Tests of a Cellular Model for Constant Branch Distribution in the Filamentous Fungus *Neurospora crassa*

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The growth of mycelial fungi is characterized by the highly polarized extension of hyphal tips and the formation of subapical branches, which themselves extend as new tips. In *Neurospora crassa*, tip growth and branching are crucial elements for this saprophyte in the colonization and utilization of organic substrates. Much research has focused on the mechanism of tip extension, but a cellular model that fully explains the known phenomenology of branching by *N. crassa* has not been proposed. We described and tested a model in which the formation of a lateral branch in *N. crassa* was determined by the accumulation of tip-growth vesicles caused by the excess of the rate of supply over the rate of deposition at the apex. If both rates are proportional to metabolic rate, then the model explains the known lack of dependence of branch interval on growth rate. We tested the model by manipulating the tip extension rate, first by shifting temperature in both the wild type and hyperbranching (colonial) mutants and also by observing the behavior of both tipless colonies and colonyless tips. We found that temperature shifts in either direction result in temporary changes in branching. We found that colonyless tips also pass through a temporary transition phase of branching. The tipless colonies produced a cluster of new tips near the point of damage. We also found that branching in colonial mutants is dependent on growth rate. The results of these tests are consistent with a model of branching in which branch initiation is controlled by the dynamics of tip growth while being independent of the actual rate of this growth.

In mycelial fungi, hyphae extend by a highly polarized process of cell extension known as tip growth. As the tip extends, periodic branches are formed at or near the apex of the tip. These branches also extend in a polarized manner as new tips. The two processes of branching and tip growth permit the organism to colonize and efficiently utilize a substrate, and they are rarely found in organisms other than fungi, leading to their being termed hallmarks of the fungal kingdom (8).

Attempts to understand tip growth and branching have employed various approaches. Cytological analysis has identified several key substances involved in the process, most notably actin and calcium (6, 8, 11, 12, 13). Ultrastructural studies have demonstrated the importance of tip-growth vesicles (1, 2, 3, 19, 22, 23). Genetic analysis of induced and naturally occurring mutants has identified over 100 loci that encode products that contribute to the extension of an individual tip. The vesicular basis of hyphal growth and branching was incorporated into a model by Trinci (22). A key element of this model was the hyphal growth unit, defined as the ratio of total hyphal length to the total number of tips. This growth unit represents the mean length of hyphae that contribute to the extension of an individual tip. The initiation of a new branch has been proposed to be controlled by changes in the cytoplasmic volume, so that branching occurs when a critical value of the mean hyphal growth unit is attained. In this way, the protoplasm considerably distant from the growing tip could have a contributing role in branch initiation. In a further elaboration of this model, Prosser and Trinci (19) proposed that the concentrations of vesicles and nuclei regulate the increase in hyphal length and the occurrence of branches and septa.

Watters et al. (25) showed that in *N. crassa* the distribution of branch intervals is independent of tip extension rate, as controlled by temperature. Although rapid cooling disturbs this distribution, the normal default distribution of branch intervals was soon restored at the new temperature. Thus, the statistical distribution of branch-to-branch intervals along a hypha seems to constitute a homeostatic set point. Prompted by this observation, our objectives in this study were to develop and test a model of lateral branch initiation that explains the apparent independence of branch interval and temperature yet permits a dramatic response to changes of temperature. This model extends previous work (1, 2, 3, 19, 22, 23) by including the kinetics of growth. In the proposed model, supply and deposition of tip extension factors henceforth assumed to be

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tip-growth vesicles, in accordance with previously published models of tip growth and branching (1, 2, 3, 19, 22, 23), are proportional to metabolic rate, resulting in a fixed set point for branch interval that is essentially temperature compensated.

**The model.** Any comprehensive model for tip growth and branching must incorporate the main phenomenology associated with these processes. Tip extension occurs via apical exocytosis of tip-growth vesicles manufactured subapically and transported to the tip (1, 2, 9, 19, 22). Thus, the tip concentration of vesicles and any other tip extension factors depends on the balance between the rates of supply (synthesis and transport) and consumption (either deposition or destruction). Branching, which is triggered by the rate of accumulation at the tip, is proportional to the excess of vesicle production over tip deposition. Branching has previously been shown to be at least partially controlled by factors at or proximal to the previous branch point (26).

We extend these ideas to explain the lack of dependence of branch distribution on temperature (or growth rate), and we have made the following assumptions: (i) that the rates of vesicle production and deposition are linearly related to each other and to the metabolic rate; (ii) that the rate of tip extension is directly proportional to the rate of vesicle deposition; and (iii) that branch initiation depends upon the accumulation of a specific number of vesicles, but the speed at which this number is attained is not relevant.

These assumptions generate a constant average branch interval, i.e., the model depends on a direct linear relationship between the number of vesicles produced and the tip extension rate, with a constant proportion of vesicles conserved for branch initiation. Hence, the default distribution of branch interval lengths should be independent of growth rate, has been previously observed (21, 25).

The first tests were performed using shifts from low to high temperatures. Although we expect the basic rates to be proportional to each other at steady state, it is likely that this relationship will fail under rapidly changing conditions during which either production or deposition might lag. For example, under conditions of increasing rate of metabolism, the supply of tip-growth vesicles might be expected to increase before an increase in their consumption. The logic of this expectation reflects the simple idea that it is relatively straightforward to deliver more components of a house to a building site, but it is not so straightforward to make use of these extra components in an accelerated building process. A lag in deposition would lead to a temporary phase in which vesicles accumulate more rapidly than usual, producing shorter branch intervals. Conversely, under conditions of decreasing metabolic rate, the supply of tip-growth vesicles would decline prior to a parallel decrease in their consumption. This decline would result in a temporary phase during which vesicles accumulate more slowly, producing longer branch intervals. In either circumstance, once the rate of consumption of vesicles catches up with supply, branching would return to the default distribution. We tested the model using isolated tips, temperature shifts, and hyperbranching colonial mutants.

**MATERIALS AND METHODS**

**Strains and media.** The standard *N. crassa* Oak Ridge wild-type 74-OR81-1a (FGSC #988 Fungal Genetics Stock Center, Microbiology Department, University of Kansas Medical Center, Kansas City, Kans.) was used in all experiments unless otherwise noted. Media and culturing procedures were those described in the work of Davis and deSerres (5). Colonial mutants used were col-4 (allele 70007c), col-8 (allele R2356), and col-16 (allele R2539).

Cultures were grown on plates for all experiments. Temperature shifts were accomplished by moving cultures between incubators. Cultures were allowed to grow to approximately 30 mm, as measured from the point of inoculation to the leading edge of the colony, before shifting. For the 10°C-to-25°C shift, this required 1 week of growth at 10°C. For the 25°C-to-4°C shift, this amount of growth was attained overnight. Tip isolation experiments were performed following overnight growth at 25°C. Tip isolation was accomplished by cutting through the colony and agar medium with a sterile blade.

**Photomicroscopy.** Cultures were photographed on TMX400 film (Eastman Kodak Company, Rochester, N.Y.) with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, N.Y.) that was fitted with a 35-mm camera. Negatives were printed to a constant magnification, and growth and branching were measured to the nearest 10 μm. Branch segments were measured following 15 to 25 mm of growth on the plate, in order to allow the colony to reach steady-state growth conditions. A single branch interval is the distance between branch points along a hypha.

**Statistical analysis.** Changes in branching were analyzed by comparing distributions of branch interval lengths. In most cases, this distribution was skewed toward shorter intervals with an extended tail representing occasional long branch intervals. In form, this distribution matched the gamma-distributed growth observed for several fungal species by Kotov and Reshetnikov (15). Because of this skew, we chose the median as a descriptor of the distribution. The data were graphed and analyzed statistically using the programs Cricket Graph III (Computer Associates Int. Inc., Islandia, N.Y.) and Statworks (Cricket Software, Philadelphia, Pa.) on a Macintosh SE/30 personal computer. The significance of the difference between pairs of distributions of branch intervals was estimated using the nonparametric Mann-Whitney test, which was chosen for its suitability for non-normal distributions.

**RESULTS**

**Temperature shifts.** The distribution of branch intervals measured at 10°C (Fig. 1) matched the default distribution (me-
In a transition phase (lasting approximately 2 h) following the shift to 25°C, the distribution of branch lengths was shortened (median = 70 μm) (Fig. 1). Following this transition, branching returned to the default distribution (P = 0.34).

The response to a temperature downshift from 25 to 4°C (Fig. 2) has been described previously (25). During the transition, the hypha initially produces a single unusually long branch interval (Fig. 2). The tip then produces a series of tightly spaced, dichotomous branches termed the starburst (25). Following the starburst phase, branching recovers, returning again to the default distribution (P = 0.70).

Hyphal damage. We also measured branch length intervals in growing hyphal tips that had been isolated from their colony (Fig. 3), as well as the resulting tipless hyphae (the segments still attached to the colony). In this experiment, all the tips of a colony were severed by cuts approximately 5 mm from the periphery of the colony. Following minor loss of cytoplasm on both sides of the cut, septa in both the isolated tip and the tipless hypha are plugged (24). Within 5 min following isolation of a tip from its colony, most tips produce a single dichotomous branch. After this initial response, the following two branch intervals are, on average, longer than usual (P = 4 × 10^{-6}) (Fig. 3). As with the temperature shift experiments, branching returns to the default distribution following a transition phase (P = 0.70).

The behavior of an older, established hyphal tube that has been deprived of its growing tip depends on the extent of damage to the remainder of the colony. If only a few tips are excised, no effect is seen. The damaged hyphae do not recover and growth proceeds in the undamaged tips. However, if the majority of the leading tips of the colony are removed, many hyphae undergo hyperbranching near the point of damage (Fig. 4). The region of hyperbranching is confined to a segment 50 to 150 μm immediately proximal to the plugged septum.

Colonial mutants. We subjected several hyperbranching mutants (col-4, col-8, and col-16 mutants) to growth at various constant temperatures (Fig. 5). In contrast to the result in wild-type N. crassa, the distribution of branch intervals in these mutants was dependent on tip extension rate. The most obvious shift in branch distribution was in the col-8 mutant (Fig. 5B), in which incubation at reduced temperature resulted in longer branch intervals. The other two hyperbranching strains (Fig. 5A and C) had more modest, but statistically significant, shifts toward longer branches when grown at reduced temperatures.

DISCUSSION

We have considered a model for the control of branching in which a branch is initiated when the concentration of tip-
growth vesicles reaches a threshold value. The model requires that the rates of supply and consumption of vesicles be proportional to the metabolic rate and that the deposition rate determine the tip growth rate. These assumptions ensure that the accumulation of vesicles that trigger a branch occurs at consistent intervals along the hypha during growth, regardless of the growth rate.

The model was tested first by observing the effects of temperature shifts that produce proportional shifts in tip extension rate. We found that after raising the temperature, the branch intervals decreased, suggesting that the rate at which vesicles are supplied exceeds the rate of consumption. Lowering the temperature results in a temporary increase in the lengths of branch intervals, implying that the supply of vesicles lags behind their consumption at the tip.

The response to temperature shifts may impact our understanding of the growth of fungi in the field. While *N. crassa* is not generally found in climates which would be expected to suffer such severe environmental shifts, the same could not be said for any number of plant pathogenic fungi found in more temperate climates, where typical day-night temperature cycles could easily reach the ranges shown to trigger a response.

The model also predicts that branching at the tip should enter into a transitional hypobranching phase following physical separation from the colony, as was observed. In contrast, proximal to the point of damage, hyperbranching is induced; we interpret this to mean that blocked septa restrict the flow of tip-growth vesicles destined for the tips, causing a buildup of vesicles and triggering branching. This result mirrors that observed by Trinci and Collinge (24). In that study, the tips of *spco-9* mutants were damaged by osmotic shock in order to observe the plugging of septa during repair. The present results show that the results of Trinci and Collinge are not a peculiarity of *spco-9* mutants.

The observation of hyperbranching proximal to the site of damage demonstrates that branches can potentially form at any point along the hyphae. Only the dynamics of tip extension cause branching to be normally confined to regions near the apex. The spacing of initiation points within the hyperbranched region is not explained by the proposed model, as the model was designed to address a growing tip. The observation of significant branching proximal to the point of damage argues...
against models in which branching is absolutely dependent on the division of some resource or structure at the tip itself (such as the Spitzenkörper).

The normally rare dichotomous branch form was induced both by temperature downshifts and in severed tips. The existence of mutations (pk, col-15) and environmental treatments that increase the frequency of dichotomous branches argues that the processes leading to the formation of lateral and dichotomous branches are distinct. Specifically, dichotomous branch points are not simply an occasional random variation on the normally lateral branch form but are triggered by a distinct set of circumstances. Both of the conditions associated with the induction of dichotomous branches in this study are those that produce longer intervals between lateral branches during their transition phases. This finding leads to the seemingly contradictory conclusion that dichotomous branch points may be induced by conditions similar to those that lead to longer intervals between lateral branches. Stated simply, dichotomous branch points may represent a failure to form a lateral branch. This observation may explain the lack of mutations that result in decreased branching. Namely, mutations that could result in longer branch point intervals instead cause closely spaced dichotomous branching and thus are not scored as "loose branch" mutations.

The proposed model also explains the observation that the distribution of branch intervals is dependent on the tip extension rate under changing conditions. The model also is consistent with the results of tip isolation experiments and explains the lack of lateral branch points may represent a failure to form a normally lateral branch but are triggered by a distinct set of circumstances. This observation may explain the lack of mutations that result in decreased branching. Namely, mutations that could result in longer branch point intervals instead cause closely spaced dichotomous branching and thus are not scored as "loose branch" mutations.

In conclusion, we have developed a model in which branching is triggered by a critical buildup of a colony-produced tip-extension-associated factor (probably tip-growth vesicles). This buildup results from the difference in the rates of supply and consumption of these vesicles. The model explains how branching can be independent of tip extension rate under steady-state conditions while responding dramatically to changing conditions. The model also is consistent with the results of tip isolation experiments and explains the lack of mutations resulting in longer branch intervals as well as the observed temperature and extension-rate dependence of branching in colonial mutants.

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REFERENCES