shown in two mutants (pro40, pro41) that the Woronin body

Table 2.1. Plasmids encoding fluorescent proteins for microscopic investigations. Plasmids encode either solely the fluorescent protein and are designed for C- or N-terminal fusion with genes of interest, or they encode an organelle-targeted fluorescent protein

Plasmid	Description	Localization	Colour	References
pEH3	hph::Pgpd-egfp-TtrpC, C-terminal	Cytoplasm	Green	Nowrousian et al. (2007a)
pYHN3	hph::Pgpd-eyfp-TtrpC, C-terminal	Cytoplasm	Yellow	Rech et al. (2007)
pCHN3	hph::Pgpd-ecfp-TtrpC, C-terminal	Cytoplasm	Cyan	Rech et al. (2007)
pRHN3	hph::Pgpd-DsRed-TtrpC, C-terminal	Cytoplasm	Red	Engh et al. (2007b)
pMHN2	hph::Pgpd-mRFP1-TtrpC, C-terminal	Cytoplasm	Red	Unpublished data
pTomato	hph::Pgpd-tdTomato-TtrpC, C-terminal	Cytoplasm	Red	Unpublished data
pmKalama1	hph::Pgpd-mKalama1-TtrpC, C-terminal	Cytoplasm	Blue	Unpublished data
pN-EGFP	hph::Pgpd-egfp-TtrpC, N-terminal	Cytoplasm	Green	Unpublished data
pN-Tomato	hph::Pgpd-tdTomato-TtrpC, N-terminal	Cytoplasm	Red	Unpublished data
pYH2A	hph::Pgpd-hh2a-eyfp-TtrpC	Nucleus	Yellow	Rech et al. (2007)
pCH2B	hph::Pgpd-hh2b-ecfp-TtrpC	Nucleus	Cyan	Rech et al. (2007)
pDsRed-SKL	nat::Pgpd-DsRed-SKL-TtrpC	Peroxisome	Red	Elleuche and Pöggeler (2008)
pGFP-SKL	phleo::Pgpd-egfp-SKL-TtrpC	Peroxisome	Green	Ruprich-Robert et al. (2002)
pCW15	his3::Pccg1-sgfp-hex-1	Woronin body	Green	Engh et al. (2007b)
pEGFP-KDEL	nat::Pgpd-Sppg1-egfp-KDEL-TtrpC	ER	Green	Nowrousian et al. (2007a)
pDsRed-KDEL	nat::Pgpd-pro41-DsRed-KDEL-TtrpC	ER	Red	Nowrousian et al. (2007a)

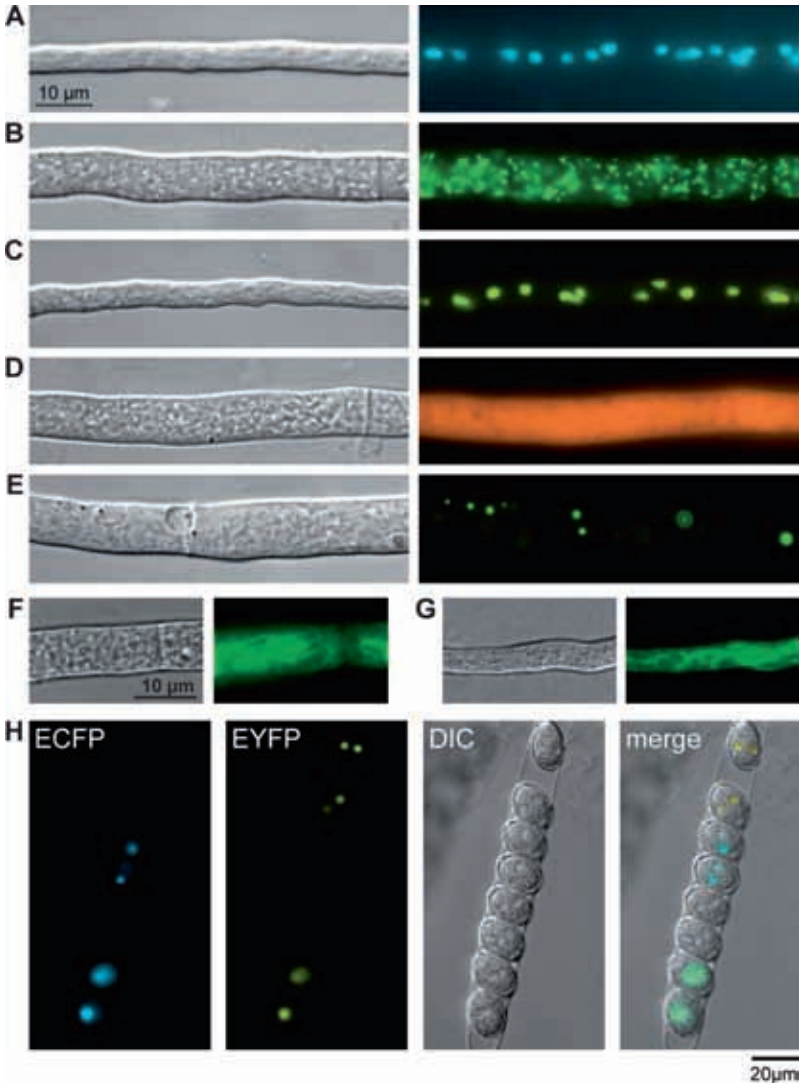

Fig. 2.6. DIC and fluorescence microscopic images of *S. macrospora*. Strains were transformed with plasmids pCH2B (**A**), pGFP-SKL (**B**), pYH2A (**C**), pTomato (**D**), pCW15 (**E**) and pEGFP-KDEL (**F**, **G**). **H** Ascus from a sexual cross of different strains transformed with pYH2A and pCH2B, respectively. Note the Mendelian segregation of the fluorescent markers. Bar size is the same for A–E and F–G, respectively

and ER appear to have a wild-type morphology, although the proteins defective in these mutants localize to these organelles (Engh et al. 2007b; Nowrousian et al. 2007a).

A particular application of fluorescence microscopy was developed for the quantification of hyphal fusion events between different *S. macrospora* strains (Rech et al. 2007). During hyphal fusion, cytoplasmic mixing occurs, which also enables mixing of genetic information by exchange of nuclei between hyphal compartments. Different strains were labelled with histone fusion proteins H2A-YFP and H2B-CFP. Following successful fusion, differently labelled nuclei are present in one hyphal compartment, and differently labelled histones are targeted to the same nucleus. Subsequently, fluorescence microscopy shows both cyan and yellow fluorescence in one nucleus as a result of hyphal fusion. Besides immunofluorescence and fluorescent proteins, vital fluorescent dyes have been used for *S. macrospora*.

An example is Calcofluor white, an exceptional dye for staining chitin in fungal cell walls. As such, it can be applied for studying morphological changes, and its use uncovered the hyperbranching phenotype of the mcm1 mutant (Nolting and Pöggeler 2006b). The DNA-staining dye 4′,6-diamidino-2-phenylindol (DAPI) was used for studying meiotic and post-meiotic events (Storlazzi et al. 2003, 2008) as well as ascus development, which is closely coupled to meiosis. For example, DAPI staining showed abnormal ascus development in several *S. macrospora*

mutants like Δste12 and the pheromone receptor/pheromone precursor double mutant Δpre2/Δppg2 (Mayrhofer et al. 2006; Nolting and Pöggeler 2006b). DAPI staining was also used to show that the mouse striatin gene is able to complement the developmental defects of the sterile pro11 mutant (Pöggeler and Kück 2004).

In summary, a great variety of tools for fluorescence microscopy has been developed for *S. macrospora* that allow not only protein localization but also specialized experiments for further studying the cell biology of this model organism.

D. Functional Genomics

In recent years, high-throughput methods for the analysis of gene expression have been established for a number of filamentous fungi. Most prominent among these new techniques are microarrays that allow the parallel analysis of gene expression for (nearly) all genes within a genome (Nowrousian et al. 2004a). Microarrays can be established from cDNA sequences or, if the genome of an organism has been sequenced, oligonucleotides as array probes can be derived from genomic sequences; and both types of arrays have been used for filamentous fungi (Breakspear and Momany 2007). In most cases, expression analyses with microarrays are performed with the organisms for which the array was developed (Nowrousian 2007). However, if two species are closely related, it is possible to do cross-species microarray hybridizations where the targets that are used for hybridization are derived from another organism than the one for which the array was originally developed. This saves the effort of setting up microarrays, especially for organisms where the genome has not yet been sequenced. *S. macrospora* was the first filamentous fungus for which cross-species array hybridizations were established (Nowrousian et al. 2005). Cross-species hybridizations were performed first using cDNA microarrays for *N. crassa*, a close relative of *S. macrospora* with ~90% nucleic acid sequence identity within coding regions (Nowrousian et al. 2004b). It was subsequently shown that these cross-species hybridizations could also be done with oligonucleotide microarrays from *N. crassa* that carry 70mer oligonucleotides as probes (Nowrousian et al. 2007a).

Gene expression analysis with microarrays can serve several purposes, for example: (1) the identification of differentially expressed genes for further studies, and (2) the analysis of genetic networks by making use of expression data as a molecular phenotype (Nowrousian 2007). Both aspects were studied in *S. macrospora* by comparing gene expression in developmental mutants and a wild-type strain (Nowrousian et al. 2005; Pöggeler et al. 2006b; Nowrousian et al. 2007a). Microarray analysis was performed using mutants pro1, pro11, pro22, pro41, and ΔSmta-1, all of which produce only protoperithecia (see Sects. IV, VI); and a number of genes were identified that are up- or down-regulated in the mutants compared to the wild type. Among these are the genes for the pheromone precursors *ppg1* and *ppg2*, the putative lectin-encoding gene *tap1*, a gene that was subsequently shown to encode the fruiting body-specific protein APP and the melanin biosynthesis genes *pks* and *sdh*. These genes were further studied to decipher the details of their expression patterns and their roles in fruiting body development (Nowrousian and Cebula 2005; Mayrhofer et al. 2006; Engh et al. 2007a; Nowrousian et al. 2007b). Additionally, overall expression patterns in the mutants were used as molecular phenotypes to establish a genetic network of *pro* genes (see also Sect. VI.E): expression patterns in mutants pro1, pro11 and pro22 are more similar to each other than to the ΔSmta-1 mutant, indicating that *Smta-1* may act in parallel to these *pro* genes (Nowrousian et al. 2005; Pöggeler et al. 2006b). Furthermore, it was shown that *pro41* acts genetically downstream of the *pro1* gene (Nowrousian et al. 2007a).

Taken together, *S. macrospora* was among the first filamentous fungi for which microarray experiments were performed; and, in recent years, the results of these high throughput analyses have identified developmentally regulated genes and aided analysis of genetic networks that control fruiting body formation.

VI. Developmental Biology and Components of Signalling Pathways

There is increasing evidence that both external and internal signals are essential for fungal fruiting body development. In several filamentous ascomycetes these signals have been shown to be transduced by conserved signal transduction pathways. The components involved can be subdivided into receptors that receive the signal, components that

transmit the signal into the cell, and nuclear transcription factors that regulate gene expression (Pöggeler et al. 2006a). The molecular mechanisms underlying sexual developmental processes in *S. macrospora* are only poorly understood. However, some of the key components of the signalling cascades from the cell surface to the nucleus have been genetically characterized in *S. macrospora*. Moreover, novel developmental proteins were discovered that might function further downstream of conserved signalling pathways, ensuring cell-specific differentiation.

A. Pheromones and Pheromone Receptors

Male and female fertility of heterothallic filamentous ascomycetes depends on interactions of pheromones with their specific receptors. Pheromone precursor genes encoding two different types of pheromones have been isolated from heterothallic filamentous ascomycetes. One of the precursor genes encodes a polypeptide containing multiple repeats of a putative pheromone sequence bordered by protease processing sites and resembles the α-factor precursor gene of *Saccharomyces cerevisiae*. The other gene encodes a short polypeptide similar to the *S. cerevisiae* a-factor precursor. The short precursor has a C-terminal CaaX motif (C = cysteine, a = aliphatic, X = any amino acid residue) expected to produce a lipopeptide pheromone with a C-terminal carboxy-methyl-isoprenylated cysteine. The two types of pheromone precursor genes are present in the same genome and expression of pheromone genes seems to occur in a mating type-specific manner (Bobrowicz et al. 2002; Coppin et al. 2005).

When pheromone genes were deleted in heterothallic ascomycetes, male spermatia were no longer able to fertilize the female partner, proving that pheromones are crucial for the fertility of male spermatia (Turina et al. 2003; Coppin et al. 2005; Kim and Borkovich 2006). Sensing of the pheromone signal depends on (G) protein-coupled seven transmembrane-spanning receptors (GPCRs). Two genes, designated *pre-1* and *pre-2*, encode pheromone receptors similar to *S. cerevisiae* a-factor receptor (Ste3p) and α-factor receptor (Ste2p), respectively, and have been shown to be essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa* (Kim and Borkovich 2004, 2006). Thus, in heterothallic filamentous ascomycetes the function of pheromones and their cognate receptor seems to be restricted to the fertilization process.

Interestingly, genes encoding two different pheromone precursors and two pheromone receptors have also been identified in the homothallic *Sordaria macrospora* (Pöggeler 2000; Pöggeler and Kück 2001). The pheromone precursor gene *ppg1* is predicted to encode a α-factor-like peptide pheromone and the *ppg2* gene an a-factor-like lipopeptide pheromone (Pöggeler 2000). The deduced gene products of the *S. macrospora pre1* and *pre2* genes are predicted to be seven transmembrane domain proteins (Pöggeler and Kück 2001). For *S. macrospora*, it has been demonstrated that disruption of the pheromone precursor *ppg1* gene prevents the production of the peptide pheromone, but does not affect vegetative growth, fruiting-body, or ascospore development (Mayrhofer and Pöggeler 2005). Similarly, no effect on vegetative growth, fruiting-body, and ascospore development was observed in the single pheromone-mutant Δ*ppg2* and single receptor-mutants Δ*pre1* and Δ*pre2*. However, double-knockout strains lacking any compatible pheromone/receptor pair (Δ*pre2*/Δ*ppg2*, Δ*pre1*/Δ*ppg1*) and the double-pheromone mutant strain (Δ*ppg1*/Δ*ppg2*) have a drastically reduced number of perithecia and sexual spores, whereas deletion of both receptor genes (Δ*pre1*/Δ*pre2*) almost completely eliminates fruiting body and ascospore formation (Mayrhofer et al. 2006). Taken together, these results suggest that pheromones and pheromone receptors are involved in post-fertilization events and are required for optimal sexual reproduction of the homothallic *S. macrospora*. Moreover, by heterologously expressing the *S. macrospora pre2* in *Saccharomycs cerevisiae MAT*a cells, lacking the Ste2p receptor, PRE2 was shown to facilitate all aspects of the *S. cerevisiae* pheromone pathway when activated by the *Sordaria macrospora* peptide pheromone (Mayrhofer and Pöggeler 2005). Therefore, one may conclude that the receptors encoded by *pre2* and *pre1* also function as G protein-coupled receptors (GPCRs) in *S. macrospora*.

B. Heterotrimeric G Proteins

Upon activation by GPRC receptors, heterotrimeric G proteins catalyse the exchange of GDP

for GTP on the G protein α subunit. This leads to dissociation of the βγ subunits. Either Gα, or Gβγ, or both are then free to activate downstream effectors. Signalling persists until GTP is hydrolysed to GDP, and the subunits re-associate (Dohlman 2002). The genome of *S. macrospora* contains three genes (*gsa1*, *gsa2* and *gsa3*) encoding Gα subunits (Kamerewerd et al. 2008). In addition genes encoding the β-subunit and the γ-subunits of the G protein are present in *S. macrospora* (Mayrhofer and Pöggeler 2005).

To explore the functional role of G protein α subunits (GSA) in the sexual life cycle of this fungus, knockout strains for all three *gsa*-genes (Δgsa1, Δgsa2, Δgsa3) and double mutants were generated. Phenotypic analysis of single and double mutants showed that the genes for Gα subunits contribute differently to sexual development. While the Δgsa2 mutant revealed wild-type-like fertility, Δgsa3 developed fruiting bodies, but the ascospores had a drastically reduced germination rate. Δgsa1 showed a delay in sexual development and a 50% reduction in the number of fruiting bodies. A more impaired phenotype can be observed in Δgsa1Δgsa2 and Δgsa1Δgsa3 double mutants. These mutants produce only protoperithecia, indicating that all Gα subunits contribute significantly to sexual development. To test whether the pheromone receptors PRE1 and PRE2 mediate signalling via distinct Gα subunits, Δpre strains were crossed with all Δgsa strains. The data obtained with double mutants carrying a disrupted *gsa* gene together with a disrupted receptor gene indicated that the pheromone receptors interact differently with GSA1 or GSA2 and that GSA1 is the main component of a signal transduction cascade downstream of the pheromone receptors. As shown in Fig. 2.7, the third Gα protein, GSA3, seems to be involved in a parallel signalling pathway but also contributes to fruiting body development and fertility (Kamerewerd et al. 2008).

C. Adenylyl Cyclase

Activated Gα or Gβγ subunits can regulate downstream effectors such as adenylyl cyclase and

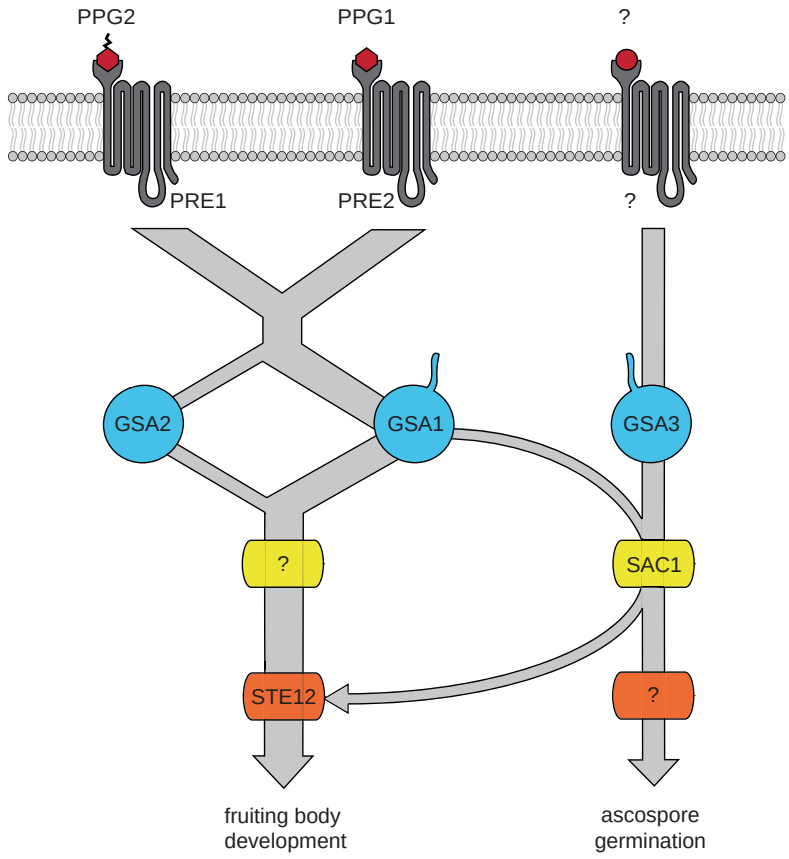

Fig. 2.7. Pheromone signalling pathway showing the function of pheromones, pheromone receptors and G protein alpha subunits, as well as downstream signalling components in the development of perithecia (modified from Kamerewerd et al. 2008)

mitogen-activated protein kinase (MAPK) cascades. The fungal adenylyl cyclase is a soluble enzyme that produces the second messenger cyclic AMP (cAMP) from ATP. The cAMP signalling pathway of fungi is involved in several important processes including nutrient sensing, stress response, metabolism, pathogenicity, and sexual development (Lengeler et al. 2000; D'Souza and Heitman 2001). Several lines of evidence indicate that many of the known fungal G proteins influence the intracellular level of cAMP by either stimulating or inhibiting adenylyl cyclase (Bölker 1998). In order to investigate a component that acts supposedly downstream of the GSAs, the *S. macrospora* gene encoding adenylyl cyclase (*sac1*) was isolated. Deletion of *sac1* prevents cAMP synthesis. Moreover, a Δsac1 mutant exhibits reduced fertility with a significant number of the fruiting bodies that are embedded in the solid media. A similar phenotype was observed in a Δgna3 mutant of *N. crassa*. In addition, ascospores derived from the Δsac1 mutant have a highly reduced germination rate and this phenotype also resembles that of Gα subunit 3 disruption mutants from *N. crassa* (Kays et al. 2000) and *S. macrospora* (Kamerewerd et al. 2008). Analysis of the three Δgsa/Δsac1 double mutants indicates that SAC1 acts downstream of GSA3, parallel to a GSA1-GSA2 mediated signalling pathway (Kamerewerd et al. 2008).

D. Transcription Factors

Signal transduction initiates morphogenesis by finally activating transcription factors which in turn activate or repress cell- or tissue-specific expression of developmental genes. To elucidate whether conserved fungal transcription factors are involved in sexual development of *S. macrospora*, *mcm1* and *ste12* were functionally analysed. The *mcm1* gene encodes a putative homologue of the *Saccharomyces cerevisiae* MADS box protein Mcm1p and *ste12* encodes a homeodomain similar to the *S. cerevisiae* Ste12p, respectively (Nolting and Pöggeler 2006a, b). Deletion of the *mcm1* gene led to a pleiotropic phenotype, including reduced biomass, increased hyphal branching, and reduced hyphal compartment length during vegetative growth, as well as sexual sterility. The *mcm1* mutant was only capable of producing protoperithecia, but was unable to form either ascospores or perithecia and, thus, phenotypically resembled pro-mutants of *Sordaria macrospora* (see Sect. IV). A two-hybrid analysis demonstrated that MCM1 may physically interact with itself and with the α-domain mating-type protein MAT1-1-1 (Nolting and Pöggeler 2006b). The pleiotropic phenotype of *S. macrospora* Δ*mcm1* suggests that the *S. macrospora* ΔMCM1 protein might be involved in a wide range of functions via interaction with diverse transcriptional regulators.

From a yeast two-hybrid screen, among several other proteins, a putative homologue of the *Saccharomyces cerevisiae* homeodomain protein Ste12p was identified as an additional MCM1 interaction partner (Nolting and Pöggeler 2006a). In *S. cerevisiae*, Ste12p is known to be a transcription factor acting downstream of Fus3p/Kss1 MAP kinases. In haploid yeast cells, Ste12p is required for the response to the mating pheromone produced by the opposite mating type and for invasive growth in response to limited nutrients. In diploid yeast cells, Ste12p regulates pseudohyphal development in response to nitrogen starvation (Madhani and Fink 1997; Gustin et al. 1998; Roberts et al. 2000). Transcriptional regulation of different classes of genes is thereby triggered through interactions with different transcriptional regulators. Ste12p forms a homodimer and binds either Mcm1p or Matα1p to regulate pheromone-responsive genes and cell-type-specific genes, respectively, and with Tec1p to activate genes required for filamentous growth (Dolan et al. 1989; Yuan et al. 1993; Bruhn and Sprague 1994; Madhani and Fink 1997).

In *Sordaria macrospora*, two-hybrid and biochemical studies showed that STE12 in addition to MCM1 is also able to interact with the mating-type protein MAT1-1-1. Analysis of a *S. macrospora* Δ*ste12* knockout mutant demonstrated that STE12 is not needed for fruiting body formation and vegetative growth but is involved in ascosporogenesis. The *S. macrospora* Δ*ste12* mutant is able to form protoperithecia and perithecia, but the latter contain a drastically reduced number of asci with predominantly non-viable ascospores. Particularly, cell walls of asci and ascospores appear to be fragile (Nolting and Pöggeler 2006a). This implies that STE12, in addition to its role in pheromone signal transduction, might be involved in cell wall integrity of asci and ascospores. To study the functional connections between the GSA subunits and the STE12

transcription factor in *S. macrospora*, the phenotypes of three ΔgsaΔste12 double mutants were analysed (Kamerewerd et al. 2008). While Δgsa2Δste12 double mutants show a wild-type-like formation of perithecia containing fragile asci and ascospores, the Δgsa1Δste12 mutant develops only a few fruiting bodies. Furthermore, perithecia of the Δgsa1Δste12 mutant contain only a few asci compared to the Δste12 single or the Δgsa2Δste12 double mutant. The Δgsa3Δste12 mutant exhibits the most severe phenotype. Protoperithecia are produced, but no mature perithecia can be detected. The sterility of the Δgsa3Δste12 mutant is a strong evidence for the STE12 transcription factor being one of the key regulators downstream of the pheromone receptors.

E. Novel Developmental Proteins

As shown in Fig. 2.4 (see Sect. IV), more than 100 *S. macrospora* mutants have been described with blocks at various stages of the developmental cycle. For seven of these mutants, the affected genes have already been identified: The wild-type phenotype was restored in the mutants by transformation with an indexed cosmid library (Pöggeler et al. 1997a). Thus it was possible to isolate the wild-type alleles of the mutated genes. Using this experimental approach, novel developmental genes were identified, most of which had not been implicated in fungal development before, thus validating a forward genetic approach.

The mutants that were complemented are pro1, pro4, pro11, pro22, pro40, pro41, and per5, all of which have a block at the stage of protoperithecium formation (pro mutants) or produce perithecia but no mature ascospores (per5). The corresponding genes can be grouped according to the functions of their gene products (Table 2.2). In mutants pro4 and per5, the *leu1* and *acl1* genes encoding β-isopropylmalate dehydrogenase and ATP citrate lyase, respectively, are defective (Nowrousian et al. 1999; Kück 2005). They are involved in amino acid and fatty acid biosynthesis, respectively, and their requirement for fruiting body formation, but not for vegetative growth may indicate an increased energy demand during sexual development. Expression analyses showed that both genes are under transcriptional control during the developmental cycle of *S. macrospora*, indicating a tight integration of metabolic activity and developmental processes.

The first *pro* gene to be identified was *pro1*, a gene encoding a zinc finger transcription factor (Masloff et al. 1999, 2002). The gene product belongs to the fungal-specific class of zinc cluster proteins, the best known member of which is the yeast transcriptional regulator Gal4 (MacPherson et al. 2006). *pro1* was the first gene encoding a transcription factor of this class that was shown to be essential for sexual development in filamentous fungi. Since then, *pro1* orthologues from several other filamentous ascomycetes have been identified that also have a role in development; however, there seems to be a great diversity in the function of *pro1* orthologues in that their roles can include functions in vegetative sporulation or growth (Colot et al. 2006; Vienken and Fischer 2006).

The last four *pro* genes that were cloned encode proteins that are either membrane or membrane-associated proteins and/or localize to organelles. Among these is PRO11, a WD40 repeat protein that associates with membranes (Pöggeler and Kück 2004). It is a functional homologue of the mammalian protein striatin as was demonstrated by the ability of the mouse orthologue to restore fertility in the pro11 mutant. This indicates that PRO11 is a member of a protein family with functionally conserved members from lower to higher eukaryotes. Another putative membrane or membrane-associated protein that is encoded by a *pro* gene is PRO22, an orthologue of the *N. crassa* protein HAM-2. The latter was shown to be necessary for hyphal fusion in *N. crassa* and, like the corresponding *N. crassa* mutant, the pro22 mutant is defective in hyphal fusion (Xiang et al. 2002; Rech et al. 2007).

Two identified organellar proteins are PRO40 and PRO41, that localize to the Woronin body and the ER, respectively (Engh et al. 2007b; Nowrousian et al. 2007a). PRO40 is a WW domain protein and the first of its class that was shown to be essential for fruiting body development. Additionally, it is the first Woronin body protein for which a role in development could be demonstrated. The Woronin body is an organelle that is specific to filamentous ascomycetes where it is necessary for septal plugging to prevent loss of cytoplasm after hyphal injury (Markham and Collinge 1987). Woronin bodies have not been previously implicated in fruiting body formation; however, detection of PRO40 (and its corresponding *N. crassa*

Table 2.2. Developmental genes from *S. macrospora*. Localizations given in brackets are derived from sequence homologies, the others were verified experimentally. The genes *acl1* and *leu1* complement mutants per5 and pro4, respectively, all other genes carry the same name as the corresponding mutant

Gene	Gene product/conserved domains	Localization	References
Primary metabolism			
acl1	Subunit of the ATP citrate lyase	Cytoplasm	Nowrousian et al. (1999)
leu1	β-Isopropylmalate dehydrogenase	(Cytoplasm)	Kück (2005)
Secondary metabolism			
fbm1	Dehydrogenase (polyketide biosynthesis)	(Extracellular)	Nowrousian (2009)
pks	Polyketide synthase (melanin biosynthesis)	(Cytoplasm)	Engh et al. (2007b)
sdh	scytalon dehydratase (melanin biosynthesis)	(Cytoplasm)	Engh et al. (2007b)
Transcription factors			
mcm1	MADS-box transcription factor	Nucleus	Nolting and Pöggeler (2006b)
pro1	C_6 zinc finger transcription factor	(Nucleus)	Masloff et al. (1999)
Smta-1	HMG domain transcription factor	(Nucleus)	Pöggeler et al. (1997b)
ste12	Homeodomain/zinc finger transcription factor	(Nucleus)	Nolting and Pöggeler (2006a)
Pheromones and pheromone receptors			
ppg1	Peptide pheromone	Extracellular	Mayrhofer and Pöggeler (2005)
ppg2	Lipopeptide pheromone	(Extracellular)	Mayrhofer et al. (2006)
pre1	Pheromone receptor	(Plasma membrane)	Mayrhofer et al. (2006)
pre2	Pheromone receptor	(Plasma membrane)	Mayrhofer et al. (2006)
Signal transduction			
gsa1	G protein α-subunit	(Membrane-associated)	Kamerewerd et al. (2008)
gsa2	G protein α-subunit	(Cytoplasm)	Kamerewerd et al. (2008)
gsa3	G protein α-subunit	(Membrane-associated)	Kamerewerd et al. (2008)
sac1	Adenylate cyclase	(Cytoplasm)	Kamerewerd et al. (2008)
Membrane proteins and membrane-associated proteins			
pro11	WD40-repeat protein	Membrane-associated	Pöggeler and Kück (2004)
pro22	Membrane protein	(Membrane)	Rech et al. (2007)
Organellar proteins			
pro40	WW domain protein	Woronin body	Engh et al. (2007a)
pro41	Membrane protein	ER membrane	Nowrousian et al. (2007)

orthologue SO that also localizes to septal plugs and is essential for sexual development) makes it tempting to speculate about as yet unsuspected roles for this fungal-specific organelle (Engh et al. 2007b; Fleißner and Glass 2007).

The last *pro* gene to be isolated was *pro41* that encodes a small protein of the ER membrane (Nowrousian et al. 2007a). PRO41 orthologues exist only in filamentous ascomycetes and the *S. macrospora* gene was the first of these to be functionally characterized. Similar to the other *pro* genes, *pro41* is dispensable for overall vegetative growth but is necessary for the formation of mature fruiting bodies. Expression studies showed that it is transcriptionally upregulated during fruiting body formation; and microarray analyses of single and double mutants indicated that it acts genetically downstream of the transcription factor gene *pro1*. The identification of the development-specific proteins PRO40 and PRO41 throws a spotlight on the role of organelles in fungal development. Future studies should include the identification of interaction partners of the proteins as well as further analysis of the genetic interactions between the genes to unravel metabolic and signalling networks that integrate subcellular functions with multicellular development.

Another interesting aspect that came to light during the analysis of the pro-mutants is the fact that there might be a connection between hyphal fusion events and the ability to form sexual structures: Mutants pro22 and pro40 are greatly impaired in their ability to form anastomoses between vegetative hyphae, and the same is true for the corresponding *N. crassa* mutants ham-2 and soft, both of which were initially identified in screens for hyphal fusion mutants (Xiang et al. 2002; Fleißner and Glass 2007; Rech et al. 2007). There are a number of hyphal fusion events necessary for sexual development. These include

fusion of sub-apical cells in crozier formation as well as (putative) fusion of hyphae that form the outer perithecial shell. In heterothallic ascomycetes like *N. crassa*, an additional fusion event has to occur to allow fertilization; however, the SO protein is not necessary for this specific type of fusion (Fleissner et al. 2005). Thus, the connection between hyphal fusion events and fruiting body development has yet to be discovered and poses one of the most challenging future questions in fungal biology.

VII Conclusions

Sordaria macrospora has a long-standing history as a classic genetic model system for conventional tetrad analysis. The application of several molecular tools, such as DNA mediated transformation, site-specific recombination or functional genomics to this filamentous fungus makes it an ideal experimental system to uncover the details of genetic interactions controlling developmental plasticity. The rapid and inexpensive genetic analysis of developmental mutants with distinct and defined morphological defects will increase our knowledge of multicellular differentiation processes in eukaryotes.

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