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Review article

Hyphal branching in filamentous fungi

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ABSTRACT

In filamentous fungi, the formation of hyphal branches is a critical process that supports the ability of mycelia to radiate across and colonize growth substrates. Branching can occur at hyphal tips (apical branching) or from subapical hyphal compartments (lateral branching). The primary focus of this review is on lateral branching. Current understanding of the physiological and molecular mechanisms that underlie the formation of lateral branches is summarized. This includes emphasis on the spatial regulation of branch formation as well as the roles of the morphogenetic machinery in branch emergence. An improved understanding of hyphal branching will provide greater insight into the morphological differentiation of fungal mycelia.

1. Introduction

A defining feature of filamentous fungi is the ability to form elongated tubular hyphae that propagate by polar extension (Steinberg et al., 2017). Depending on the phylum, hyphae may or may not be partitioned into cells by cross-walls known as septa (Stajich et al., 2009). Furthermore, individual hyphal compartments may either be uninucleate or multinucleate (Dorter and Momany, 2016). Regardless of the morphological details, hyphae typically originate from a single spore via the process of germination, whereby a dormant spore hydrates and undergoes isotropic expansion until a polarity axis is specified (Steinberg et al., 2017; Dorter and Momany, 2016; Riquelme et al., 2018). Subsequent growth along this axis leads to the emergence of a germ tube that transitions into a hypha. A single spore possesses the capacity to generate multiple hyphae that form a network referred to as a mycelium. Mycelia usually radiate outwards from the founding spore, such that nutrients are acquired at hyphal tips and then transported into interior regions to support development and formation of the next generation of spores. Accordingly, there is arguably some benefit to maximizing the number of hyphal tips within a mycelium, which is achieved through the formation of branches (Harris, 2008). Hyphal branching occurs when an existing hypha generates a new hypha via mechanisms thought to share features with spore germination. Perhaps the most significant of these features is the specification and maintenance of a new polarity axis. The resulting secondary hypha can in turn branch to form tertiary hyphae, and so on, thus dramatically increasing the density and number of tips within a mycelium.

The objective of this review is to describe the different patterns of hyphal branching in filamentous fungi and to summarize what is known about the underlying mechanisms. In addition, a new protocol for quantitative assessment of hyphal branching will be presented, along with data acquired using this approach that implicates autophagy in branching.

2. Patterns of hyphal branching and underlying physiology

Fungal hyphae display two general patterns of branching (Riquelme and Bartnicki-Garcia, 2004). Apical branching occurs when an extending hyphal tip splits into two distinct tips. This pattern is often observed in rapidly extending hyphae and could conceivably be triggered by an excessive flux of secretory vesicles towards the hyphal tip, though there is currently no direct evidence to support this view. It has been shown in *Neurospora crassa* that apical branching is preceded by slowing of hyphal extension and deformation of the tip, which is accompanied by disappearance of the Spitzenkorper (Riquelme and Bartnicki-Garcia, 2004). The latter is an apical body that is composed of vesicles, ribosomes, and microfilaments (Harris et al., 2005); it is thought to serve as a vesicle trafficking center that guides the direction of rapidly extending hyphae. Subsequent to its disappearance, two or more apical branches emerge from the deformed tip, with each possessing its own Spitzenkorper. The resulting hyphae initially extend along the same general axis of the original parent hypha. The second pattern is lateral branching, whereby new branches emerge from sub-apical compartments that by definition are separated from the hyphal tip by one or more septa (Harris, 2008). Although vesicle flux from the interior of a hypha towards the tip may also trigger the initiation of lateral branches (see 8,9), the separation of these branches from the tip implies that the events underlying their formation are independent of those occurring at the hyphal tip. Consistent with this view, lateral branching requires formation of a new Spitzenkorper to support extension of the nascent hypha (Watters et al.,

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2013). Moreover, the ability of diverse mutational and environmental triggers to differentially induce apical vs. lateral branching supports the notion that they are distinct patterns as opposed to subtle variations of a common process (Watters et al., 2013).

Filamentous fungi such as *Aspergillus nidulans* display a parasynchronous mode of nuclear division whereby mitosis in apical hyphal compartments occurs in a wave (Fiddy and Trinci, 1976a). In addition, sub-apical (or intercalary) hyphal compartments in these fungi exhibit a remarkable uniformity of size and nuclear content (e.g. (Wolkow et al., 1996)). These features enabled the study of the physiological coordination of growth, nuclear division, septation (i.e., the formation of incomplete cross-walls that separate adjacent hyphal compartments), and branch formation in these fungi (Fiddy and Trinci, 1976a). The overall process was termed the duplication cycle in reference to analogous events that occur during the cell cycle of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Harris, 1997). During the duplication cycle, the rate of lateral branch formation parallels the specific growth rate of hyphae and is also tightly correlated with number of septa (Fiddy and Trinci, 1976a). Accordingly, branching is normally confined to subapical hyphal compartments that have entered a phase of nuclear quiescence. Furthermore, the emergence of a lateral branch is closely associated with the resumption of nuclear division in these compartments. The site of branch emergence is slightly biased towards the proximal region (i.e., closest to the dominant hyphal tip) of subapical hyphal compartments (Fiddy and Trinci, 1976a). Initially, these cells only form a single lateral branch, though a second will occasionally emerge once the first branch has been separated from the subapical hyphal compartment by formation of a septum. The extent to which these observations can be broadly applied to all filamentous fungi is unclear. For example, fungi capable of rapid hyphal extension (i.e., *N. crassa*, *Ashbya gossypii*) possess far greater numbers of nuclei per hyphal compartment and much less synchrony of nuclear division compared to *A. nidulans* (e.g., (Roper et al., 2011), (Gladfelter et al., 2006)). Accordingly, coordination of branch emergence with nuclear division and septum formation is less evident, though in some cases the site of branch emergence is highly biased in that it is adjacent to an existing septum (Harris, 2008).

3. Mechanisms that underlie branch formation

3.1. General regulation of branching

Several filamentous fungi are ideally suited for the application of classical genetic approaches for gene discovery and functional characterization (The Fungal Kingdom, 2017). Nevertheless, large-scale screens for mutants defective in hyphal branching have not been undertaken because branch formation is highly sensitive to subtle environmental variables such as media composition (Watters et al., 2013). This poses challenges because mutant phenotypes often appear unstable and difficult to track. A few interesting mutants with either hyper-branching or hypo-branching phenotypes have been recovered from much broader screens for morphogenetic mutants in *A. nidulans* and *N. crassa*. For example, analysis of hyper-branching mutants (i.e., *hbr*) in *A. nidulans* has revealed potential roles for membrane synthesis and vacuolar protein sorting in the control of branching frequency, whereas analysis of hypo-branching mutants (*ahb*) confirmed the importance of nuclear division in the control of branch formation (Lin and Momany, 2004; Harris et al., 2009). In *N. crassa*, phenotypes observed during a systematic screen for morphological mutants helped to define four discrete genetically separable steps that underlie branch formation (Seiler and Plamann, 2003); (i) selection of the branch site, (ii) expansion of the growth zone at that site, (iii) formation of a thin incipient branch, and (iv) maturation of the new hyphal tip. Attempts to further refine this genetic pathway and to functionally characterize the gene products implicated at each step are clearly warranted, as are studies that focus on determining the extent to which the pathway is conserved beyond *N. crassa*.

In addition to large-scale mutant screens, gene products implicated in hyphal branching have been identified through the functional characterization of specific genes. For example, deletion of genes encoding components of heterotrimeric G proteins (i.e., Gα, β, and γ) results in a near absence of lateral branches in *A. nidulans* and other filamentous fungi (e.g., (Harris, 2008), (Yu et al., 1996), (Ganem et al., 2004)). Although this observation would seemingly suggest that G-protein coupled receptors (GPCRs) regulate branch formation, no single GPCR deletion mutant has yet been shown to possess an apparent branching defect in, for example, *A. nidulans* or *A. flavus* (Brown et al., 2018; Affeldt et al., 2014). Because the *A. nidulans* genome encodes at least 16 distinct GPCRs, it remains possible that functional redundancy is masking such a role.

The mechanisms that coordinate hyphal branching with growth during the duplication cycle remain largely unknown. However, studies using *N. crassa* have suggested that a homeostatic control system acts to maintain a constant interval between neighboring lateral branches regardless of growth conditions (Watters et al., 2013; Watters and Griffiths, 2001). The potential existence of this system is notable because other morphological traits such as hyphal diameter, compartment volume, and growth rate are not maintained at a constant level regardless of growth rate. The analysis of *N. crassa* morphological mutants provided preliminary evidence that as yet undefined events at the hyphal tip regulate branch density. A broader screen of deletion mutants identified a more diverse set of functions implicated in the coordination of lateral branching with growth, including protein kinases and circadian clock associated proteins (Watters et al., 2011, 2013). It will be important to extend these studies to further understand how these functions regulate branch formation, and to determine whether this control system is more widely conserved.

3.2. Spatial regulation of branching

Remarkably little is known about the how spatial patterns of lateral branching are determined. Other than examples where lateral branches emerge adjacent to existing septa (Harris, 2008; Fiddy and Trinci, 1976b), there are no reports of branch formation occurring with high frequency at specified lateral sites. There is some evidence that the emergence of lateral branches is slightly biased towards the region of the sub-apical compartment that is nearest to the hyphal tip (Trinci, 1974). Nevertheless, one defining feature of lateral branching is avoidance – that is, the presence of an active hyphal tip generally precludes the formation of another tip in the local region. This feature is particularly evident in apical compartments, where it is known as apical dominance (Semighini and Harris, 2008). The term hyphal growth unit refers to the average length of a hypha that supports a growing tip (Trinci, 1974). This property presumably reflects the volume of vesicles, membrane material, and cell wall precursors needed to support a growing tip. Accordingly, lateral branching is generally blocked near the hyphal tip (as noted above, apical branching appears to be a distinct process triggered by tip deformation). The mechanisms that enforce apical dominance remain unknown, but do involve signalling mediated by reactive oxygen species (ROS) and calcium (Ca^{++}).

In fungi such as *A. nidulans* and *Epichloe festucae*, localized production of ROS by the NADPH oxidase (NOX) complex mediates the suppression of hyphal branching (Takemoto et al., 2009). Mutations that compromise NOX function permit spurious branch formation and abrogate apical dominance. Notably, amongst the key regulators of NOX are the GTPase Rac1 (Tanaka et al., 2008), as well as the guanine nucleotide exchange factor Cdc24 and the scaffold protein Bem1 (Takemoto et al., 2011). Homologues of the latter two are key components of the *S. cerevisiae* polarity establishment machinery (Bi and Park, 2012).

In fungi such as *N. crassa*, the presence of a tip-high Ca^{++} gradient appears to also play a critical role in suppressing branch formation (e.g. (Silverman-Gavrila and Lew, 2003)). However, assessing the importance of this gradient for apical dominance is complicated by the broader role of Ca^{++} in polarized growth, as pharmacological or mutational disruption of the gradient impacts the rate of hyphal tip extension (e.g. (Lew, 2011)). Nevertheless, it remains an intriguing possibility that the roles of ROS and Ca^{++} in the regulation of lateral branching might be intertwined, as localized ROS production controls calcium channel activity in plant root hairs and thereby facilitates polar growth (Carol and Dolan, 2006).

3.3. Roles of the morphogenetic machinery

In general, the formation of a hyphal branch represents a *de novo* polarity establishment event (Harris, 2008). Exceptions include apical branching, which largely reflects the splitting of an existing polarity axis, and lateral branch formation adjacent to septa, where the polarity axis used to deliver membrane and cell wall material to the septum is presumably co-opted to initiate branch formation. The morphogenetic machinery refers to the cytoskeletal elements and vesicle trafficking components involved in localized cell surface expansion and cell wall deposition in fungi (Steinberg et al., 2017). Although this machinery plays a reasonably well-characterized role in the emergence of germ tubes from spores, its role in emergence of a lateral branch is less understood. To some extent, this is because mutants in which the morphogenetic machinery is compromised rarely undergo sufficient growth to form sub-apical hyphal compartments capable of branching. On the other hand, the use of immunofluorescence microscopy has shown that cytoplasmic microtubules and microfilaments are directed into newly emerged lateral branches (e.g., (Sampson and Heath, 2005), (Torralba et al., 1998), (Mourino-Perez et al., 2006), (Berepiki et al., 2010)). The difficulty in predicting future branch sites poses a significant challenge to determining whether the localization of these cytoskeletal elements precedes branch emergence. However, analyses of mutants that eliminate function of the sole formin in *A. nidulans* (i.e., SepA) show that lateral branch formation is largely abolished in the absence of detectable microfilaments (Harris et al., 1997). A key objective for future study will be the development of approaches that will enable better experimental control of branching so that the full role of the morphogenetic machinery can be properly evaluated. A particularly intriguing question is whether the requirements for lateral branch formation are identical to those needed for germ tube emergence.

The septins form heteropolymers that possess multiple functions during hyphal morphogenesis (Khan et al., 2015). This includes the regulation of lateral branch formation, as for example in *A. nidulans*, mutants in which individual septins are deleted display a hyper-branching phenotype amongst other morphogenetic defects (Lindsey et al., 2010; Hernandez-Rodriguez et al., 2012). In both *A. nidulans* and *A. gossypii*, septin rings and collars localize to the base of new lateral branches (DeMay et al., 2009). Strikingly, live imaging of *A. gossypii* hyphae shows that formation of these septin structures is concurrent with emergence of the new branch (DeMay et al., 2009). Thus, as with bud formation and growth in *S. cerevisiae* (Bi and Park, 2012), the septins may delineate growth zones that define incipient branches. In doing so, they may contribute to the spatial patterning of lateral branch formation by prohibiting the formation of new branches adjacent to existing ones.

4. Proposed framework for the regulation of branch formation

As noted by Fiddy and Trinci (1976a), hyphal branching occurs within the context of the duplication cycle, and is thus roughly analogous to processes such as budding in the yeast *S. cerevisiae*. It then follows that the formation of a branch from a hyphal cell could conceivably be subject to similar temporal and spatial controls as bud formation in yeast (Bi and Park, 2012). At the same time, however, most fungal hyphae are multicellular with some degree of communication between cells within a hypha (Rayner, 1991). Accordingly, an additional level of control over branching is presumably exerted at the level of a hypha, or even a mycelium (Fig. 1, **Hyphal**). As noted above, an example of this level of control would be the role of ROS or Ca^{++} in enforcing apical dominance. More importantly, branch formation must somehow be integrated with hyphal growth. Amongst key parameters that might be monitored as part of this integration are the rate of hyphal extension, the length of the duplication cycle, and the ratio of nuclei per cytoplasm (Trinci, 1974; Fiddy and Trinci, 1976a; Harris, 1997). Signalling modules that could potentially integrate branch formation with growth include the protein kinase A (PKA) and Target-of-Rapamycin (TOR) pathways. Both the PKA and TOR pathways are involved in the coordination of budding with growth in *S. cerevisiae* (Broach, 2012). Ultimately, the timing and location of branch formation are seemingly regulated at the level of the individual hyphal cell (Fig. 1, **Compartmental**). Key events subject to regulation at this scale are likely to be nuclear division and polarity establishment (Fig. 1). Evidence suggests that lateral branch formation is coordinated with nuclear division (Fiddy and Trinci, 1976a), and cyclin-dependent kinase (CDK) is an excellent candidate to mediate this function (Lin and Momany, 2004). In this manner, nuclear division would set the timing of branch formation. Moreover, localized nuclear division within a multinucleate hyphal cell could also specify the location of branch formation. Alternatively, stochastic asymmetries in the distribution of hypothetical cortical factors might mark branch cites. Regardless, as in *S. cerevisiae* (Bi and Park, 2012), reciprocal feedback between nuclear division and the signalling modules that direct polarity establishment could consolidate spatial information to enable emergence of a new branch. Subsequently, the septins and associated proteins (Bi and Park, 2012; Khan et al., 2015; DeMay et al., 2009) would stabilize the new polarity axis to ensure continued branch growth and prohibit emergence of new branches at adjacent sites. It should be noted that the framework for regulation of branch formation proposed here is generic,

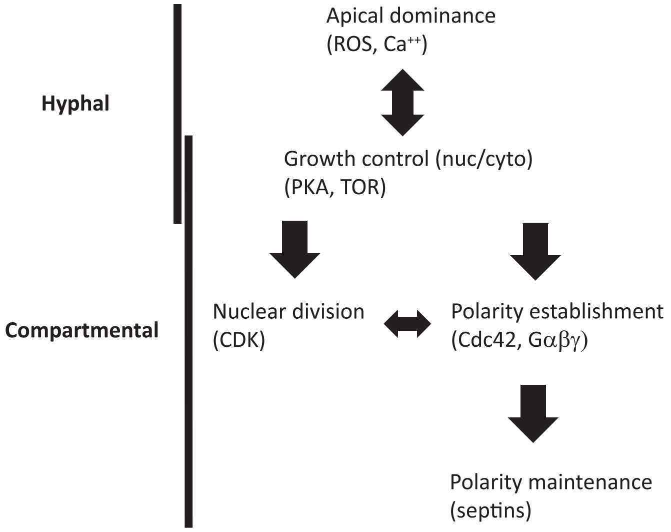

Fig. 1. Proposed framework for the regulation of branch formation. Regulation at two levels is depicted: hyphal and compartmental. The vertical lines demarcate the features subject to control at each level. Growth control is potentially regulated at both hyphal and compartmental levels. See text for additional detail. ROS = reactive oxygen species. PKA = protein kinase A. TOR = target-of-rapamycin. CDK = cyclin-dependent kinase. Nuc/cyto = ratio of nuclei per cytoplasmic volume.

and is undoubtedly subject to modification based on modes and patterns of hyphal extension. For example, in fungi such as *N. crassa* that undergo rapid hyphal extension, greater compartmental autonomy and reduced coordination of branch emergence with nuclear division might expedite the emergence of new branches.

5. Branch formation in response to external signals

Arbuscular mycorrhizal fungi (AMF) engage in widespread beneficial mutualisms with plants whereby they provide essential nutrients in exchange for reduced carbon (Lanfranco and Young, 2012). The initial stages of this interaction require signalling between roots and fungal hyphae. In particular, the plant hormone strigolactone is used to recruit potential partners. Application of strigolactones or their synthetic derivatives has shown that they stimulate hyphal branching (Akiyama et al., 2005). The mechanism underlying this response remains unknown, though a potential role for ROS has been suggested (Belmondo et al., 2017). In addition, it is not clear how broad the branch-inducing activity of strigolactones is. More importantly, it is not known whether other fungi that do not participate in specialized interactions with plants (i.e., saprophytes) respond to branch inducing factors. Although there is evidence that compounds such as cyclic nucleotides and choline can modify branching patterns (Robson et al., 1991; Markham et al., 1993), evidence for branch inducing factors is generally lacking. The identification of such factors would be notable, if for no other reason than they would facilitate the study of branch formation by providing a controlled approach to trigger the process.

6. Approaches for the study of branch formation

In filamentous fungi, several fundamental questions about hyphal branching remain unanswered. These include;

- How are branch sites selected?
- What is the role of adjacent nuclei in the regulation of branch formation?
- How does an actively growing tip suppress branch formation at adjacent sites?
- How similar are the mechanisms that underlie emergence of a new branch to those involved in germ tube emergence?

The ability to address these questions and others related to hyphal branching require facile experimental approaches that can be applied to diverse fungi. This could include, for example, the use of an external signal (e.g., strigolactones?) able to stimulate branch formation in a synchronous manner, thus permitting the use of different omics-based platforms to identify cellular processes and functions that are associated with branching. Alternatively, deletion libraries that cover an entire genome (e.g., *N. crassa*; (Park et al., 2011)) or a specific cellular function (e.g., protein kinases; (De Souza et al., 2013)) are available and could be leveraged to collect large amounts of phenotypic data related to branching, thereby identifying relevant genes in an unbiased manner. Whether these or other approaches are employed, an improved understanding of hyphal branching is essential to providing deeper insight into the morphological differentiation of fungal mycelia.

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Appendix A. Supplementary data

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