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CONTROL OF BRANCH INITIATION IN *NEUROSPORA*

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ABSTRACT. In a previous study of branching in *Neurospora*, it was determined that branching at the tip is not a tip autonomous process, but that branching is controlled at least in part by a factor or factors at or near the previous branch point. This was determined by the demonstration of a statistical correlation between lengths of branch intervals (the distance between two tandem branch points) having a common origin. That study was unable to determine the nature of that common factor. Namely, the correlation could have been due to the physical division of a structure or resource present at the time and location of the common branch point. It could have alternatively been caused by the division, at the common branch point, of the flow of an undetermined factor toward the growing tips. This study distinguishes between these two alternatives by extending the examination of branch interval correlation and comparing branch intervals that share a common origin, one step removed. The observation of branch length correlation at this level suggests that the previously observed correlation primarily results from the division of an undetermined factor flowing toward the tip and not the singular division of a structure at the time of branch formation.

Keywords: *Neurospora crassa*, morphology, branching, hypha

Filamentous fungi grow by hyphal tip extension and by making new hyphal tips (branching). These two processes are fundamental aspects of fungal biology and have attracted much attention in research. Of the two processes, tip growth has been more extensively studied, leading to reasonable models for the cellular mechanisms involved (Bartnicki-Garcia 1973; Collinge & Trinci 1974; Heath 1990; Howard 1981; Riquelme & Bartnicki-Garcia 2004). There are a number of mathematical models for tip growth and morphogenesis (Hutchinson et al. 1980; Kotov & Reshitnikov 1990, Yang et al. 1992), but these do not attempt to explain branch initiation, and usually assume branching to be a random event. A branch, once formed, must abide by the same growth mechanisms as the original apex. However, first a critical event presumably takes place that commits the cell to form a new branch. Among published models for branch initiation, there are two basic categories. These differ fundamentally in the proposed source of the branching signal.

The focus of one category of models is on branch initiation by factors originating proximal to the branch event itself, from somewhere within the colony. In *Neurospora crassa*, the protoplasm for tip extension can come from regions of the mycelium more than 12 mm from the colony margin (Zalokar 1959). Katz et al. (1972) proposed that a new branch would be initiated when the extension capacity of the hypha overcomes the extension capacity of existing tips. Trinci (1974) and Prosser & Trinci (1979) proposed that tip growth vesicles are the key elements whose accumulation triggers branching.

In the second category of branching models, the focus is on events that are controlled independently at the tip. Bartnicki-Garcia et al. (1989a, b) suggested that there might also be a tip-based pulling mechanism that displaces the vesicle supply center (VSC) for branching. Possible molecular candidates for this role are microfilaments (Bartnicki-Garcia et al. 1989a, b), integrin (Kaminskyj & Heath 1996) and spectrin (Degousee et al. 1997). Calcium has been invoked as a factor important in tip growth and branching (Heath 1990). A cytoplasmic Ca^{2+} gradient is thought to play a role in maintaining apical dominance by inhibiting branch formation at the apex of a hyphal tip (Schmid & Harold 1988). This idea is supported by the work of Reissig & Kinney (1983), Prokisch et al. (1997), Capelli et al. (1997), Levina et al. (1995) and Grinberg & Heath (1997).

Parallel to the question of control of branching generally, is the issue of lateral *vs.*

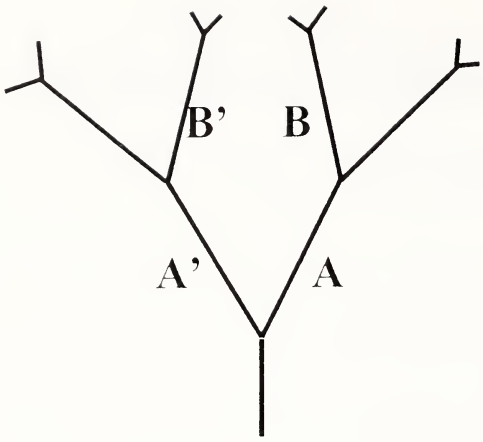

Figure 1.—Definition of branch interval pairs discussed in the text. A branch interval is defined as the distance between tandem two branch points. The pairs of intervals discussed in the text either compare intervals having a common origin (A *vs.* A′) or those who immediately follow intervals having a common origin (B *vs.* B′). For the purpose of this study, the B and B′ intervals were defined either as those intervals occurring along the main hypha (for lateral branches) or the central two branch intervals (for apical branches).

apical branching. It is unclear at present if the two morphological forms are simply variations of a single process or if they represent the results of fundamentally different events. Observations of Spitzenkörper behavior by Riquelme & Barnicki-Garcia (2004) show very different behavior during apical and lateral branch formation. In addition, growth can be induced to switch from lateral to apical branching by either mutation or environmental insult (Scott 1976; Watters et al. 2000a; Watters & Griffiths 2001). These lines of evidence suggest lateral and apical branch points are fundamentally distinct from each other. However, statistical examination of branching (Watters et al. 2000a, b; Watters & Griffiths 2001) has suggested that apical and lateral branching share much of their control, with apical and lateral branch arrays both showing symmetry (branch intervals having a common origin show correlation for their lengths) and similar distributions (in terms of the shape of the curve, but not the means) of spacing between branch points.

Watters et al. (2000b) demonstrated that the lengths of branch intervals sharing a common origin (i.e., A *vs.* A′ in Fig. 1) are correlated for their lengths. This demonstrates that although branching takes place at the tip, the decision to form a branch is not controlled independently by the tip itself. The branch decision is instead controlled, at least in part, by a factor associated with or determined by the previous branch point. Briefly, something about a branch point controls when the next branch point will form along a growing hypha. There are two alternative models that could explain the observed correlation and explain this control.

Under the first suggested model, tip growth and branching is purely under the control of a structure or resource, such as the Spitzenkörper located at the tip of a growing hypha. The Spitzenkörper is a collection of tip growth vesicles located at the tip that has been shown to play a critical role in both tip growth and branching (Bartnicki-Garcia 2002; Riquelme & Bartnicki-Garcia 2004). This model is consistent with the suggestion that branching is controlled directly by the Spitzenkörper and its collection of tip growth vesicles. The degree of symmetry following a given branch event would then be proposed to be controlled by the degree of symmetry by which the resources of the Spitzenkörper are divided during a branch event.

Under the second suggested model, branching is proposed to be triggered by either a signal or by the flow of resources from the colony to the growing tip. While this could take the form of a more direct signal, it is also consistent with previously suggested models (Katz et al. 1972; Watters & Griffiths 2001) where branching is triggered by the accumulation of factors fed by the colony to the tip. These factors would most likely be material important in tip growth supplied to the tip either in vesicles or simply in the cytoplasm as it streams toward the tip. This material is generated deep in the colony and is supplied to the growing tips via cytoplasmic streaming. It has been seen in *Coprinus disseminatus* that tips do not slow their extension following a branch event (Butler 1961), so it can be concluded that the supply of this material to the tip must exceed the rate of consumption. This suggests the accumulation of a reserve of tip growth material at the tip. Under this model, once the supply in this reserve reaches a set threshold, a branching event is triggered.

The two models differ in one unique pre-

diction, namely their expectation for the lengths of branch intervals following the initial intervals observed previously (i.e., B *vs.* B′ in Fig. 1). In the first model, the critical event (the division of a structure or resource, such as the Spitzenkörper) is limited in time. When the two forks emerging from the common branch point form new branches at their apexes, the correlation should be at an end. The divisions that mark these two new branch points would be expected to be independent events, so there is no reason to expect to observe correlation past this point. In the second model, however, the critical event (the division of the flow of resources at a branch point) is on-going and is thus expected to continue to exert an influence on growth (and branching) in subsequent branching events.

In the present work, we have tried to distinguish between these two basic models by extending the study of branch correlation. Here, we examine the correlation between branch intervals that do not share a common origin, but those where the intervals immediately prior to those under consideration share a common origin. If branching is controlled solely by a resource or structure that is divided at the time of a branch event, the intervals under comparison result from independent divisions and thus should not be correlated. If, however, branching is controlled by resources flowing to the tip from deeper in the colony that are divided at branch points, the common branch point should still exert an influence and result in detectable correlation. The results presented below support the suggestion that branch symmetry results from a roughly equally divided flow of material and thus that branching is triggered somehow by resources streaming to the growing hyphal tip from within the colony. This correlation is seen for both apical and lateral branch arrays, arguing in favor of the suggestion that both branch morphologies are regulated by related mechanisms.

METHODS

Strains and media.—The standard *Neurospora crassa* Oak Ridge wild-type 74-OR81-1a (FGSC #988) was used for most experiments (McCluskey 2003). Media and culturing procedures were those described in Davis & deSerres (1970), except as noted. Cold shock in *Neurospora* induces a temporary phase during which branching is exclusively apical (Watters et al. 2000a). For the study of branch symmetry involving apical branches, colonies were subjected to cold shock; wild type was grown overnight on Vogel's medium at 25 °C, then shifted to 4 °C. Measurements were made following a 24 h incubation at 4 °C.

Photomicroscopy.—Cultures were photographed on TMX400 film, using a Olympus BH-2 microscope fitted with a 35 mm camera. Branch intervals (distances between branch points) were determined by measuring rear projected negatives. These measurements were limited by: the degree of photographic enlargement, the thickness of the average hyphae and the degree to which any individual hyphae deviated from perfect linear growth. This allowed measurement of branch interval lengths to the nearest 10 μm.

Statistical analysis.—The distribution of branch intervals in most strains is markedly skewed toward the short end of the range. Consequently, normal statistics are not appropriate. Thus, the non-parametric Spearman correlation coefficient (*Rs*) was used for all comparisons. The branch data were analyzed statistically using the program SPSS (SPSS Inc., Chicago, Illinois).

RESULTS

To distinguish between the two models presented above, a survey was done of branching in *Neurospora crassa* to detect any possible second order symmetry (B *vs.* B′, Fig. 1). Lateral and apical branches were examined separately as it was previously observed (Watters et al. 2000b) that the strength of the correlation was dependent on the morphology of the branch point. The results of the current survey (Table 1) confirm the first order (A *vs.* A′, Fig. 1) correlation previously observed, and also show a clear second order (B *vs.* B′, Fig. 1) correlation present for both lateral and apical branch patterns.

DISCUSSION

The first order (A *vs.* A′, Fig. 1) symmetry observed previously (Watters et al. 2000b) was confirmed. This reinforces the previous conclusion that branching is somehow controlled by events at the previous branch point. In addition, second order (B *vs.* B′, Fig. 1) symmetry was observed for both lateral and

Table 1.—Correlation between branch interval lengths. Statsitical analysis of paired branch interval lengths confirms the previous observation of the correlation of branch interval lengths sharing a common origin (A *vs.* A′), as well as the second order (B *vs.* B′) symmetry currently under test. All correlations were observed with *P* values below 0.01. *Rs* = Spearman rank correlation coefficient, *n* = Sample size.

Branch morphology		A *vs.* A′	B *vs.* B′
Lateral branches	*Rs*	0.124	0.115
	P value	0.003	0.007
	n	549	549
Apical branches	*Rs*	0.686	0.585
	P values	<0.001	<0.001
	n	206	206

apical branch arrays. This supports the assertion that the primary cause of the observed symmetry is the division of resources flowing through branch points. The strength of the second order correlations observed parallels that observed for the first order symmetry (Watters et al. 2000b). Specifically, the symmetry is much stronger among apical branches than it is for lateral branches. This argues that the flow of resources is divided more evenly at apical branch points than it is at lateral branch points. This might not be surprising given the obvious spatial symmetry of an apical branch *vs.* a lateral branch.

The observation of symmetry relationships in both lateral and apical branches supports the argument that despite their obvious differences, they are regulated and triggered by similar mechanisms. The fact that apical branching can be induced by environmental insults (Watters et al. 2000a), genetic mutations (Scott 1976; Perkins et al. 2001) and cytoplasmic contractions (Reynaga-Peña et al. 1995; Riquelme & Bartnicki-Garcia 2004) would, however, argue they were induced via different mechanisms. This conclusion is reinforced by the direct observations of Spitzenkörper behavior during lateral and apical branch formation (Riquelme & Bartnicki-Garcia 2004). If apical and lateral branches are triggered differently, the proposed mechanisms must be able to explain how both triggering mechanisms produce the symmetry observed above and previously (Watters et al. 2000b). They must also explain the similar distributions of branch intervals observed previously (Watters et al. 2000a).

Lateral branches have previously been suggested to be induced by the accumulation of a critical factor at the tip (Katz et al. 1972; Watters & Griffiths 2001). These factors (generally suggested to be vesicles containing material for tip growth) have their origins in the colony and are fed to the tip via cytoplasmic streaming. The flow of this material would by necessity be divided at branch points. If this flow is indeed critical to the decision to form a lateral branch, it would result in the correlation observed above.

Apical branches have long been suspected to be induced by an alternative process. Riquelme & Barnicki-Garcia (2004) recently observed that apical branch formation was immediately preceded by a rapid cytoplasmic contraction that resulted in the dissolution of the Spitzenkörper followed by the reformation of a pair of Spitzenkörper forming the two tips of an apical branch. If such contractions have their origins deep enough in the colony, any contraction would propagate equally through branch points, sending this 'signal' through both hyphae emerging from a branch point to be received by growing tips at the same time. This alternative branching signal would still have its origin in the colony and show the observed symmetry, providing an alternative mechanism of control that still displayed symmetry.

Although the mean branch point to branch point distance is quite different for apical and lateral branch series, the shapes of the overall distribution of such intervals (Watters et al. 2000a) are the same. Although dual models for triggering lateral and apical branch points can both effectively produce symmetrical arrays, it is unclear, however, why such distinctive mechanisms for branch induction would produce such similarly shaped distributions of branch interval lengths.

In conclusion, the above observations demonstrate that branching is triggered by a signal(s) that has its origin in the colony. This signal flows to the tips roughly equally to result in the observed symmetry of growth. Although this symmetry is observed for both lateral and apical branches, these observations are consistent with the possibility that lateral and apical branches are induced by different signals, so long as both signals originate with-

in the colony and propagate to the tips similarly.

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