REVIEW

Conidiation in *Neurospora crassa*: vegetative reproduction by a model fungus

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Abstract

Asexual development, conidiation, in the filamentous fungus *Neurospora crassa* is a simple developmental process that starts with the growth of aerial hyphae. Then, the formation of constrictions and subsequent maturation gives rise to the mature conidia that are easily dispersed by air currents. Conidiation is regulated by environmental factors such as light, aeration and nutrient limitation, and by the circadian clock. Different regulatory proteins acting at different stages of conidiation have been described. The role of transcription factors such as FL, and components of signal transduction pathways such as the cAMP phosphodiesterase ACON-2 suggest a complex interplay between differential transcription and signal transduction pathways. Comparisons between the molecular basis of conidiation in *N. crassa* and other filamentous fungi will help to identify common regulatory elements.

Keywords Asexual development · Conidiation · *Neurospora* · Sporulation

Introduction

Neurospora crassa is an ascomycete heterothallic filamentous fungus that grows as branched multinucleated hyphae with perforated septa. In nature, species of *Neurospora* have been found in a wide range or areas that include tropical, subtropical and temperate regions, and the fungus is easily detected growing on the surface of fire-scorched vegetation because of its ability to metabolize cellulose and the activation by heat of the sexual spores (ascospores) (Jacobson et al. 2006; Jacobson et al. 2004; Luque et al. 2012; Turner et al. 2001). *N. crassa* has been used as a model organism for the research on several aspects of eukaryotic biology, including the mechanism of recombination, genome defence by RNAi, circadian clock regulation and light sensing (Davis and Perkins 2002;

Perkins and Davis 2000; Roche et al. 2014) The life cycle of *N. crassa* includes asexual reproduction and the development of vegetative conidia that are easy to disperse. The developmental processes that lead to conidiation in *N. crassa* are, however, very different from those regulating conidiation in *Aspergillus nidulans*, despite both being members of the Ascomycota. Here, we review our knowledge of the regulation, the genetics and molecular basis of conidiation in *N. crassa*. We propose that the simplified mode of vegetative reproduction in *N. crassa* and related fungi may help to understand asexual development in other ascomycetes with more complex conidiation pathways.

Morphological events during conidiation in *Neurospora crassa*

There are two pathways for asexual reproduction in *N. crassa*: macroconidiation and microconidiation. Macroconidiation (henceforth conidiation) is induced by the transfer from a liquid to an air interface, desiccation and nutrient depletion, and is influenced by several environmental factors such as light and CO_2 levels, and by the circadian rhythm (Springer 1993). Conidiation is a very simple developmental process. The induction of conidiation promotes a change in the direction of growth of the vegetative hyphae that grow away from the substrate, and leads to the formation of a mass of aerial

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hyphae. About 4 h after conidial induction, hyphal growth changes from apical elongation to apical budding leading to the formation of chains of proconidia that are divided by minor constrictions. Budding continues in proconidial chains, and major constrictions appear approximately 8 h after the induction of conidiation. This results in the separation of each proconidia during their maturation into full conidia (Fig. 1A). Interconidial junctions are cleaved several hours later, but fragile connective threads hold conidia together until they are dispersed by wind currents (Springer 1993; Springer and Yanofsky 1989). The entire process of conidiation takes between 12 and 24 h. However, conidia undergo a biochemical maturation period of several days before they are able of efficient germination (Fig. 1B).

The other asexual reproduction pathway, microconidiation, is often observed in old cultures. Microconidia are uninucleated spores morphologically and developmentally different from macroconidia. It has been shown that mutants affected in macroconidiation are not affected in microconidiation, suggesting that both pathways are independent and should have few common regulatory elements, if any (Maheshwari 1999). Microconidia emerge from a protuberance in the vegetative hyphae which constricts until microconidia are liberated. Externally, microconidia are smaller than conidia and do not contain carotenoids in their cell wall. They are less viable than macroconidia (Springer

1993; Springer et al. 1992; Springer and Yanofsky 1989). In addition, microcycle conidiation has been observed in wild type isolates of *N. crassa*, but the genetics and morphological characterization have been limited (Maheshwari 1991).

The regulation of conidiation

The process of conidiation is regulated by a number of environmental factors and endogenous signals that should be properly coordinated by the fungus in order to produce conidia when environmental conditions are appropriate for vegetative reproduction. Conidia formation is normally observed when the fungus is exposed to air, but carbon starvation can induce conidiation in submerged mycelia (Madi et al. 1994; Madi et al. 1997). In *N. crassa* and other fungi, developmental cycles are often initiated by the same environmental stresses that induce the accumulation of high levels of trehalose, including carbon starvation, increased temperature and desiccation (Li et al. 1997; Sargent and Kaltenborn 1972; Springer and Yanofsky 1992; Sun et al. 2011). Moreover, the process of development itself is considered to cause physiological stress on organisms. In mature conidia, higher levels of trehalose and stress response proteins may be required for resistance and survival (Häfker et al. 1998; Rensing and Monnerjahn 1998). Thus, trehalose may play a dual role in the cell, functioning as a reserve carbohydrate for future hyphal growth and

Fig. 1 (A) Developmental timeline of conidiation in *Neurospora crassa*. (B) Different stages of conidial development visualized by scanning electron microscopy (a, b) and fluorescence microscopy (c, d, e, f): histone H1-GFP for nuclei visualization and calcofluor white for cell wall staining. Arrows indicate major and minor constrictions (b), hyphal septa (a, e) and interconidial junctions (f)
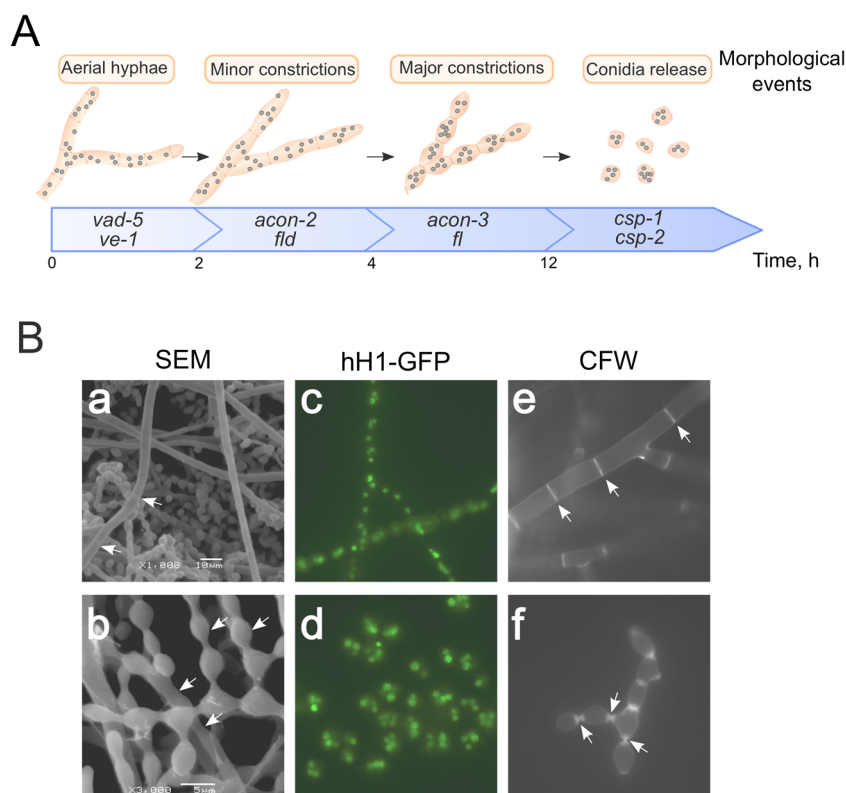
as a stress protectant. The mechanism of induction of conidiation after the transition to an air interphase is not well understood but it has been proposed that the development of conidia helps the fungus to cope with the excess of damaging oxygen radicals (Hansberg et al. 1993; Peraza and Hansberg 2002).

Light regulates conidiation (Springer 1993), and the regulation by light of conidiation requires the activity of proteins WC-1 and WC-2 (Lauter and Russo 1990). WC-1 contains a zinc finger, a chromophore-binding domain (LOV) and PAS domains for protein-protein interactions (Ballario et al. 1996; Crosthwaite et al. 1997). The chromophore-binding domain binds the flavin FAD, allowing WC-1 to act as a blue-light photoreceptor (Froehlich et al. 2002; He et al. 2002). The protein WC-2 contains a zinc finger and a PAS domain, and interacts with WC-1 (Linden and Macino 1997) to form a White Collar Complex (WCC). This complex, upon light exposure, binds transiently to the promoters of light-inducible genes to activate their transcription (Belden et al. 2007; Froehlich et al. 2002; He and Liu 2005; Smith et al. 2010), including the promoter of *fl* (*fluffy*), a gene that encodes a key regulator of conidiation. Conidiation increases in *N. crassa* cultures exposed to light suggesting that light may activate the transcription of key regulatory genes which indeed accumulate after light exposure as it has been shown for *fl* or *csp-1* among others (Belden et al. 2007; Chen et al. 2009; Olmedo et al. 2010a; Olmedo et al. 2010b).

Conidiation is regulated by the endogenous circadian clock resulting in bands of conidia every 22.5 h of growth in the dark (Dunlap and Loros 2017). The mechanism by which the clock regulates conidiation has been characterized in detail and is based on a negative feedback loop in which the positive element is the WCC which activates transcription of *frq*. FRQ is the negative element of the clock and together with its partners the RNA helicase FRH and the casein kinase-1 (CK-1) blocks its own transcription as well as the transcription of the other WCC-regulated genes (Crosthwaite et al. 1997; Dunlap and Loros 2004; Heintzen and Liu 2007). The oscillatory amount of FRQ during the circadian cycle promotes changes in the abundance, phosphorylation and activity of the WCC during growth in the dark (Brunner and Schafmeier 2006; Dunlap 2006; Dunlap and Loros 2006).

Genetics of conidiation: regulatory genes and proteins

Genetics has helped to identify some of the key elements that regulate conidiation in *N. crassa*. Several genes required for conidiation have been described and their genetic interactions have been established. Strains with mutations in *aconidiate-2* (*acon-2*) or *fluffyoid* (*fld*) are blocked in the transition from filamentous to budding growth. Mutations in *aconidiate-3* (*acon-3*) or *fluffy* (*fl*) allow the production of minor

constrictions, but development is blocked and few major constrictions between proconidia are detected. Mutations in two conidial separation genes (*csp-1* and *csp-2*) prevent the separation of the cross walls in matured conidia to release free conidia (Springer 1993). Of these genes, attention has focused on the products of genes *fl* (NCU08726), *csp-1* (NCU02713) and *csp-2* (NCU06095) that are transcription factors that should act by regulating transcription of genes that participate in the development of conidia. These genes are all light-inducible providing a connection between light regulation and conidiation (Bailey and Ebbole 1998; Chen et al. 2009; Lambreghts et al. 2009; Paré et al. 2012; Smith et al. 2010).

The *fl* gene has been characterized in detail. The FL protein is a 792-amino acid polypeptide containing a Zn_2Cys_6 binuclear zinc cluster domain belonging to the Gal4p family (Bailey and Ebbole 1998). Mutations in *fl* block conidiation at the formation of minor constrictions, few hours after the induction of conidiation (Springer and Yanofsky 1989). *fl* mRNA accumulates in aerial hyphae where conidiation-specific genes are expressed suggesting an important role for FL in the expression of those genes (Bailey-Shrode and Ebbole 2004). However, it has been also found that *fl* mRNA accumulates shortly after induction of conidiation suggesting an additional role for FL in the formation of aerial hyphae (Correa and Bell-Pedersen 2002). The importance of FL as one of the major regulators of conidiation in *N. crassa* relies on the observation of conidial development when *fl* is overexpressed in submerged vegetative hyphae (Bailey-Shrode and Ebbole 2004). The overexpression of *fl* in vegetative hyphae leads to the expression of *eas* (Bailey-Shrode and Ebbole 2004), the gene for the hydrophobin rodlet protein located on the surface of matured conidia (Bell-Pedersen et al. 1992; Lauter et al. 1992). This observation supports the described binding of FL to the *eas* promoter (Rerngsamran et al. 2005). Other regulatory genes have been found to be upregulated when *fl* is overexpressed in vegetative mycelia, including the conidiation-specific genes *con-6* and *con-10* (Rerngsamran et al. 2005) supporting the proposal of FL as a conidiation-specific transcription factor. It has been described that the aconidial phenotype of a *fl* mutant can be partially suppressed by mutation in the gene *vib-1*, a transcription factor that is involved in the regulation of heterokaryon incompatibility. This would suggest that FL may regulate conidiation through the repression of VIB-1 (Xiang and Glass 2002). The *fl* gene is directly activated by light through the binding of the WCC to a light regulatory element on its promoter located at position −640 from initiator ATG (Olmedo et al. 2010a). *fl* mRNA accumulates rhythmically in an ACON-2-dependent manner (Correa and Bell-Pedersen 2002), supporting the proposal that the rhythmic production of conidial bands requires the rhythmic accumulation of *fl* mRNA.

Other conidiation mutants have been isolated and characterized. ACON-2 is a cAMP phosphodiesterase, but its role in the regulation of conidiation remains to be investigated in detail (Greenwald et al. 2010), and FLD has been proposed to be a transcription factor (Carrillo et al. 2017; McCluskey et al. 2011). ACON-3 is a protein without any identified domain and is the homologue of MedA, a protein that participates in the regulation of conidiation of *Aspergillus nidulans* (Chung et al. 2011). It is interesting to note the abundance of transcription factor mutants that are blocked in conidiation. CSP-1 is a light-inducible zinc finger transcription factor (Lambreghts et al. 2009; Smith et al. 2010) and CSP-2 was identified as a light-regulated grainy head-like transcription factor (Colot et al. 2006; Paré et al. 2012). CSP-2 is involved in the development and remodelling of the cell wall and plays a role in the activation of genes related with defence and virulence (Paré et al. 2012). A third transcription factor, FLB-3, has been recently characterized. It is the *N. crassa* homologue of FlbC, a transcription factor that participates in the regulation of *A. nidulans* conidiation. The *flb-3* mutant is blocked in conidiation, shows altered sexual development and is unable to complete the sexual cycle (Boni et al. 2018). These results suggest that FLB-3 plays a key role in the transcriptional coordination between asexual and sexual reproduction.

Transcriptional regulation during conidiation

The discovery that several transcription factors regulate conidiation suggested a key role in transcriptional regulation during conidiation. In addition, several genes have been found to be highly expressed during conidiation (Roberts et al. 1988) although their mutants do not have a clear developmental phenotype. The *con* genes of *N. crassa* are preferentially expressed during conidiation but some of them are also induced by light in vegetative mycelia in a WC-dependent manner (Corrochano et al. 1995; Lauter and Russo 1991; Madi et al. 1994). The genes *con-10* and *con-6* contain several regulatory elements in their promoters that are responsible for their transcriptional regulation by development, light and the circadian clock (Corrochano et al. 1995; Lee and Ebbole 1998; Olmedo et al. 2010b). Similar complex regulation has been observed for genes *con-5* and *con-13*, since they are regulated by light and conidiation although their promoters have not been extensively characterized.

The availability of the *N. crassa* genome sequence made possible the creation of a gene knockout mutant collection for almost all the genes identified in the genome (Colot et al. 2006; Dunlap et al. 2007). The viable knockout mutants have been analysed and their developmental phenotypes have been characterized (Borkovich et al. 2004; Carrillo et al. 2017; Colot et al. 2006). Several strains bearing mutations in genes coding for transcription factors have shown defects in

different stages of the *N. crassa* life cycle. Most of them were found to have impaired asexual sporulation although the correlation between the phenotypes and gene expression during conidiation has not been stablished for most genes yet (Carrillo et al. 2017).

In a few cases, the role of transcriptional regulators on conidiation has been investigated. Mutations in the Gal4-like transcription factor VAD-5 or the velvet protein VE-1 reduce the growth of aerial hyphae (Bayram et al. 2008; Sun et al. 2012). The gene *chc-1* encodes a protein with a helix-loop-helix binding domain and the mutant shows enhanced conidiation, in particular under high CO_2 (Sun et al. 2011).

Signal transduction pathways that regulate conidiation

The key role of the cAMP phosphodiesterase ACON-2 in conidiation indicated that other signal transduction pathways participated in the regulation of conidiation in addition to the transcriptional regulation of conidiation genes.

Conidiation in *N. crassa* is also regulated by signalling pathways based on heterotrimeric G proteins and G protein-coupled receptors (GPCR). G proteins play key roles as signalling proteins in eukaryotes where they form heterotrimers composed of α, β and γ subunits which are associated with the plasma membrane (Neves et al. 2002; Won et al. 2012). The Gα subunit binds GTP and GDP and hydrolyses GTP to GDP, and the Gβ and Gγ subunits form a dimer. In the inactive state, the Gα subunit binds GDP and the three subunits are present in a complex in association with a GPCR. Ligand binding to the GPCR leads to exchange of GTP for GDP on the Gα protein and dissociation of the Gα and Gβγ dimer. Both the Gα-GTP and Gβγ moieties regulate downstream effector proteins in various systems, including ion channels, adenylyl-cyclases, phosphodiesterases and phospholipases. GTP hydrolysis on the Gα subunit allows the GDP-bound Gα to reassociate with the Gβγ dimer and the GPCR at the membrane, ready to reinitiate the signalling cycle (Li et al. 2007).

Neurospora has three Gα subunits (GNA 1–3), one Gβ protein (GNB-1), one Gγ protein (GNG-1) and several predicted GPCRs (Li et al. 2007). GNA-1 was the first identified G protein subunit in filamentous fungi, and participates together with the GPCR GPR-4 and the protein kinase A (PKA) in the regulation of carbon source-dependent apical growth and asexual development (Ivey et al. 1996; Ivey et al. 2002; Ivey et al. 1999; Li and Borkovich 2006; Turner and Borkovich 1993). GNA-3 regulates conidiation via modulating a cAMP-dependent pathway and acts as a negative regulator of conidiation (Kays and Borkovich 2004; Kays et al. 2000). The GNB-1 (Gβ)/GNG-1(Gγ) dimer is required for the stability of Gα proteins and acts as a repressor of

conidiation (Krystofova and Borkovich 2005; Yang et al. 2002). Recent studies have demonstrated that RIC-8 (resistant to inhibitors of cholinesterase) positively regulates Gα subunits, GNA-1 and GNA-3, and deletion of *ric-8* results in defects in growth and asexual development. Mutations that activate the GNA-1 and GNA-3 proteins in the Δ*ric-8* background partially suppress Δ*ric-8* phenotypes. RIC-8 interacts with GNA-1 and GNA-3 in the yeast two-hybrid assays and acts as a GEF (guanine nucleotide exchange factor) for GNA-1 and GNA-*3* in vitro (Wright et al. 2011). The phenotypes of mutants in 36 GPCRs have been analysed. Mutations in 14 GPCR genes lead to alterations in conidiation, specifically in the formation of aerial hyphae. About half of the mutants showed a reduction, and half of the mutant had an increase in the amount of aerial hyphae. Several mutants had pleiotropic effects on development suggesting cross regulations between hyphal growth, sexual and asexual development in *N. crassa* (Cabrera et al. 2015).

Mutants in components of other signal transduction pathways show alterations in conidiation. Mutants in genes coding for calcium signalling components suggest a role for calcium signalling in conidiation (Barman and Tamuli 2017). The GTP-binding cytoskeletal proteins septins participate in cell polarity and the strains with deletions in septin genes show alterations in conidiation, among other developmental phenotypes (Berepiki and Read 2013).

In addition, kinases participate in the regulation of conidiation. The NDR kinase COT-1 regulates hyphal branching and interacts with several proteins that modulate its regulatory activities. The interacting proteins include MOB2A/B, the arginine methyltransferase SKB1 and the regulatory subunits of the protein phosphatase 2A. Mutations in these proteins lead to changes in the regulation of COT-1 activity and alterations in conidiation, suggesting that they play a regulatory role in this developmental process (Dvash et al. 2010; Feldman et al. 2013; Shomin-Levi and Yarden 2017; Ziv et al. 2013). The mutant in the second NDR kinase, DBF-2, shows alterations in the growth of aerial hyphae and conidiation (Dvash et al. 2010), and a similar phenotype is observed in the mutant in the putative histidine kinase DCC-1 (Barba-Ostria et al. 2011).

Comparison of conidiation in *N. crassa* with the asexual program of other ascomycetes

The widespread occurrence of conidiation in ascomycetes highlights the importance of this process during the evolution of this group of fungi (Berbee and Taylor 2001). *N. crassa* and *A. nidulans* diverged more than 300 million years ago (Taylor and Berbee 2006; Taylor and Ellison 2010). Conidiation in *A. nidulans* has been investigated in detail and several mutants affected on conidiation have been characterized (Park and Yu 2012). These mutants provide the tools to address questions about the evolution of conidiation. Morphologically, asexual development in *A. nidulans* is significantly different from that in *N. crassa*. In *A. nidulans*, conidiation begins with the formation of a foot cell and is followed by successive emergence of the stalk, vesicle, metulae, phialides and finally aerial spores or conidia (Park and Yu 2012). The master regulator of conidiation in *A. nidulans* is encoded by the gen *brlA* (*bristle*). Although it has no sequence similarity with *fl*, the activation of *brlA* transcription is sufficient to induce conidiation, and in a similar manner to *fl*, *brlA* is activated by light in a WC-dependent manner (Mooney and Yager 1990; Ruger-Herreros et al. 2011). BrlA activates transcription of another regulatory gene, *abaA* (abacus). Unlike *brlA*, overexpression of *abaA* does not induce conidiation but does cause an arrest of vegetative growth and induction of genes normally observed during conidiation (Adams et al. 1990; Adams et al. 1998). There are many other genes acting upstream of BrlA and whose mutation gives rise to aconidial phenotypes. Most of these genes are conserved in the Aspergilli (Ojeda-Lopez et al. 2018). Some of these genes are also found in the *N. crassa* genome (Fig. 2) and have been found to complement the defect on sporulation on the corresponding mutants in *A. nidulans* (Table 1) (Chung et al. 2011; Shen et al. 1998). Despite the presence of several homologous genes between *A. nidulans* and *N. crassa*, their conidiation pathways differ in both their regulation and morphology, highlighting the simplicity of the conidiation program in *N. crassa* (Fig. 2).

Conclusion

Conidiation in *N. crassa* has a complex regulation but is a simple developmental process when compared with vegetative reproduction in other filamentous fungi. The isolation of aconidial mutants and the identification of the genes involved showed that differential transcription and signal transduction pathways have key roles in conidiation. The role of environmental regulation in conidiation is less understood. The role of light on the regulation of conidiation by controlling transcription of *fl* and other key regulatory genes has been proposed, but the connection between nutrient sensing and conidiation, and how the transition from a liquid to an air interphase activates conidiation remain to be investigated in detail. It is possible that some of these environmental signals are sensed by any of the GPCRs that have been described in *N. crassa*. The *N. crassa* genome sequence and the collection of single mutants with deletions in most of the genes in the genome have provided a large collection of mutants with altered conidiation. The large number of conidiation mutants suggests that altering conidiation is not very difficult, but identifying key regulators, like FL or ACON-2, required genetic screens and
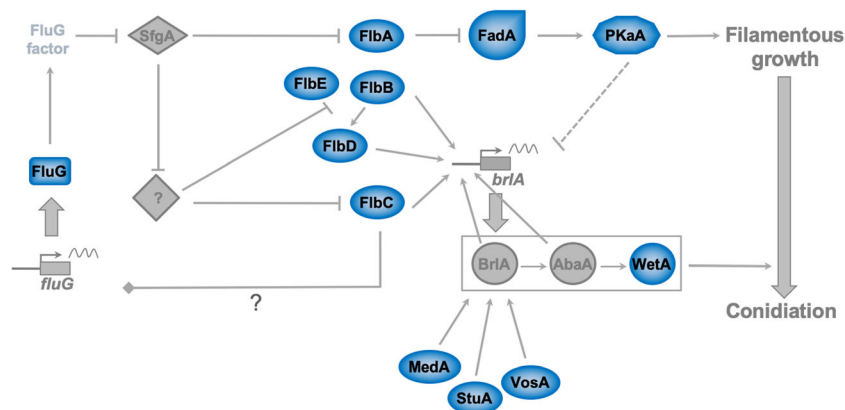
Fig. 2 Comparison between the regulatory genes controlling conidiation in *A. nidulans* (in grey) vs. *N. crassa* (in blue). Conidiation in *A. nidulans* is controlled by the master regulator *brlA* which is necessary and sufficient to induce asexual development. Different pathways lead to the transcriptional activation of *brlA*: a protein kinase A-dependent cascade which promotes filamentous growth is blocked by the action of FluG factor. At the same time, several *fluffy* genes (*flb* genes) directly activate transcription of *brlA*. In this scenario, many of the genes important for conidiation in *A. nidulans* are also present in *N. crassa* genome although the morphological and regulatory processes in both fungi substantially differ.

strong mutant phenotypes, not a reduction or increase in conidiation. Future work should focus on identifying new key regulators of conidiation perhaps by looking for conditional aconidial mutants.

Table 1 Homologous genes between *A. nidulans* and *N. crassa* and the corresponding phenotype of the knockout mutants

Aspergillus nidulans	Neurospora crassa	Phenotype of the N.crassa mutant
sfgA	–	–
fluG	NCU04264	Wild type
flbA	NCU08319	Wild type
flbB	NCU07379, tcf-5	Wild type
flbC	NCU03043, flb-3	Aconidial
flbD	NCU01312, rca-1	Wild type
flbE	NCU05255	Unknown
fadA	NCU06493, gna-1	Wild type
pkaA	NCU06240, pkac-1	Pleiotropic
stuA	NCU01414, asm-1	Short aerial hyphae
vosA	NCU05964, vos-1	Reduced conidiation
medA	NCU07617, acon-3	Aconidial
brlA	–	–
abaA	–	–
wetA	NCU01033	Wild type
–	NCU8726, fl	Aconidial
–	NCU9739, fld	Aconidial
AN1251	NCU2713, csp-1	Conidial separation defective
AN4878	NCU06095, csp-2	Conidial separation defective
pdeB	NCU00478, acon-2	Aconidial

The comparison of the conidiation pathways in *N. crassa* and *A. nidulans* shows a few common features like the regulation by light of key transcriptional regulators, and the key role of transcriptional regulation, but many differences like those in morphology and the use of specific proteins in each pathway. It seems that there is not a basic conidiation pathway shared by all ascomycetes but different developmental solutions based on differential transcription and common signal transduction pathways to the problem of how to develop conidia. However, we expect that further characterization of conidiation in *N. crassa* and comparison with other filamentous fungi will help to identify common elements in the regulation of fungal development.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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