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HYPOBRANCHING INDUCED BY BOTH ENVIRONMENTAL ANTIOXIDANTS AND ROS METABOLISM GENE KNOCKOUTS IN *NEUROSPORA CRASSA*

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ABSTRACT. Previous work suggested a role of reactive oxygen species (ROS) metabolism on branch density, the statistical distribution of physical distances between branch points along a growing hypha in *Neurospora*. Here we report the results of experiments designed to ask more generally about the relationship between ROS and branch density by examining the branching effects of selected ROS metabolism gene knockout mutants as well as the impact on branching of exogenously added antioxidants. In all ROS metabolism mutants tested, growth was shown to branch less densely (hypobranching) when grown at lower temperatures, a shift not observed in the wild-type. Interestingly, this holds true for knockouts of genes expected to reduce ROS as well as those expected to produce them. In addition, in tests on wild type *Neurospora*, added ascorbic acid produced unusual branching patterns. Hypha exposed to exogenous antioxidants display dose dependent hypobranching with hypha becoming more hypobranched as doses increase. At higher doses, however, the branch distribution becomes bimodal with one maximum continuing to shift toward hypobranching and the second maximum representing a spike of very closely spaced branch points.

Keywords: *Neurospora*, morphology, branching, hypha, reactive oxygen species

INTRODUCTION

Growth in filamentous fungi proceeds via the continuously extending tip of a hypha which sends off periodic branches which are capable of extension and branching. Tip growth results from the polarized flow and exocytosis of 'tip growth' vesicles at the apex of the growing tip (Heath et al. 1971; Katz et al. 1972; Trinci 1974; Steinberg 2007; Riquelme et al. 2011); however, the role of these vesicles in the control of branching is unclear. The genetic system underlying tip growth and branching is complex. Studies on the epistasis of morphological mutants have revealed a complex range of interactions (Gavric & Griffiths 2003). Previous studies of tip growth and branching have included statistical/mathematical modeling studies of branching patterns (Prosser 1995; Watters et al. 2000b; Davidson 2007) and visualization of system components (Riquelme & Bartnicki-Garcia 2004; Mouriño-Pérez et al. 2006; Riquelme et al. 2007). Genetic approaches to understanding tip growth and branching have proven fruitful, including the analysis of suppressors of classical mutations (Plamann et al. 1994; Minke et al. 1999), broad screens for new mutants (Seiler & Plamann 2003), making use of established paradigms from yeast budding (Momany 2002; Harris & Momany 2003; Knechtle et al. 2003; Harris et al. 2005), and the cloning and characterization of classical morphological mutations.

It has been suggested that branching is induced when the concentration of tip-growth vesicles reaches a critical density at the apex (Trinci 1974). Although several studies have presented results that are consistent with this hypothesis (Katz et al. 1972; Trinci 1974; Watters & Griffiths 2001), none demonstrate it definitively. The results presented by these previous studies, in fact, are entirely consistent with the possibility that branching is triggered by the accumulation of some other undefined factor (other than tip-growth vesicles) associated with tip growth. The experiments described below were designed to begin to explore the possibility that the triggering factor was the accumulation of reactive oxygen in the hypha.

The observation of a temporary response to growth rate shifts followed by a return to normal branch density (Watters et al. 2000a; Watters & Griffiths 2001; Watters 2013) supported the

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Table 1.—ROS control mutants result in growth rate sensitive branching not seen in wild-type *Neurospora*. N values for the comparison varied between samples. Typical N values were roughly 200 branch lengths at each of the two temperatures. Reported are the means ± standard deviation of the distribution of distances between branch points for each strain under the two temperatures tested. Also reported are P values for a T-test comparing branching at the two temperatures. As with previous studies, wild-type shows no significant difference in branching between the two conditions. All tested ROS metabolism mutants however show significant differences (below p = 1%) between branching under the two conditions. The ROS metabolism mutants' branching, while typically close to wild-type at 33° C, show a strong shift toward much longer than normal branching when grown at 10° C.

Accession #	Disrupted gene	Branching (μm) at 33° C (Mean ± SD)	Branching (μm) at 10° C (Mean ± SD)	P value 33° C vs 10° C
	Wild-type	155 ± 104	169 ± 136	0.21
NCU08791	catalase-1	140 ± 87	239 ± 132	5.1×10^{-12}
NCU05770	catalase-2	155 ± 134	254 ± 174	1.5×10^{-11}
NCU00355	catalase-3	159 ± 109	257 ± 135	1.7×10^{-9}
NCU05169	catalase-4	169 ± 109	268 ± 175	4.2×10^{-13}
NCU02110	NADPH oxidase-1	197 ± 132	239 ± 149	2×10^{-3}
NCU10775	NADPH oxidase-2	169 ± 131	239 ± 154	3.6×10^{-7}
NCU07850	NADP oxidase regulator-1	140 ± 89	239 ± 156	4×10^{-17}
NCU02133	superoxide dismutase-1	154 ± 136	211 ± 112	5.1×10^{-7}
NCU01213	superoxide dismutase-2	155 ± 140	211 ± 123	2.4×10^{-7}
NCU07386	Fe superoxide dismutase	155 ± 117	254 ± 142	5.8×10^{-14}
NCU07851	superoxide dismutase 1 copper chaperone	113 ± 62	211 ± 134	1.3×10^{-28}
NCU09560	superoxide dismutase	183 ± 112	225 ± 148	4.6×10^{-5}

hypothesis that tip growth and branching are connected, but that this connection is compensated for in the wild-type, resulting in a consistent branch density. Thus, it was proposed (Watters & Griffiths 2001) that *Neurospora* morphology was controlled, in part, by a homeostatic system responsible for branch initiation. This system compensates for growth rate and is responsible for the maintenance of a constant branch density under a wide range of growth conditions (Watters & Griffiths 2001).

Mutations which appear to affect the proposed growth rate/branch density compensation system have been identified among both older *Neurospora* mutants (Watters et al. 2008) as well as among the current knockout library (Watters et al. 2011). Among the gene knockouts seen to affect the previously proposed growth rate/branch density compensation system were two (catalases) involved in the metabolism of reactive oxygen species (ROS). ROS are highly reactive molecules, commonly generated in biological organisms as a byproduct of normal oxygen metabolism. ROS production typically increases in fungi due to various stresses (reviewed by Gessler et al. 2007). Reactive oxygen species have been found to play a role in sexual and asexual development as well as hyphal growth in fungi (Cano-Dominguez et al. 2008; Semighini & Harris 2008). Reactive oxygen species have also been linked to developmental determination in a broad range of organisms (Finkel 2003; Foreman et al. 2003; Lambeth 2004; Aguirre et al. 2005; Carol & Dolan 2006). The relationship between cold stress and ROS has been studied extensively in plants, where there appears to be a multi-step response to exposure to cold where the plant initially experiences an increase in the production of ROS, and damage associated with that increase, followed by an increase in the production of antioxidants which counter that initial increase allowing the plant to better acclimatize to the cold (Beck et al. 2007, Airaki et al. 2012, Miura et al. 2012). The observation that ROS control was playing a role in branching, suggests the possibility that the accumulation of ROS could be the trigger for branch initiation. The experiments reported here were designed to test that hypothesis.

We report here on an expansion of our earlier study (Watters et al. 2011) that examined the *Neurospora* knockout library (Colot et al. 2006) for mutants with defects in the maintenance of

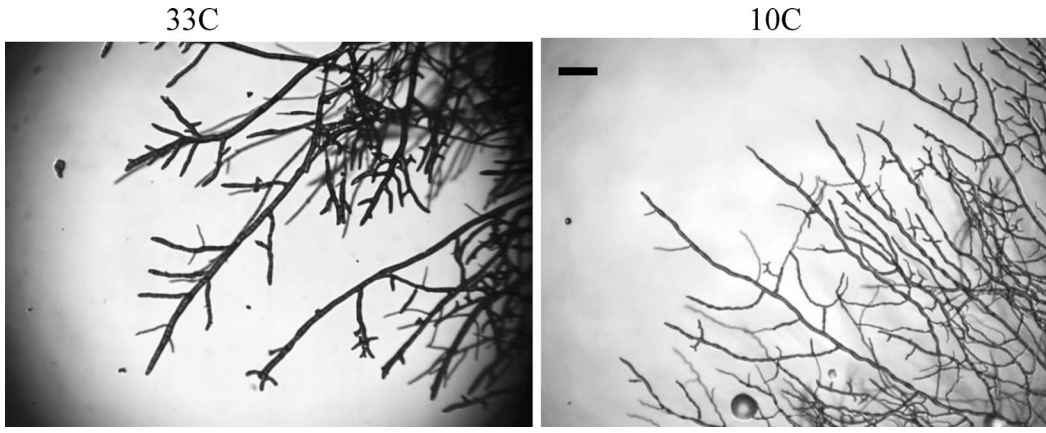

Figure 1.—Wild-Type at 33° C and 10° C. In wild-type *Neurospora*, branch density is kept constant during a wide range of growth conditions, including temperature variation. Scale bar = 100 μm.

branch density at different rates of growth. We expanded on the previous study by closely examining mutations in a wide variety of genes known to be important to ROS metabolism for their effects on branch density homeostasis. In addition, the effect on branching of exposure to the water soluble antioxidants ascorbic acid and glutathione were examined. Both lines of study point to a relationship between ROS control and branching in *Neurospora*. These also represent the first report of sustained hypobranching in *Neurospora*.

MATERIALS AND METHODS

Strains and media.—A library of knockout strains containing disruptions in presumptive genes has been constructed (Colot et al. 2006). Strains from this library are available from the Fungal Genetics Stock Center (McCluskey 2003). Our attention for this study was focused on a group of selected strains with knockouts in genes (listed in Table 1) related to the metabolism of reactive oxygen species. Vogel's media and culturing procedures were those described in Davis & deSerres (1970). Water soluble antioxidants ascorbic acid and glutathione as well as lipid soluble antioxidants beta-carotene and alpha-tocopherol were independently added to media.

The accession numbers listed in Table 1 represent the locus number of the gene subject to inactivation in the knockout strain under test.

Figure 2.—Cat-4 at 33° C and 10° C. The ROS metabolism mutants tested (cat-4 shown, others similar) responded to differences in incubation temperature. In all cases, the branch density at reduced incubation temperatures (10° C) was significantly hypobranched relative to growth at 33° C. Scale bar = 100 μm.

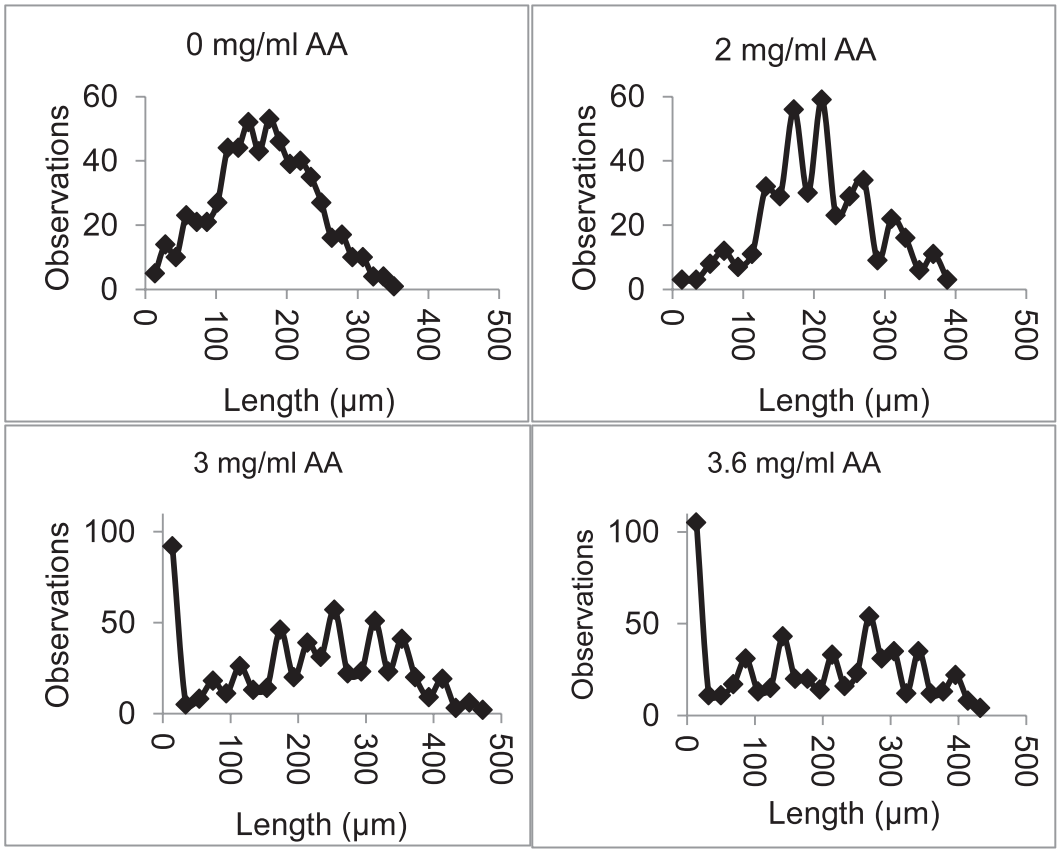

Figure 3.—Influence of ascorbic acid on branch density. Exposure to ascorbic acid at 33° C causes a dose dependent shift to hypobranching. At sufficiently high concentrations, however, a shift to frequent, very tight branching with some wider spaced branching is observed. Shown are curves of frequencies of branch interval lengths at 0, 2, 3 and 3.6 mg/ml ascorbic acid (AA). Sample sizes are 608, 413, 606 and 602 branch intervals, respectively.

Every annotated gene in *Neurospora crassa* has been assigned a locus number of the form NCU#### by the Broad Institute. The gene functions associated with the knockout strains reported in Table 1 are based solely on the annotations currently associated with those strains and have not been independently confirmed by the authors of this study. The functions reported are those associated with the genes as annotated on the Broad Institute's *Neurospora crassa* database: http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html.

Photomicroscopy.—Growing cultures were examined and photographed using a Diagnostic Instruments, Inc. SPOT RTke digital camera attached to an Olympus BH-2 microscope. Photographs were taken of well separated, leading hyphae in order to determine the branch density (distances between branch points along hyphae as they extend). Photos were used to measure distances between branch points. Measurements of distances between branch points were collected into databases of several hundred individual distances for statistical comparison of branching at different growth conditions. Growth at 33° C was photographed after 24 hrs growth. Growth at 10° C was photographed after seven days growth in order to allow sufficient growth to measure. This is the same procedure used previously (Watters et al. 2011)

Statistical comparison of branch density.—Comparison of branch density followed the procedure used previously (Watters et al. 2000a; Watters & Griffiths 2001; Watters et al. 2011).

Figure 4.—Altered morphology induced by high doses of antioxidants. Branching variation induced in wild type *Neurospora* by exposure to 3 mg/ml ascorbic acid. The altered branching displays a combination of regions of very tightly spaced lateral branches separated by regions of longer than normally spaced branches. Scale bar = 100 μm.

Measurements of the distance between branch points were made by measuring directly off photographs of growth and converting these lengths to distances on the plate. These measurements were then used to build a dataset of lengths between branch points. N values for the comparison varied between samples. Typical N values were roughly 200 branch lengths at each of the two temperatures. The resulting distributions were compared and analyzed using the Student's t-test.

RESULTS

Wild strains and strains with knockouts of selected genes involved in the metabolism of ROS in *Neurospora* were grown on minimal media at both 33° C and 10° C. As with previous studies (Watters et al. 2000a; Watters and Griffiths 2001; Watters 2013), wild-type strains showed no significant difference in branching between the two temperatures (Fig. 1; Table 1). All tested ROS metabolism mutants, however, showed significant differences (below $p = 1\%$) between branching under the two conditions (Fig. 2; Table 1). The ROS metabolism mutants' branching, while typically close to wild-type at 33° C, show a strong shift toward much longer than normal branching when grown at 10° C. In fact, statistically longer inter-branch distances (i.e., hypobranching) were seen in every ROS mutant tested compared with wild-types. For every ROS metabolism mutant tested however, the results were similar, regardless of the mutant's predicted impact on ROS levels.

In addition to the tests of the influence of ROS on branching, we examined the effect of the addition of reducing agents (antioxidants) to the media. At modest concentrations (0.04 to 2.0 mg/ml) of added ascorbic acid, the distribution of inter-branch intervals shifted toward

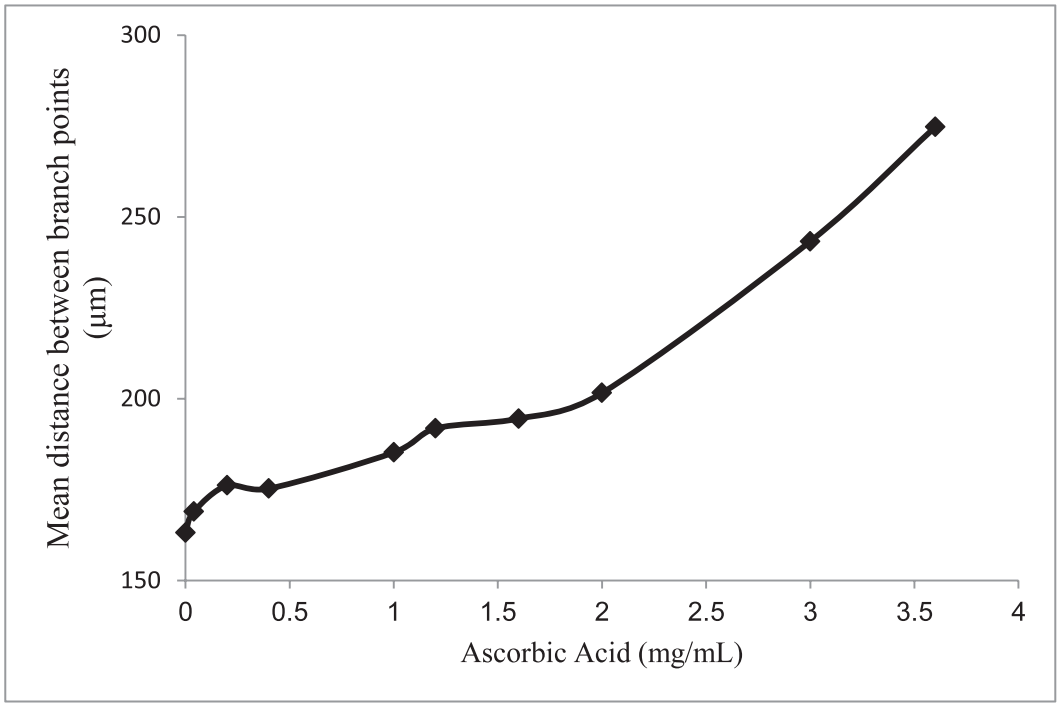

Figure 5.—Progressive shift toward hypobranching induced by increasing ascorbic acid concentration in media. Hypobranching induced by antioxidants is dose dependent. Increasing ascorbic acid in the medium leads to stronger hypobranching until the critical concentration, causing more serious disruptions, is reached. Each point on the curve is the mean of a branch distribution curve (as above) and represents the mean of between 400 and 650 branch intervals. For this curve, the smallest branch intervals have been eliminated in order to remove the impact of the spike of tightly spaced lateral branches and focus solely on the behavior of the more substantial portion of the branch distribution curves. Displayed is the response of wild-type *Neurospora* grown at 33° C.

longer distances (Fig. 3). At larger concentrations (>3.0 mg/ml), however, a new effect was observed. A "spike" of very short (10–20 μm) inter-branch distances begins to be observed. Past 3.0 mg/ml ascorbic acid, the frequency of the very short branches which form the spike increased with increasing dose of ascorbic acid. A representative photograph showing the morphology resulting from high-dose antioxidant exposure is shown in Fig. 4. In order to track the impact of antioxidant exposure beyond the high-dose spike, we deleted the shortest branches from each dataset, calculated the mean of the remaining inter-branch distance measurements and plotted these means against the concentration of ascorbic acid they were subjected to, resulting in Fig. 5. This shows a clear dose-dependent shift toward longer inter-branch distances as the dose of ascorbic acid in the media was increased.

DISCUSSION

Reactive Oxygen Species (ROS) have been linked to developmental determination in a broad range of organisms (Finkel 2003; Lambeth 2004; Aguirre et al. 2005; Carol & Dolan 2006). In *Neurospora*, reactive oxygen species play a role in sexual and asexual development as well as hyphal growth (Cano-Dominguez et al. 2008). This current study further explored a relationship between ROS metabolism, growth rate, and branch density previously suggested in *Neurospora* (Watters et al. 2011). This relationship remained masked in the wild-type and under standard growth conditions due to a homeostatic system which compensates for diverse growth rates to produce a consistent branch density. The mutants thus identified in this study may prove useful in the further exploration of the branch density homeostasis system

as well as the general relationship between tip growth and branching.

The observation that branching responds to exogenous antioxidant (ascorbic acid) exposure provides additional evidence of the role of ROS in branching control. The added ascorbic acid resulted in a dose-dependent shift toward hypobranching. This response becomes complex at higher concentrations, however, as more extreme hypobranching is induced as well as a spike of hyperbranched growth. Similar results were seen with glutathione (data not shown). Exposure to lipid-soluble antioxidants (beta-carotene and alpha-tocopherol) had no detectable impact on branching (data not shown).

Mutations or certain environmental conditions which result in hypobranching are rare. Hypha from germinating ascospores or conidia in the mutant *ipa* are reported (Perkins et al. 2001) to branch less frequently, but quickly revert to normal branch density. The morphological response to cold shock (Watters et al. 2000a) includes a brief hypobranched phase, but that is followed by tightly spaced apical branching and a subsequent return to normal branch density. The response of growing tips to hyphal damage (Watters & Griffiths 2001) likewise includes a hypobranching component, but the response is transient, and branch density rapidly returns to normal. In *Aspergillus*, the temperature sensitive *ahbA1* mutant was reported (Lin & Momany 2004) to result in hypobranching at the restrictive temperature, but continued incubation of the mutant under those conditions proved lethal. In contrast to hyperbranching, hypobranching appears to be rare and unstable. This report marks the first observation of sustained hypobranching.

The results observed are difficult to interpret in the context of the proposed hypothesis that ROS concentration at the tip serves as the appropriate trigger for branch formation. This hypothesis would have predicted that mutants that result in decreased ROS concentrations (NADPH oxidase-1, -2, and NADP oxidase regulator-1 in Table 1) would display hypobranching while those which cause increased ROS concentrations (remaining mutants tested) would display hyperbranching. What is observed is that both cause hypobranching under slow growth conditions. Furthermore, the hypothesis would predict that exogenous antioxidants would also cause hypobranching. While this is observed to be the case, in some extreme concentrations they also cause hyperbranching at the same time. These interesting and seemingly contradictory results suggest that the relationship between ROS and branching is complex. This relationship could be explored further in future using ROS sensitive dyes in actively growing and branching hypha.

LITERATURE CITED

Aguirre, J., M. Rios-Momberg, D. Hewitt & W. Hansberg. 2005. Reactive oxygen species and development in microbial eukaryotes. Trends in Microbiology 13:111–118.

Airaki, M., M. Leterrier, R.M., Mateos, R. Valderrama, M. Chaki, J.B. Barroso, L.A. Del Rio, J.M. Palma & F.J. Corpas. 2012. Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low temperature stress. Plant, Cell and Environment 35:281–295.

Beck, E.H., S. Fettig, C. Knake, K. Hartig & T. Bhattarai. 2007. Specific and unspecific responses of plants to cold and drought stress. Journal of Biosciences 32:501–510.

Cano-Domínguez, N., K. Álvarez-Delfín, W. Hansberg & J. Aguirre. 2008. NADPH oxidases NOX-1 and NOX-2 require the regulatory subunit NOR-1 to control cell differentiation and growth in *Neurospora crassa*. Eukaryotic Cell 7:1352–1361.

Carol, R.J. & L. Dolan. 2006. The role of reactive oxygen species in cell growth: lessons from root hairs. Journal of Experimental Botany 57:1829–1834.

Colot, H.V., G. Park, G.E. Turner, C. Ringelberg, C.M. Crew, L. Litvinkova, R.L. Weiss, K.A. Borkovich & J.C. Dunlap. 2006. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proceedings of the National Academy of Sciences of the United States of America 103:10352–10357.

Davidson, F.A. 2007. Mathematical modeling of mycelia: a question of scale. Fungal Biology Reviews 21:30–41.

Davis, R.H. & F.J. deSerres. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods in Enzymology 17:79–143.

Finkel, T. 2003. Oxidant signals and oxidative stress. Current Opinion in Cell Biology 15:247–254.

Foreman, J., V. Demidchik, J.H. Bothwell, P. Mylona, H. Miedema, M.A. Torres, P. Linstead, S. Costa, C. Brownlee, J.D. Jones, J.M. Davies & L. Dolan. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442–446.

Gavric, O. & A.J.F. Griffiths. 2003. Interaction of mutations affecting tip growth and branching

in *Neurospora*. Fungal Genetics and Biology 40: 261–270.

Gessler, N.N., A.A. Aver'yanov & T.A. Belozerskaya. 2007. Reactive oxygen species in regulation of fungal development. Biochemistry (Moscow) 72: 1091–1109.

Harris, S.D. & M. Momany. 2003. Polarity in filamentous fungi: moving beyond the yeast paradigm. Fungal Genetics and Biology 41:391–400.

Harris, S.D., N.D. Read, R.W. Robertson, B. Shaw, S. Seiler, M. Plamann & M. Momany. 2005. Polarisome meets Spitzenkörper: microscopy, genetics, and genomics converge. Eukaryotic Cell 4:225–229.

Heath, I.B., J.L. Gay & A.D. Greenwood. 1971. Cell wall formation in the Saprolegniales: cytoplasmic vesicles underlying developing walls. Journal of General Microbiology 65:225–232.

Katz, D., D. Goldstein & R.F. Rosenberger. 1972. Model for branch initiation in *Aspergillus nidulans* based on measurements of growth parameters. Journal of Bacteriology 109:1097–1100.

Knechtle, P., F. Dietrich & P. Philippsen. 2003. Maximan polar growth potential depends on the polarisome component AgSpa2 in the filamentous fungus *Ashbya gossypii*. Molecular Biology of the Cell 14:4140–4154.

Lambeth, J.D. 2004. NOX enzymes and the biology of reactive oxygen. Nature Reviews Immunology 4:181–189.

Lin, X. & M. Momany. 2004. Identification and complementation of abnormal hyphal branch mutants ahbA1 and ahbB1 in *Aspergillus nidulans*. Fungal Genetics and Biology 41:998–1006.

McCluskey, K. 2003. The Fungal Genetics Stock Center: from molds to molecules. Advances in Applied Microbiology 52:245–262.

Minke, P.F., I.H. Lee, J.H. Tinsley, K.S. Bruno & M. Plamann. 1999. *Neurospora crassa* ro-10 and ro-11 genes encode novel proteins required for nuclear distribution. Molecular Microbiology 32: 1065–1076.

Miura, K., A. Sato, H. Shiba, S. Won Kang, H. Kamada & H. Ezura. 2012. Accumulation of antioxidants and antioxidant activity in tomato, *Solanum lycopersicum*, are enhanced by the transcription factor SlICE1. Plant Biotechnology 29: 261–269.

Momany, M. 2002. Polarity in filamentous fungi: establishment, maintenance and new axes. Current Opinion in Microbiology 5:580–585.

Mouriño-Pérez, R.R., R.W. Roberson & S. Bartnicki-Garcıa. 2006. Microtubule dynamics and organization during hyphal growth and branching in *Neurospora crassa*. Fungal Genetics and Biology 43:389–400.

Perkins, D.D., A. Radford & M.S. Sachs. 2001. The *Neurospora* Compendium: Chromosomal Loci. Academic Press, San Diego, California U.S.A. 325 pp.

Plamann, M., P.F. Minke, J.H. Tinsley & K.S. Bruno. 1994. Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. Journal of Cell Biology 127:139–149.

Prosser, J.I. 1995. Mathematical modelling of fungal growth. Pp. 319–335. *In* The Growing Fungus. (N.A.R. Gow & G.M. Gadd, Eds.). Springer, Netherlands.

Riquelme, M. & S. Bartnicki-Garcia. 2004. Key differences between lateral and apical branching in hyphae of *Neurospora crassa*. Fungal Genetics and Biology 41:842–851.

Riquelme, M., S. Bartnicki-Garcıa, J.M. Gonzalez-Prieto, E. Sanchez-Leon, J.A. Verdin-Ramos, A. Beltran-Aguilar & M. Freitag. 2007. Spitzenkörper localization and intracellular traffic of GFP-labeled CHS-3 and CHS-6 chitin synthases in living hyphae of *Neurospora crassa*. Eukaryotic Cell 6:1853–1864.

Riquelme, M., O. Yarden, S. Bartnicki-Garcia, B. Bowman, C.L. Ernestina, S.J. Free, A. Fleissner, M. Freitag, R.R. Lew, R. Mouriño-Pérez, M. Plamann, C. Rasmussen, C. Richthammer, R.W. Roberson, E. Sánchez-León, S. Seiler & M.K. Watters. 2011. Architecture and development of the *Neurospora crassa* hypha - a model cell for polarized growth. Fungal Biology 115:446–474.

Seiler, S. & M. Plamann. 2003. The genetic basis of cellular morphogenesis in the filamentous fungus *Neurospora crassa*. Molecular Biology of the Cell 14:4352–4364.

Semighini, C.P. & S.D. Harris. 2008. Regulation of apical dominance in *Aspergillus nidulans* hyphae by reactive oxygen species. Genetics 179: 1919–1932.

Steinberg, G. 2007. Hyphal growth: a tale of motors, lipids, and the Spitzenkörper. Eukaryotic Cell 6:351–360.

Trinci, A.P.J. 1974. A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. Journal of General Microbiology 81:225–236.

Watters, M.K., C. Humphries, C.I. deVries & A.J.F. Griffiths. 2000a. A homeostatic set point for branching in *Neurospora crassa*. Mycological Research 104:557–563.

Watters, M.K., A. Virag, J. Haynes & A.J.F. Griffiths. 2000b. Branch initiation in *Neurospora* is influenced by events at the previous branch. Mycological Research 104:805–809.

Watters, M.K. & A.J.F. Griffiths. 2001. Tests of a cellular model for constant branch distribution in the filamentous fungus *Neurospora crassa*. Applied and Environmental Microbiology 67:1788–1792.

Watters, M.K., E.R. Lindamood, M. Muenich & R. Vetor. 2008. Strain-dependent relationship

between growth rate and hyphal branching in *Neurospora crassa*. Proceedings of the Indiana Academy of Science 117:1–6.

Watters, M.K., M. Boersma, M. Johnson, C. Reyes, E. Westrick & E. Lindamood. 2011. A screen for *Neurospora* knockout mutants displaying growth rate dependent branch density. Fungal Biology 115:296–301.

Watters, M.K. 2013. Control of branching in *Neurospora crassa*. Pp. 23–44. *In Neurospora* Genomics and Molecular Biology. (D.P. Kasbekar & K. McCluskey, Eds.). Caister Academic Press, Norfolk, United Kingdom.

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