
5 Genetic and Environmental Influence on Development of the Filamentous Fungus *Neurospora crassa*

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5.1 Introduction¹

Fungi are eukaryotic, heterotrophic organisms with an absorptive mode of nutrition. Most fungi grow in yeast-like or filamentous forms which are characteristically multicellular and multinucleate. The fungal cell is, in most cases, protected by a rigid polysaccharide wall. Recently, in recognition of the unique nutritional and morphological attributes of fungi, as expressed in the immensely diverse habitats and niches various fungi accommodate, taxonomists have elevated their taxonomic ranking to a separate kingdom – The Fifth Kingdom, on equal footing with plants and animals. The genetics of naturally occurring inter- and intraspecific fungal diversity and the speciation in fungi have been reviewed by Perkins (1991).

The basic plan of filamentous fungi is a microscopic tubular form that grows at the tip and becomes multicellular by septation. The linear hyphal growth confers an important advantage in or on solid substrates. In contrast to unicellular forms, filamentous fungi can invade new niches rather than stay confined as a colony to less favorable substrates. The effective asexual and sexual reproduction and propagule dispersal systems present in most (but not all) fungi complement the proliferative capacity of the fungal mycelium.

The proliferative success of many fungal species can be attributed to the fact that fungi are modular organisms. Carlile has summarized the modular characteristics of filamentous fungi, which include (1) growth by iteration of modular units (2) open-ended growth (while conditions permit) (3) variation in organismal size (4) common lack of motility, thus resources are reached via growth (5) localized (rather than organismal)

response to the environment (6) almost absolute organismal tolerance to local damage (7) reproductive potential increases indefinitely (8) clonal reproduction is common (9) senescence and death may be locally confined, with loss of unwanted modules.

Even though the modular nature of fungi differs from more complex organisms, fungi serve as highly suitable models for analysis of basic genetic and cellular events common to almost all eukaryotes. Moreover, the fact that some fungi are easy to grow, analyze, and manipulate has made them convenient model systems of choice for many researchers. As the time and efforts devoted to the study of fungal biology have progressed, occurrence of mammalian homologues to fungal genes has been frequently identified, further supporting the relevance of findings obtained by the study of fungal systems to the scientific community.

5.2 *Neurospora crassa* as a Model for Filamentous Fungi²

In this chapter, we have chosen *Neurospora crassa* to demonstrate some of the developmental features of filamentous fungi, yet some major differences between *N. crassa* and other fungi will be discussed. Overall, though diversity among fungi can be immense, many of the basic concepts of fungal growth and development are common.

N. crassa is a eukaryotic organism which belongs to the phylum Ascomycotina. In nature, it grows on decaying or burnt vegetation in tropical or subtropical areas. Since the 1840s, when Louis Pasteur was called to advise the French army regarding the cause of infestation of bread from the

Paris bakeries by an orange bread mold (cham-pignons rouges du pain), the fungi of the genera *Neurospora* have been the object of biological experiments. One century later, the era of biochemical genetics was opened by the pioneering work of Beadle and Tatum, who obtained, using *N. crassa*, the first biochemical mutants and proposed the one-gene-one-enzyme hypothesis. Since then, *Neurospora* has aided in making fundamental contributions in a broad spectrum of biological research. Much of the knowledge accumulated by the study of *N. crassa* has often been shown to be highly relevant to other eukaryotes, spanning the entire evolutionary spectrum. Examples of studies initially carried out in *Neurospora* include: nutritional mutants (including the identification of the first conditional biochemical mutants), translational suppression, cross-pathway control of amino acid biosynthetic enzymes, membrane transport systems, circadian rhythms, vegetative incompatibility, mating types, etc. (for a detailed perspective see Perkins 1992).

Today, a large amount of information is available on the genetic, biochemical, ultrastructural, and morphogenetic aspects of this organism. Hundreds of auxotrophic, morphological and developmental mutant strains exist, and are maintained at the Fungal Genetics Stock Center (FGSC) in Kansas City (USA). The center also maintains an internet site (<http://www.kumc.edu/research/fgsc/main.html>) which contains short articles on techniques, new strains, genetic maps, recent bibliography, and links to other mycological resources. Mutants available so far define more than 600 different genes (in addition to other genes which have been defined on the basis of structural similarity to genes analyzed in other organisms). About half of these genes have effects on morphology. The strain collection also has a group of *Neurospora* isolates collected from nature.

Besides the well-advanced classical genetics and molecular biology, there are many features that make *Neurospora* an attractive model system to study morphogenesis and development. The first is that this fungus is structurally much simpler than multicellular eukaryotes, producing only about a dozen different morphological structures in its life cycle. Sufficient quantities of several of

these structures can be produced in the laboratory. Thus, it is one of the simplest among the complex eukaryotic organisms. Second, the ability to grow on simple, well-defined medium is very useful for studies relating to biochemistry and nutritional effects on morphogenesis. Third, it grows very fast and under laboratory conditions; the entire life cycle can be completed in 2 weeks when including sexual crosses, while the vegetative cycle requires only a few days. Fourth, it reacts to many environmental stimuli like CO₂, nitrogen and carbon starvation, blue light, and gravity.

5.2.1

The Life Cycle of *Neurospora crassa*

N. crassa has an asexual and a sexual life cycle (Fig. 1). In the asexual cycle, a conidium or an ascospore germinates on a suitable substrate and forms the mycelium, a mat of branched intertwined hyphae. The hyphae have perforated cross-walls which, in addition to creating cytoplasmic continuity throughout long stretches of the fungal mycelium, allow organelles such as nuclei or mitochondria to pass through. The mycelium is therefore coenocytic. Under optimal conditions, the mycelial front advances on solid medium at the rate of more than 10 cm per day. After a few days, aerial hyphae and macroconidia are formed. Macroconidia have from one to five nuclei. A different class of spores, microconidia, are formed directly from mycelia in old cultures. Microconidia are smaller than macroconidia and have only one nucleus.

Some species of *Neurospora* (including *N. crassa*) are heterothallic, namely, they have two mating types. The two mating types, A and a, which are idiomorphs (two alternative DNA sequences at the same chromosomal locus), are identical in morphology and distinguishable only in their mating reaction. Under laboratory conditions, several days after inoculation of media with either mating type (under suitable conditions, see later), the newly formed female structures (protoperithecia) can be fertilized with cells of the other mating type. About 2 weeks from fertilization the

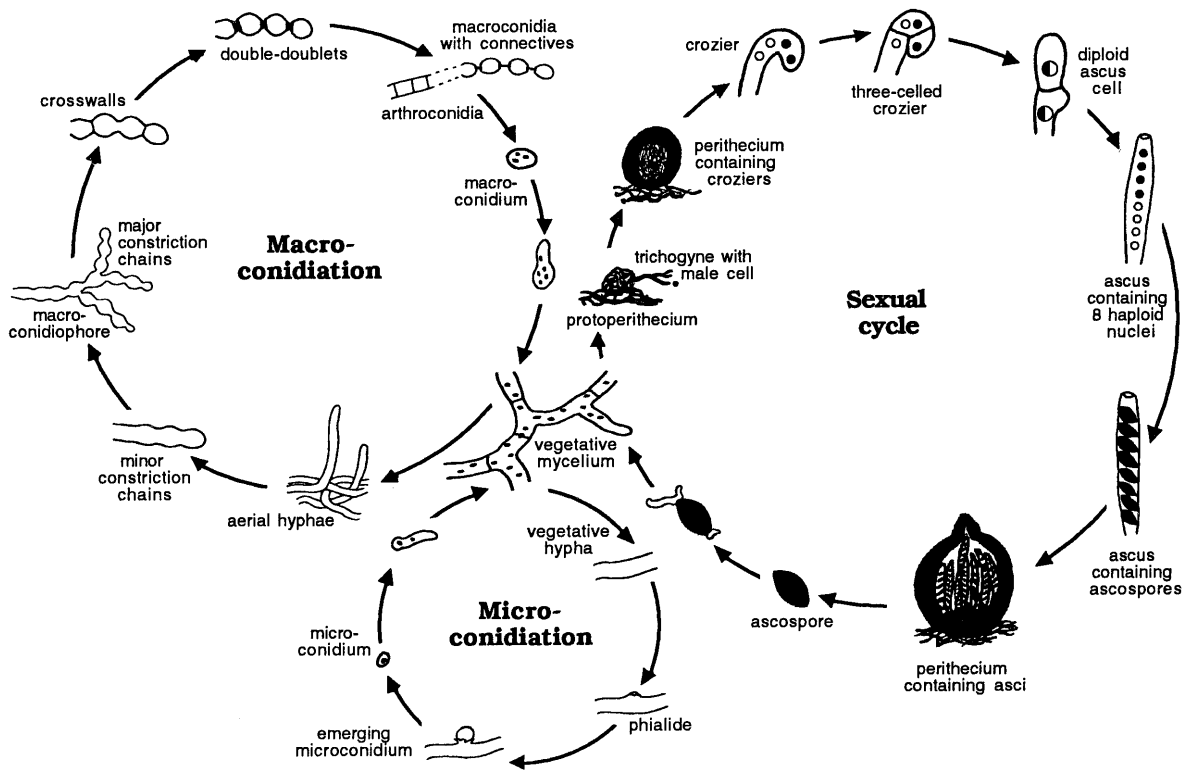


Fig. 1. *Neurospora crassa* life cycle. Depending on environmental conditions, the vegetative mycelium can undergo the asexual sporulation process of macroconidiation or microconidiation. It can enter the sexual cycle by forming

protoperithecia, which, upon fertilization, can initiate development leading to the production of meiotically derived ascospores. (After Springer 1993)

mature perithecia start shooting ascospores in groups of eight. This process continues for several days.

5.2.2 Microbiological and Genetic Techniques³

Neurospora is haploid except for a transient phase which precedes meiosis during the sexual life cycle. The haploid genome contains about 4×10^7 base pairs, roughly corresponding to ten times the size of the *E. coli* genome. The young ascospores have several genetically identical nuclei. Most of the genetic or biochemical work is done using conidia or mycelia grown from them. Technically the fungus is handled in a manner similar to a prokaryotic organism like *E. coli*.

N. crassa protoplasts (fungal cells lacking the rigid cell wall) can be prepared and transformed efficiently to yield about 10^4 – 10^5 transformants per μg DNA. The transformants arise by integration of incoming DNA into the chromosomes. Similar, as well as other, transformation procedures have been developed for a growing number of fungal species. Reverse genetics approaches, allowing for the analysis of gene function on the basis of isolated DNA fragments, are possible via homologous gene disruption experiments as well as by using a natural gene inactivation process termed RIP (repeat-induced point mutations). This process occurs during the sexual cycle of *N. crassa* and introduces multiple GC to AT transitions, accompanied by methylation of many cytosine residues, in duplicated DNA segments. As a

result of RIP, duplicated DNA segments are structurally altered and functionally inactivated in some of the progeny obtained from a cross in which one or both parental strains harbor duplicated DNA sequences.

More than 250 genes have already been cloned, and cosmid and cDNA libraries are available from the FGSC. Any chromosomal segment, even when cloned only as cDNA, can be mapped with a restriction polymorphism kit also available from the FGSC. The identification and availability of various genes and gene fragments has been significantly accelerated since the initiation of the *Neurospora* genome project. Information on this project (as well as the parallel *Aspergillus nidulans* genome sequencing effort) can be obtained at the FGSC site. The culmination of the ongoing effort to sequence the genomes of *N. crassa* and *A. nidulans* will dramatically enhance the possibilities to identify genes, study their function, and manipulate fungal traits.

5.3 Morphological Studies⁴

5.3.1 From Spore to Mycelium

N. crassa can produce three different types of spores, asexual microconidia and macroconidia, and sexual spores (ascospores). Two asexual sporulation pathways, involving different mechanisms of budding from hyphae, lead to the formation of the micro- and macroconidia. The ascospores are produced by a more complex reproductive pathway which involves fruiting body formation, meiosis, and the concomitant formation of the spores within sac-like structures (asci). The germination of asexual versus sexual spores is also very different. Asexual spores will grow at once when put into growth medium; they are not long-lived at normal temperatures and conditions. Ascospores, on the other hand, which are protected by thick, melanin-rich coats, will remain viable in the dormant state for many years, resistant to a number of environmental stresses. They do not grow simply in response to availability of nutrients. The ascospores

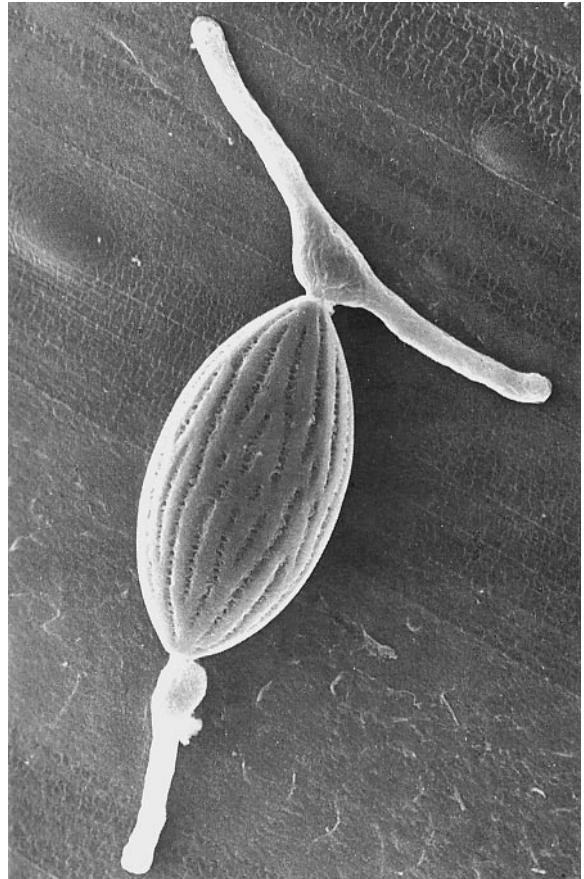


Fig. 2. Germinating ascospore. The ascospore is 25 μm long. (Courtesy of Rudi Lurz and Wolfgang Heimler)

can be germinated by a heat shock or by furfural treatment. The sequence of visible morphological events is the same as that for conidia once dormancy is broken. A germ tube is formed after about 3 h and elongates by apical extension (Fig. 2). After a few hours, branching and continued elongation results in a fully grown multinuclear mycelial mass (Fig. 3 a, b).

5.3.2 From Mycelium to Conidia⁵

As mentioned above, the basic growing unit of the filamentous fungus is the hyphal cell. The shape of this cell (as in other structures) is de-

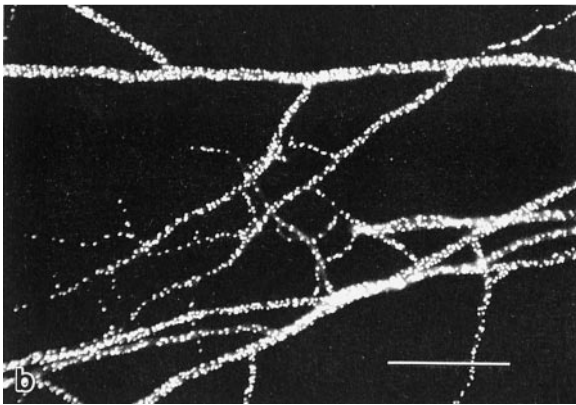
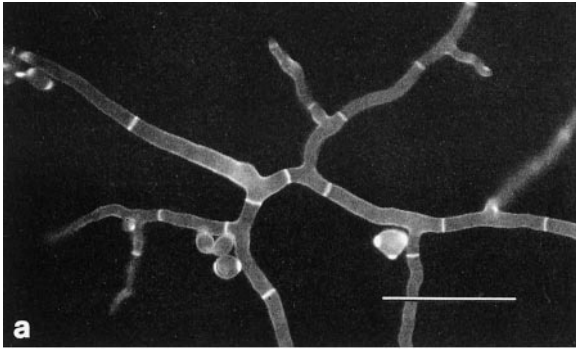


Fig. 3 a, b. Fluorescence micrographs of mycelial hyphae. **a** Hyphal cross-walls visualized with Calcofluor. **b** Multi-nucleate hyphae stained with Hoechst 33258. Bars **a** 50 μ M, **b** 50 μ M. (After Springer and Yanofsky 1989)

pendent on the proper spatial and temporal assembly of the multicomponent cell wall. The cell wall is composed predominantly of sugar polymers (glucan and chitin) as well as proteins, lipids, and salts.

Hyphal elongation and branching rates will, in many cases, determine the linear proliferation and biomass accumulation of a fungal colony. These processes require that the biosynthetic events which give rise to the structural components be properly concerted so as to successfully accomplish the formation of the required fungal structure. The presence of several chitin synthases in *N. crassa* (and in other filamentous fungi) and the differential expression and functional consequence of chitin synthase gene inactivation have

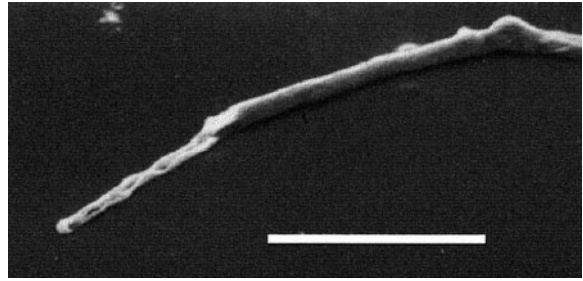


Fig. 4. Collapsing hyphal tip region in a *N. crassa* mutant in which the chitin synthase 1 gene had been inactivated. Bar: 50 μ M

demonstrated that different chitin synthases have different roles during fungal growth and development. Some chitin synthases are essential for maintaining proper hyphal rigidity and form and are required for hyphal elongation (Fig. 4). The specific roles of other members of this gene family have yet to be elucidated. Figure 5 is a representation of some of the processes involved in apical growth of the fungal filament. The cell wall biosynthetic (chitin and glucan synthases) as well as cell wall lytic (chitinases and glucanases) enzymes required for cell elongation and branching events are conveyed to the required location and, at least in some cases, compartmentalized trafficking is carried out in membranous vesicles. The wall at the tip is plastic, allowing the extension of the cell by insertion of new material. As extension progresses, the material at the former position of the apex is progressively rigidified as it becomes the lateral wall of the growing cell. This rigidification is brought about by the covalent cross-linking of wall materials, especially chitin and $\beta(1\rightarrow3)$ glucans, and the hydrogen bonding of adjacent polysaccharide chains, especially chitin, to give microfibrils.

Conidiation, the formation of asexual spores, typically takes place on solid medium or above a stationary liquid culture, but can occur in liquid under certain conditions (see below). The most complete studies on conidiation using wild-type and conidiation-defective mutants support a timeline of events shown in Fig. 6. During conidiation, the aerial hyphae cease to elongate by apical extension, and the process of growth by successive

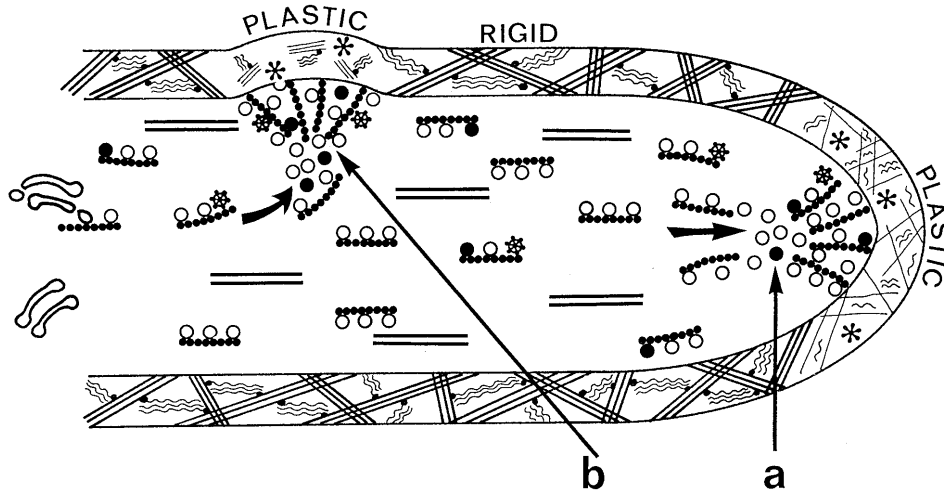


Fig. 5. Model of some suggested features of apical growth and branching of hyphae. Microvesicles of various types (empty, solid, and starred circles) are being produced by Golgi and being transported to two sites, the sub-apical tip region (a) and the new branch (b). The microvesicles are associated with cytoskeleton, particularly microfilaments (beaded lines), but also perhaps with microtubules (parallel lines). The plastic wall at the apex is shown with nascent

fibrils of chitin (straight lines) and glucan (wavy lines). The wall becomes progressively rigidified by their crystallization, and cross-linked by covalent bonds (black dots). At the branch site, the action of lytic enzymes (stars) has created a soft spot in the wall. It is likely that lytic enzymes are also secreted at the apex, but their role there is unclear. (After Gooday 1995)

apical budding is initiated. This results in the formation of proconidial chains (Fig. 7a). Cross-walls are laid down between each proconidial element (Fig. 7b). These cross-walls then thicken and separate, leaving the newly formed conidia held together by a fragile connective (Fig. 7c), which can break and release wind borne conidia at the slightest disturbance.

5.3.3

The Sexual Cycle⁶

A prerequisite for the sexual cycle is the production of protoperithecia. These spherical structures, roughly 50 micrometer in diameter, have several hyphae protruding from their surface (Fig. 8). One of these hyphae, the trichogyne, is connected with the ascogonial cell (the female gametangium in ascomycete fungi) within the protoperithecium. Protoperithecia can be formed only on mycelia that are not submerged. Fertilization begins when a conidium or a piece of myce-

lium of the opposite mating type comes in contact with the trichogyne. The trichogyne grows towards the cell of the opposite mating type guided by attractants which have not yet been defined. Nuclei of opposite mating type pass into the trichogyne by wall fusion and are then transported to the ascogonium. Here, nuclei of both mating types undergo several divisions before karyogamy (Fig. 9). Nuclei of opposite mating type finally fuse in the crozier, a hook-shaped structure composed of three cells, and immediately undergo meiosis. In the meantime, the protoperithecium darkens, grows in size, and develops a beak-shaped structure with an aperture (ostiole) through which the ripe ascospores are eventually discharged. The resulting structure, now called the perithecium, contains hundreds of asci, each one coming from a different crozier. Each ascus has eight ascospores representing an outcome of meiotic divisions. Of the eight ascospores, each pair of adjacent ascospores has an identical genotype which may be different from the neighboring pair of ascospores. Each young

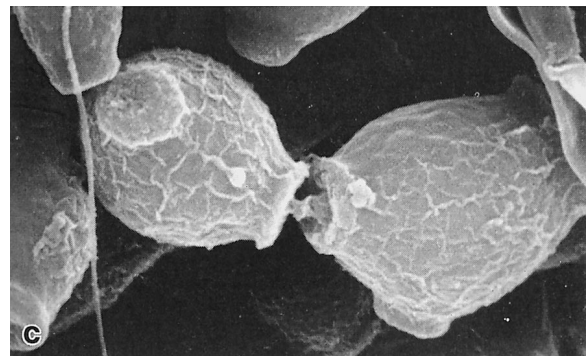
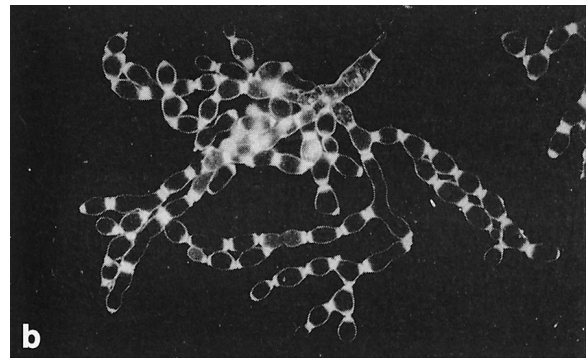
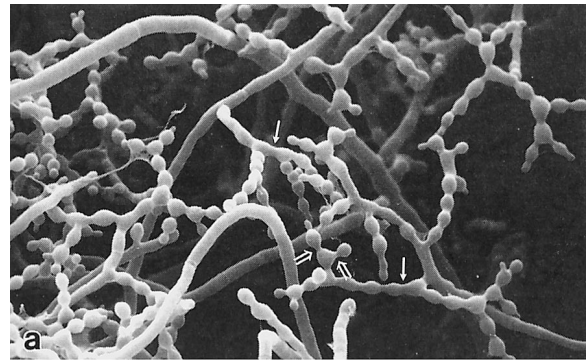
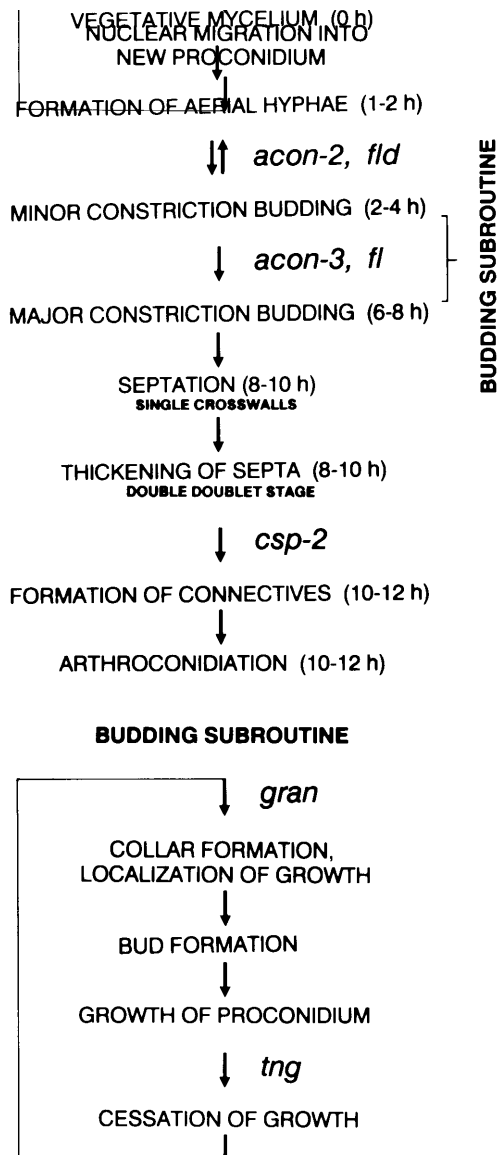


Fig. 7a-c. Scanning electron micrographs of developing wild-type conidiophores. a Major constriction chains. *Double line arrows* point to major constrictions; *small arrows* point to minor constrictions. b Combined dark field and Calcofluor fluorescent images of a conidiophore with thickened cross walls (double-doublet stage). c Connective between two mature conidia. (After Springer and Yanofsky, 1989)

Fig. 6. Developmental timeline of conidiation. The timeline is divided into two parts: the conidiophore timeline, which applies to the entire conidiophore as it is developing, and the budding subroutine, which occurs every time a proconidial chain buds. The approximate time at which each step in the conidiophore timeline begins after induction is indicated in parentheses. The step at which conidiation is blocked in the mutant strains *acon-2*, *acon-3*, *csp-2*, *fl*, *fld*, *gran* and *tng* is indicated. (After Springer and Yanofsky 1989)

ascospore has two nuclei formed by a mitotic division inside the ascospore; the genotype of the two nuclei is therefore identical. The four potentially different genotypes of the ascospore pairs are the result of two meiotic divisions. The ascospore

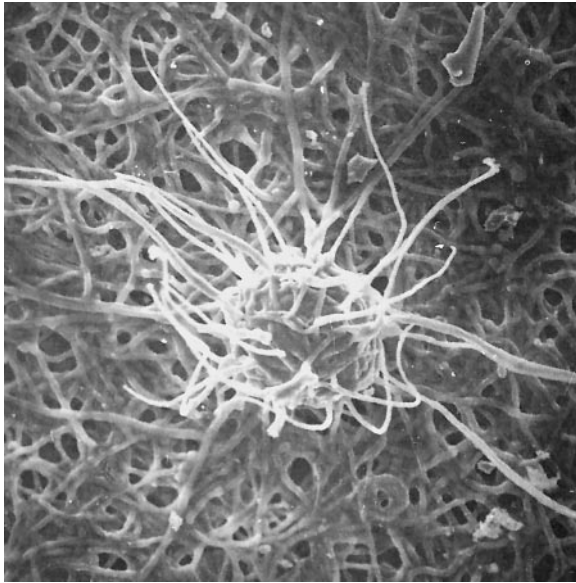


Fig. 8. Scanning electron micrograph of a protoperithecium. (Courtesy of Rudi Lurz and Wolfgang Heimler)

pores of each ascus are ejected together through the ostiole of the perithecium.

What are the factors which influence and orchestrate hyphal elongation/branching, the eventual formation of the asexual dispersal units or structures involved in the sexual reproductive cycle? It is now clear that these developmental changes are governed by both genetic and environmental factors and that the contribution of the genetic program and the environmental cues vary among the developmental phases and among fungal species. Some of the factors influencing development will be discussed in the second part of this chapter.

5.4

Genetic Influence on Development

5.4.1

Developmental Genes of *Neurospora*⁷

More than 250 genes which affect one or more of the morphological processes in *N. crassa* have been identified. Furthermore, genes involved in

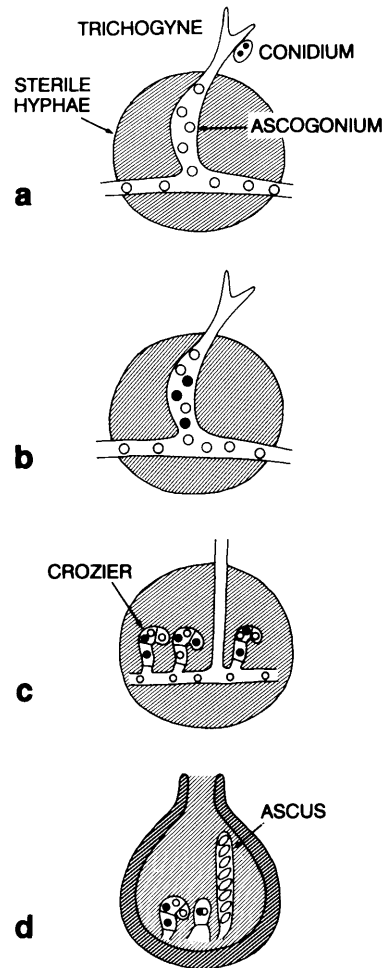


Fig. 9a-d. Schematic representation of the major stages in the development of a perithecium. a Conidium attaches to the trichogyne. Circles represent nuclei. b Nuclei of the conidium enter the ascogonium. c Croziers are formed. d Asci are formed from croziers. The drawings are not to scale

almost every aspect of fungal development are being isolated and analyzed. Clustering of some development-specific (as well as metabolic pathway) genes occurs in filamentous fungi, and repression of clustered genes through an inactive state of chromatin as a control mechanism has been suggested, yet the majority of work has focused mainly on gene structure and function and less on the significance of the physical distribu-

tion of different loci in the genome. The developmental process best characterized is conidiation (Fig. 6).

5.4.2

Gene Regulation During Conidiation⁸

As asexual spore formation is one of the most obvious and central developmental events occurring in many filamentous fungi (not all fungi have been shown to produce conidia), much effort has been invested in studying this process. Significant changes in gene expression have been detected by the comparative analyses of mRNA and protein species present in mycelial and conidiating cultures of *N. crassa*. Several genes which are transcriptionally regulated at different times after the conidiation stimulus, have been cloned. However, inactivation of most of the conidiation-specific genes isolated has not brought about observable phenotypes. It has become evident that the some of conidiation (*con*) genes examined in detail are regulated by factors other than those specific for macroconidiation. Perhaps the most extensively studied is *con-10*, which encodes a polypeptide induced during major constriction chain formation. The polypeptide accumulates in all three *N. crassa* spore types. On the one hand, *con-10* undergoes genetic control, as its expression is repressed in mycelia and activated during conidiophore development. However, it is clear that environmental factors such as light also play a role in regulation of *con-10* expression.

A second well-characterized conidiation-related gene is *eas/bli-7/ccg-1* which encodes the major coat (rodlet) protein of the conidium. The genetic isolation of the *easily-wettable* mutant preceded the later, parallel, identification of the *blue-light-regulated* and *clock-controlled* genes, all of which are allelic. Thus, this is an example of a gene which is developmentally as well as environmentally (by light or by nitrogen starvation) regulated. The protein encoded by *eas* belongs to the hydrophobin family, shown to be involved in both morphogenetic as well as pathogenetic events in filamentous fungi.

5.4.3

Protein Phosphorylation in Developmental Processes⁹

The regulation of the cellular processes includes transcriptional, translational, and post-translational modifications. It is now clear that at least some of the intracellular as well as intercellular signaling leading to fungal morphogenesis is based on hierarchies involving protein phosphorylation. The identification of these regulators and elucidating the interactions between them is one of the current foci of fungal biology.

Among the multitude of *N. crassa* mutants isolated over the decades, about 25% exhibit altered morphologies. Among them are a series of mutants which display a colonial temperature-sensitive (*cot*) phenotype. The *cot* mutants grow as tight, compact colonies at elevated (restrictive) temperatures, while at the lower, permissive, temperature conditions, they grow at variable degrees of similarity to the wild type (Fig. 10). Genetic analysis of the *cot-1* strain has shown that the *cot-1* gene encodes a ser/thr protein kinase. It has since then been determined that COT1 most probably belongs to a new subfamily of protein kinases which group together the mammalian, *Drosophila* and *C. elegans*, myotonic dystrophy-like protein kinases. The identification of a regulator of hyphal elongation is an initial step in the eventual unraveling of the entire regulatory circuit(s) involved in controlling hyphal elongation. Based on analysis of partial suppressors of *cot-1* (which have been determined to encode cytoskeletal elements), it has been suggested that COT1 kinase may be involved in the transport of precursors and enzymes needed for cell wall biosynthesis to hyphal tips. If the kinase is a regulator of vesicle transport, then it is conceivable that a mutation affecting the endocytic pathway may partially compensate for a reduction in COT1 activity.

The developmental changes occurring in some pathogenic fungi induced by a specific environment (e.g., plant surface architecture) are a prerequisite for successful pathogenesis. It is now clear that phosphorylation cascades play a role in such pathogenesis-related developmental changes. It has been suggested that sensing of rice leaf architecture by the rice blast fungus *Magnaporthe grisea* is followed by activation of adenylate

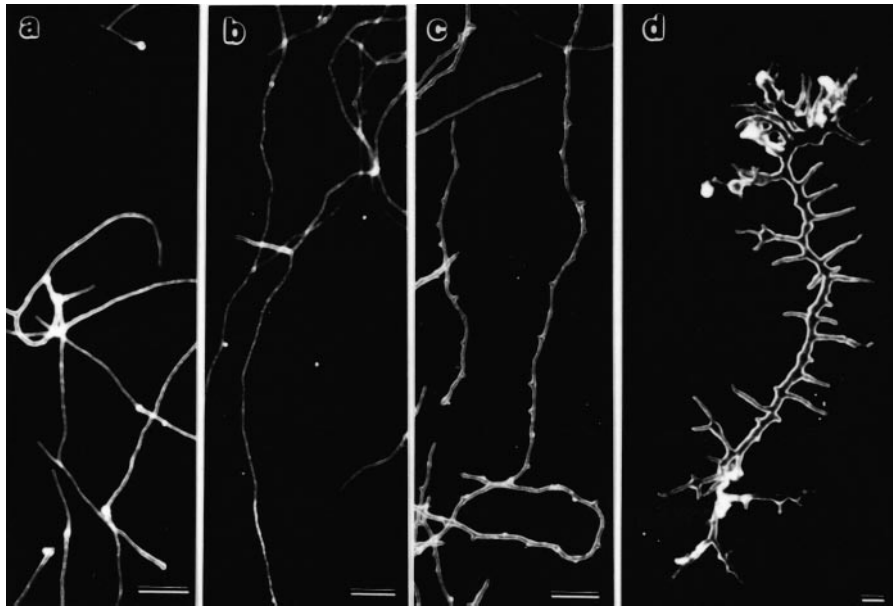


Fig. 10a–d. **a** Wild type *N. crassa* grown for 18 h at 25°C (shaking liquid culture). **b** *cot-1* grown for 18 h at 25°C (the permissive temperature for this temperature-sensitive mutant). **c** *cot-1* grown for 16 h at 25°C, followed by 2 h of growth at 37°C (the non-permissive temperature). Note the uniform initiation of multiple branches along the hyphal

filaments, resulting in a “barbed-wire” morphology. **d** A 30-h culture of *cot-1* grown in liquid on a microscope slide at 37°C. In time, hyphal tips originating from the newly formed branches (shown in **c**) will cease to elongate. In some case, secondary and tertiary branching occurs prior to arrest of hyphal tip growth. (After Yarden et al. 1992)

cyclase which elevates the intercellular levels of cAMP. The increase in cAMP levels stimulates early infection structure development events modulated by a cAMP-dependent kinase. The later events in the penetration and infection processes are stimulated by a mitogen-activated protein (MAP) kinase cascade, presumably through a MAP kinase module that may respond to the cAMP signal.

5.5 Epigenetic Regulation of Fungal Gene Expression¹⁰

Developmental gene expression and environmental influence on gene expression can be altered, permanently or reversibly, by changes in the DNA. As early as the mid 1950s, Waddington used the term epigenetics to indicate the presence of a developmental regulation mechanism which

functions beyond that of known hereditary genetics. Some 20 years later, cytosine methylation was suggested to be involved in gene regulation in a manner which can be mitotically inherited, thus providing a mechanism for the involvement of epigenetics in development. It has since then been established that epigenetic mechanisms are present in a variety of eukaryotes (fungi, plants, animals). In organisms lacking DNA methylation (e.g., yeasts, *Drosophila*) the epigenetic mechanisms are obtained through higher-order chromosome structure. The presence of epigenetic mechanisms has, in part, been revealed along with the increased use of DNA-mediated transformation procedures, during which external DNA is introduced into the cell. It is through a number of repon suppression of transgene expression that the role of epigenetic influence on gene expression has gained attention also in fungi. This phenomenon, linked with the apparent requirement for multiple gene

copies was described in *Neurospora* and called reversible inactivation. In plants it was termed cosuppression or RIGS (repeat-induced gene silencing). In some cases, RIGS appears to occur at the transcriptional level and is correlated with cytosine methylation (on DNA methylation see also Chaps. 25, 26, and 27). Differential cytosine methylation during vegetative and sexual growth stages of *N. crassa* have been reported. A similar phenomenon of suppression of transcription by methylation during asexual growth phases of the phylogenetically related fungus *Ascobolus immersus*, has also been demonstrated. The acronyms RIP (repeat-induced point mutations) and MIP (methylation induced premeiotically) have been coined to describe DNA methylation-related phenomena in *N. crassa* and *A. immersus*, respectively.

Two genes encoding methyltransferases have been analyzed in *A. immersus*: a maintenance methyltransferase which methylates hemimethylated DNA and a de novo methyltransferase (the first of its kind isolated from any eukaryote) which acts on unmethylated DNA. Disruption of the latter has demonstrated that activity of this transferase is not required for the normal vegetative growth of the fungus, yet is required for development of the sexual fruiting bodies which are essential for sexual phase development. Thus, it appears that methylation can play a role during fungal development.

The RIP phenomenon is not limited to conferring epigenetic effects. In contrast to MIP, RIP can be accompanied by irreversible gene inactivation due to mutational changes introduced to all copies of the duplicated DNA fragments, demonstrating that RIP involves both epigenetic as well as mutational effects. As mentioned above, MIP and RIP were identified following the duplication of DNA in the fungal genome by transformation. What is the natural role of these processes? A possible explanation is the transient (via methylation) or permanent (via mutation) safeguarding of the haploid genome from selfish and redundant DNA, while at the same time generating new genetic material for evolution. Even though the major emphasis in fungal epigenetics research has been put on the role of methylation, not all transgene silencing in *N. crassa* is due to this phenomenon. Recently, DNA-RNA or RNA-RNA interac-

tions have also been suggested to be involved in affecting transgene expression.

There is ample evidence for the presence of the machinery which can confer epigenetic effects on fungal gene expression. Whether or not epigenetic influence is limited to interference with transgene DNA expression or also plays a direct role in gene expression during development, has yet to be determined.

5.6 Environmental Influence on Development¹¹

The microenvironment in which microorganisms grow can change rapidly in time and space. It is therefore understandable that microorganisms have evolved mechanisms which sense a changing environment. Nutrient depletion, for example, is a stimulus for differentiation in a variety of organisms including *Bacillus subtilis*, *Streptomyces coelicolor*, *Dictyostelium discoideum*, yeasts, *Aspergillus*, plants, and animals. Two main strategies have evolved to survive adverse conditions: first, the ability to produce dormant cells which resist environmental stresses and initiate growth once favorable conditions are available; and second, the ability to sense the conditions which would favor wide dispersion of such spores, thereby escaping from a potentially unfavorable environment. Compared to mycelium, both conidia and ascospores are more resistant to a variety of environmental stresses such as adverse temperature, radiation, chemicals, and starvation. It is therefore understandable that changes in environmental conditions can serve as cues for changes in sexual as well as asexual differentiation.

Normally, *N. crassa* mycelium does not differentiate in submerged culture. A mycelial culture can be induced to conidiate by filtering it onto filter paper and letting it stand to dry. With this method, aerial hyphae and conidia are produced in about 6 h. The actual stimuli are not known. Using this technique, it is possible to show that humidity and, to some extent, light have an influence on conidiation. It is possible to induce conidiation in liquid culture by nitrogen or carbon limitation up to the point exhibited in Fig. 7a. Under such conditions the conidia may be

formed directly on the hyphae with a lag time of 2 h. Conidiogenesis on solid medium is inhibited by as little as 0.2% CO₂. Aging of mycelium induces formation of microconidia on vegetative hyphae. Nitrogen starvation is essential for this differentiation process while a short pulse of blue light in nitrogen limiting conditions can accelerate the process by a factor of 30. Blue light is a signal which induces a variety of physiological responses not only in *N. crassa* but also in several other microorganisms, plants, and animals. The major physiological responses in *N. crassa* include carotenoid production, phototropism, and developmental changes.

The regulation of the developmental switch governing the change from hyphae to conidiation is not uniform in all fungi and is far from being fully understood. The interrelationship between genetic and environmental mechanisms which influence such switches varies among fungi. In *N. crassa*, the growth (mycelial phase) and the major asexual reproductive phase (macroconidiation) processes are separated in time, whereas in *A. nidulans*, both events can occur in the same colony at the same time (yet only spatially separated). In addition, the developmental competence (composed of genetic and environmental prerequisites) required for initiation of conidiation in *A. nidulans* has not been observed in *N. crassa*. Thus, initiation of macroconidiation in *N. crassa* is strictly controlled by environmental conditions.

5.6.1

Nutrient Source¹²

In *Neurospora*, nutritional changes can trigger switches in fungal development. Carbon source deprivation induces conidiation as well as protoperithecia production and nitrogen deprivation inhibits conidiation and stimulates the initiation of the sexual sporulation pathway. Several genes are regulated by blue light as well as by nitrogen starvation.

5.6.2

Temperature

The diversity of the fungal kingdom is also evident by the broad spectrum of temperatures at which various fungal species can survive, grow, and reproduce. The minimal, maximal, and optimal temperatures vary among species and for the different aspects of the fungal life cycle. Thus, in the case of *N. crassa*, which is a mesophilic fungus (in contrast to fungal species exhibiting optimal growth at extreme temperatures), the growth profile spans a range of 4–45 °C with a maximal linear growth rate at 34 °C. However, variation in temperature (between 4 and 37 °C) has no effect on conidiation. In contrast, production of protoperithecia is sensitive to temperature, the optimum being between 18 and 26 °C. Perhaps the most pronounced heat-dependent developmental phenomenon which occurs during the *N. crassa* life cycle is the heat-induced germination of the sexual propagules (ascospores), which are dormant until the proper environmental cue (heat or exposure to certain chemicals) is available. The sighting of *Neurospora* in the wild has often been associated with fire. The heat-induced activation of ascospore germination in nature is mimicked under laboratory conditions by exposing the spores to 60 °C for a period of 45 min.

5.6.3

Light¹³

Fungal photosensitivity was documented as early as the middle of the 19th century. Payen, and later Pasteur, described the outbreak of the orange bread mold in Paris bakeries and reported a linkage between light and the presence of an orange color in what may have been one of the first experiments in photobiology. Some 50 years later, Went, a Dutch plant physiologist, discovered that the orange color was due to the presence of carotenoids and that blue light was the active region of the light spectrum that triggered carotenoid accumulation. Since then, the involvement of light in growth and development (e.g., hyphal cell branching frequencies, asexual spore formation, and fruiting body formation, and development) of a variety of fungal species has been documented.

In *Neurospora* and many other microorganisms, blue light regulates a series of physiological responses. In *N. crassa*, blue light responses include carotenoid production, the circadian rhythm of conidiation (in the *band* mutant it can be synchronized and shifted), phototropism of perithecial necks, and protoperithecia production. Blue light is a signal which can be given in a short pulse (60 s) to induce protoperithecia formation. The fungus is ideal for studying gene regulation during differentiation because the illuminated and dark-grown cultures can be compared. Another fungus, *A. nidulans*, conidiates freely only after exposure of hyphae to red light. Induction is partially reversible by exposure to far red light, similar to phytochrome-mediated responses in higher plants.

In *N. crassa*, where regulation of transcription by blue light has been extensively studied, 3 to 4% of genes that are expressed during vegetative growth are blue light-regulated within 30 min following irradiation. This represents 70–200 of the 2000–6000 genes estimated to be expressed in the vegetative mycelium. The regulation is time-staggered, the first gene being regulated after a 2-min lag, the last one after 20 min, with increases of up to 100-fold in the abundance of light-induced gene transcripts. Over a dozen genes which are blue light-regulated have already been cloned and sequenced. Among them is the COT1 kinase-encoding gene (described above), in which light affects both transcript abundance and transcript size.

Carotenoid biosynthesis was a rational choice to use as an initial tool for the molecular analysis of the light response in fungi. The isolation of mutants with altered carotenoid biosynthesis and the identification of the genes involved provide access to the genetic elements mediating light signal transduction. Even though in *N. crassa* carotenoids are produced both in hyphae and in conidia, only the production of pigment in hyphae is light-dependent. Thus, mutants which produce pigmented conidia yet lack carotenoids in hyphae, even when grown in the light, are prime candidates for identifying genes which are likely to be involved in regulation of light signal transduction. Such mutants, designated *white collar* (*wc*), were initially isolated in David Perkin's laboratory at

Stanford University and were shown later to be essential for photocarotenogenesis, phototropism, and photomorphogenesis in *N. crassa*. It has since then been determined that the *wc-1* and *wc-2* genes, both global regulators of photoresponses in *Neurospora*, encode DNA-binding proteins that contain structural motifs which are known to be shared among polypeptides involved in light signaling and circadian rhythmicity, and are believed to act as transcriptional activators. Analogous genes from other fungi as well as additional *N. crassa* mutants which show a reduced sensitivity to light have also been isolated. The presence of multiple light-responsive genes which are not necessarily set in a single hierarchical structure (consisting of a single pathway) and involve a variety of different cellular activities suggest that different photoreceptors and different (though possibly converging) transducing pathways are present in filamentous fungi.

One of the most striking examples of a light-mediated developmental response is the circadian rhythm. This topic will be discussed in Chapter 32.

5.7 Outlook

Morphogenetic pathways of *N. crassa* can be activated through external stimuli and the same stimuli regulate several genes or pathways. Thus, nutrient composition, air, light, and heat are involved in the vegetative as well as sexual developmental stages of this organism. In other fungal species, such as *A. nidulans*, an existing developmental program is superimposed on environmental control (even though environmental conditions can influence it). We know that hundreds of genes are necessary for proceeding through the different developmental pathways. The functions of the majority of the genes involved are not yet known. Furthermore, the mechanisms of environmental stimulus perception and eventual transduction of the signals within (and among) fungal cells is far from clear. *Neurospora* could prove invaluable in the process of identifying and analyzing the nature of the blue light photoreceptor(s). As the physical mapping and genomic sequencing of fila-

mentous fungi will be completed in the foreseeable future, a major research focus will be to analyze the functions of the isolated genes. Obtaining a better understanding of the role(s) of epigenetics during fungal growth and development would advance our overall perception of gene expression regulation. As both the environmentally induced as well as the preprogrammed developmental processes (which can be environmentally influenced) are complex and at times converge or interlink, elucidating the hierarchies, feedback loops and cross-pathway interactions will provide an exciting challenge for fungal biologists. The fruits of such studies will most probably span well beyond the fungal kingdom.

5.8 Summary

The fungal kingdom is comprised of a plethora of diverse species yet with basic attributes common to most eukaryotes. *Neurospora crassa* is a filamentous fungus with both an asexual and a sexual life cycle. The nucleus is haploid with a DNA content of approx. 4×10^7 bp, about ten times more than *E. coli*. There are more than a dozen different morphological structures which are produced following environmental or preprogrammed stimuli. More than 700 genes are mapped and about 250 nuclear genes influence one or more steps in morphogenesis. Differentiation of both cycles can be regulated by environmental stimuli, i.e., carbon and nitrogen source composition, heat, desiccation and blue light. Some environmental signals which induce conidiation or protoperithecia formation regulate hundreds of genes. The identification, isolation of genes involved in development, following the structural and functional analyses of these genes has set the stage for understanding fundamental processes in fungi and higher eukaryotes.

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