Molecular Microbiology (2012) 86(6), 1334–1344 ■

doi:10.1111/mmi.12077
First published online 5 November 2012

Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies

Robert-Jan Bleichrodt,[1,2] G. Jerre van Veluw,[1,2] Brand Recter,[1,2] Jun-ichi Maruyama,[3] Katsuhiko Kitamoto[3] and Han A. B. Wösten[1,2]*

[1]*Department of Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.*
[2]*Kluyver Centre for Genomics of Industrial Fermentation, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.*
[3]*Department of Biotechnology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.*

Summary

Hyphae of higher fungi are compartmentalized by septa. These septa contain a central pore that allows for inter-compartmental and inter-hyphal cytoplasmic streaming. The cytoplasm within the mycelium is therefore considered to be a continuous system. In this study, however, we demonstrate by laser dissection that 40% of the apical septa of exploring hyphae of *Aspergillus oryzae* are closed. Closure of septa correlated with the presence of a peroxisome-derived organelle, known as Woronin body, near the septal pore. The location of Woronin bodies in the hyphae was dynamic and, as a result, plugging of the septal pore was reversible. Septal plugging was abolished in a Δ*Aohex1* strain that cannot form Woronin bodies. Notably, hyphal heterogeneity was also affected in the Δ*Aohex1* strain. Wild-type strains of *A. oryzae* showed heterogeneous distribution of GFP between neighbouring hyphae at the outer part of the colony when the reporter was expressed from the promoter of the glucoamylase gene *glaA* or the α-glucuronidase gene *aguA*. In contrast, GFP fluorescence showed a normal distribution in the case of the Δ*Aohex1* strain. Taken together, it is concluded that Woronin bodies maintain hyphal heterogeneity in a fungal mycelium by impeding cytoplasmic continuity.

Introduction

Cellular heterogeneity within an isogenic cell population is common in prokaryotic and eukaryotic organisms. Heterogeneity of cells can be beneficial for organisms in many ways. For instance, it is important for cell specialization (Kaech and Wherry, 2007) and it may function in the survival of (members of) the population under adverse conditions (Nobile and Mitchell, 2007; Veening *et al.*, 2008). It has been shown that hyphae within a fungal mycelium are also heterogeneous. Hyphal heterogeneity has been observed in the case of gene expression, growth and secretion (Wösten *et al.*, 1991; Moukha *et al.*, 1993a,b; Teertstra *et al.*, 2004; Vinck *et al.*, 2005; 2011; Masai *et al.*, 2006; Levin *et al.*, 2007a,b; Kasuga and Glass, 2008; Etxebeste *et al.*, 2009; de Bekker *et al.*, 2011a,b). For instance, only part of the hyphae at the periphery of a vegetative mycelium of *Aspergillus niger* secrete glucoamylase (Wösten *et al.*, 1991). This is due to heterogeneous expression of *glaA* in this part of the colony. In fact, two populations of hyphae can be distinguished; one that highly and one that lowly expresses this gene (Vinck *et al.*, 2005). Hyphae that show high *glaA* expression also highly express other genes encoding secreted proteins. Moreover, they have a high rRNA content and highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* (Vinck *et al.*, 2011). From these studies it was concluded that two populations of hyphae can be distinguished at the outer part of the vegetative mycelium, one with a 'high' and one with a 'low' transcriptional and translational activity. The low activity would be sufficient to support growth but a high activity would be needed to support secretion of high amounts of protein.

Hyphae of Ascomycota and Basidiomycota are compartmentalized by porous septa. The diameter of the septal pore varies between 50 and 500 nm, which allows for passage of cytosol and even organelles (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Upon mechanical injury, septa of ascomycetes are plugged by Woronin bodies to prevent excessive cytoplasmic bleeding (Trinci and Collinge, 1974; Collinge and Markham, 1985; Jedd and Chua, 2000; Tenney *et al.*, 2000; Soundararajan *et al.*, 2004; Maruyama *et al.*, 2005; Dhavale and Jedd, 2007). The Woronin bodies of *Aspergillus nidulans* are usually located at the septal pore or in apical regions (Momany *et al.*, 2002). In the case of *Neurospora crassa*, they are tethered via WSC and Leashin to the cell cortex (Ng *et al.*, 2009; Jedd, 2011). Woronin

Accepted 15 October, 2012. *For correspondence. E-mail h.a.b.wosten@uu.nl; Tel. (+31) 30 253 3448; Fax (+31) 30 251 3655.

© 2012 Blackwell Publishing Ltd

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

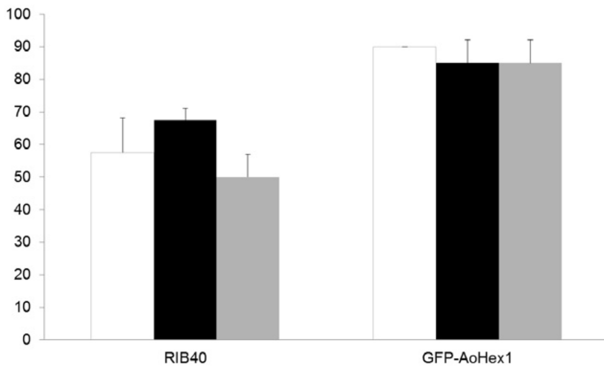


Fig. 1. Percentage of open septa of leading hyphae of the wild-type *A. oryzae* strain RIB40 and the Δ*Aohex1* strain that had been complemented with EGFP–AoHex1. White, black and grey bars show percentages of the first, second and third most apical septa respectively. Experiments were carried out with 20 hyphae in duplicate. Bars represent standard deviations.

bodies originate from peroxisomes by Pex11- and WSC-mediated budding (Jedd and Chua, 2000; Liu *et al.*, 2008; Escaño *et al.*, 2009). Pex14 functions in biogenesis of Woronin bodies in *N. crassa* by playing a role in import of HEX1 in the organelle (Managadze *et al.*, 2007). The lumen of Woronin bodies is filled with hexagonal rods of the HEX1 protein (Hoch and Maxwell, 1974; Jedd and Chua, 2000; Managadze *et al.*, 2010). Deletion of *HEX1* in *N. crassa*, *Magnaporthe grisea* and *Aspergillus oryzae* results in the absence of Woronin bodies (Jedd and Chua, 2000; Tenney *et al.*, 2000; Soundararajan *et al.*, 2004; Maruyama *et al.*, 2005).

Here, it is shown that Woronin bodies of *A. oryzae* plug septa not only in damaged hyphae but also in intact growing hyphae. By doing so, they maintain hyphal heterogeneity in a fungal mycelium by impeding cytoplasmic continuity.

Results

Septal closure during vegetative growth does not depend on environmental conditions

It was assessed whether septa in intact growing hyphae are open or closed. To this end, the wild-type *A. oryzae* RIB40 strain was grown for 2 days at 30°C in a glass bottom microscopy dish in CD + Met medium. The apical compartments of hyphae at the most outer part of RIB40 colonies were dissected using a UV laser and simultaneously it was monitored whether cytoplasm from the sub-apical, adjacent compartment was streaming through the septum of the damaged compartment. The septum was scored as 'open' when cytoplasm was streaming through the septum towards the ruptured compartment (see Movie S1). When cytoplasmic movement quickly ceased within seconds, the septum was scored 'quickly closed' (Movie S2). In the case cytoplasmic streaming was not observed, the septum was scored as 'closed' (Movie S3). Using these criteria it was found that 57.5 ± 10.6% of the apical septa of the wild-type *A. oryzae* strain were open (this included the septa that were scored quickly closed) (Fig. 1). In more detail, 5 ± 7.1%, 52.5 ± 17.7% and 42.5 ± 10.6% of the septa were scored as 'open', 'quickly closed' and 'closed' respectively. Similar percentages of open septa were found for the second and third septa (67.5 ± 3.5% and 50 ± 7.1% respectively; Fig. 1). To assess whether the septal plugging state of neighbouring septa correlates, the first three compartments of hyphae were sequentially dissected. It was observed that neighbouring septa of closed compartments can be either open or closed (Table 1). Taken together, these data show that there is no difference in septal plugging incidence between the first three septa of intact growing hyphae at the periphery of an *A. oryzae* RIB40 colony and that closure of neighbouring septa does not correlate.

Aspergillus oryzae RIB40 was grown on CD + Met medium for 2 days at 30°C, after which it was subjected to 4°C or 45°C, hypo- or hypertonic conditions, or pH 2.0 or 11.0. Alternatively, RIB40 was grown for 2 days with C or N limitation. Laser dissection showed that none of the conditions significantly influenced the septal plugging incidence of the first (i.e. the apical) septum (Fig. 2). After incubating the mycelium of RIB40 for 30 min at 4°C or 45°C, 72.5 ± 3.5% and 65 ± 0% of the septa were open respectively (Fig. 2). When it was subjected to 1 M $MgSO_4$ or to H_2O for 1.5 h, 57.5 ± 3.5% and 77.5 ± 3.5% of the septa were open respectively. Similarly, 62.5 ± 10.6% and 67.5 ± 10.6% of the septa were open after incubation of the mycelium at pH 2.0 or pH 11.0 for 1.5 h. These numbers were 57.5 ± 10.6% and 70 ± 7.1%, when the mycelium was exposed to carbon and nitrogen starvation for 2 days (Fig. 2).

Table 1. Absence of association between the plugging state of the three most apical septa with a growing hypha of *A. oryzae*.

Hypha number	First septum	Second septum	Third septum
1	1	1	1
2	0	0	0
3	1	1	1
4	0	1	0
5	0	0	0
6	0	0	1
7	0	1	0
8	1	0	0
9	1	0	1
10	0	0	0

1 = septum open, 0 = septum closed.

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

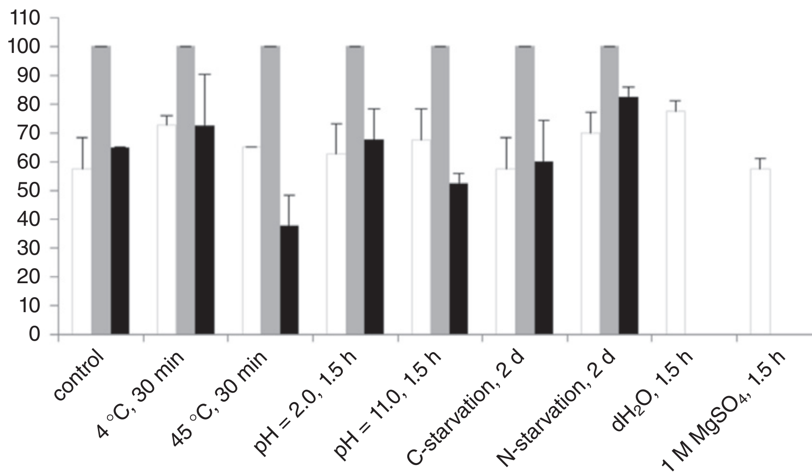


Fig. 2. Percentage of open apical septa of leading hyphae of the wild-type *A. oryzae* strain RIB40 (white bars), control strain NSRKu70-1-1AS (black bars) and the Δ*Aohex1* strain NSRK-ΔHx5 (grey bars) under various environmental conditions. Strains were grown for 2 days on CD + Met medium (RIB40) or M medium (NSRKu70-1-1AS and NSRK-ΔHx5), after which they were exposed to 4°C or 45°C, pH 2.0 or 11.0, C or N limitation, and in the case of RIB40, to hypo- or hypertonic conditions. Experiments were carried out with 20 hyphae in duplicate. Bars represent standard deviations.

Woronin bodies are responsible for septal closure during vegetative growth

The *A. oryzae* Δ*Aohex1* deletion strain NSRK-ΔHx5 was grown for 2 days at 30°C on M medium and was exposed to standard environmental conditions and to 4°C, 45°C, pH 2.0, pH 11.0, and to carbon and nitrogen starvation. In all cases, 100 ± 0% of the apical septa were open (Fig. 2). In contrast, on average 62 ± 3.5% of the septa of the control strain NSRKu70-1-1AS were open during these conditions. Notably, cytoplasmic movement was observed throughout at least seven compartments after damaging the apical compartment of leading hyphae of NSRK-ΔHx5. In contrast, cytoplasmic movement was only observed in the second and sometimes the third compartment of strain NSRKu70-1-1AS.

Septal closure in strain NSRK-ΔHx5 was partially rescued after introduction of a vector encompassing a gene encoding EGFP–AoHex1 under control of the *amyB* promoter. In the resulting strain 11NSR-NAGHs 90 ± 0%, 85 ± 7.1% and 85 ± 7.1% of the first, second and third septa of the leading hyphae, respectively, were open under standard growth conditions (Fig. 1). The partial complementation of AoHex1 by EGFP–AoHex1 may be caused by a lower binding of the fusion protein to WSC when compared to the native AoHex1 protein. This binding is needed for proper localization of the Woronin body at the septal pore (Ng *et al*., 2009). Septa of the 11NSR-NAGHs strain with a Woronin body localized at the septal pore (visualized by the reporter protein EGFP–AoHex1) were always closed (Fig. 3A). In contrast, in the absence of a Woronin body, the septum was open in 100% of the cases. These results show that Woronin bodies of *A. oryzae* close septa during vegetative growth under various environmental conditions. Confocal laser scanning microscope (CLSM) live cell imaging of three hyphae showed that Woronin body localization at the septum is dynamic. Woronin bodies moved away from the septum between 15 min and several hours of growth (Fig. 3B).

Heterogeneous distribution of GFP *resulting from glaA- and aguA-driven expression is abolished in a ΔAohex1 strain*

Constructs pAN52-10S65TGFPn/s (Siedenberg *et al*., 1999) and P*aguA*sGFP+ (Vinck *et al*., 2011) encompassing the *sGFP* gene under control of the *glaA* and *aguA* promoters of *A. niger*, respectively, were introduced in strains NSRKu70-1-1AS and NSRK-ΔHx5 of *A. oryzae*. Transformants were screened by fluorescence microscopy and two representative strains of each transformation were selected. These strains were called RB#140.1 and RB#140.2 (*glaA*, Δ*ku70* background), RB#154.3 and RB#154.4 (*aguA*, Δ*ku70* background), RB#141.3 and RB#141.4 (*glaA*, Δ*ku70*Δ*Aohex1* background), and RB#156.2 and RB#156.4 (*aguA*, Δ*ku70*Δ*Aohex1* background). Hyphal heterogeneity of GFP accumulation at the outer part of colonies of these strains was assessed by modelling log transformed fluorescence intensity distributions assuming the existence of two populations of hyphae (i.e. one with high and one with low GFP fluorescence) (Fig. 4). For this, 50–100 μm sections of apical compartments of hyphae were measured. The confidence intervals of the mean in populations with low (μ_1) and high (μ_2) GFP fluorescence were not overlapping in *A. oryzae* strains RB#140.1, RB#140.2; RB#154.3 and RB#154.4 that all contain Woronin bodies (Table 2). This showed that *glaA*- and *aguA*-driven expression of GFP is heterogeneous in these strains. In contrast, the two confidence intervals of the mean in strains RB#141.3 and RB#141.4, and RB#156.2 and RB#156.4, all having the *Aohex1* deletion, did overlap. Thus, the assumption that GFP distribution is

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

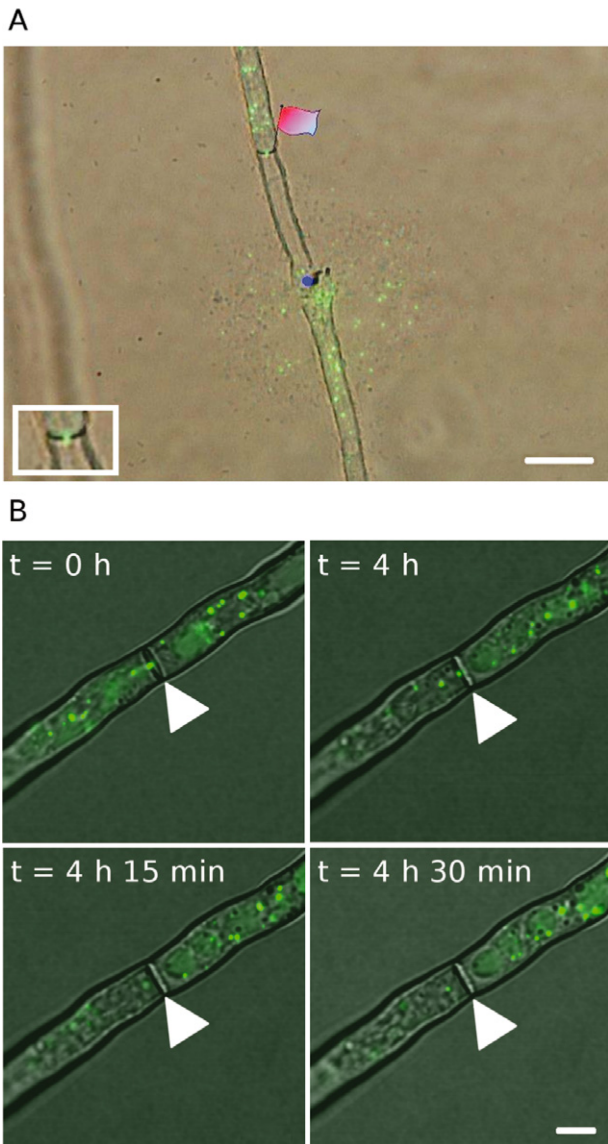


Fig. 3. Woronin body closes a septum reversibly. Strain 11NSR-NAGHs was grown for 2 days on CD + Met medium. A septum with a Woronin body (tagged with EGFP–AoHex1) was selected.
A. No cytoplasmic streaming was observed through the septum (indicated by the flag) after dissection of the hypha at the spot indicated by the blue dot. Inset shows a magnification of the Woronin body plugging the septum.
B. The location of a Woronin body at the septum (arrowhead) was monitored in time using CLSM. After 4 h the Woronin body was still localized at the septum but 15 min later the organelle had lost its position.
Bar represents 25 (A) and 5 (B) µm respectively.

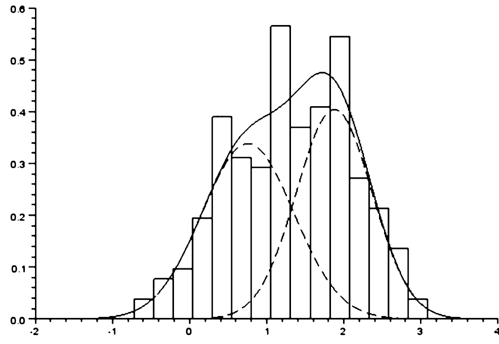
heterogeneous in these strains was falsified (Fig. 4, Table 2). Taken together, these data show that Woronin bodies maintain hyphal heterogeneity in the *A. oryzae* mycelium.

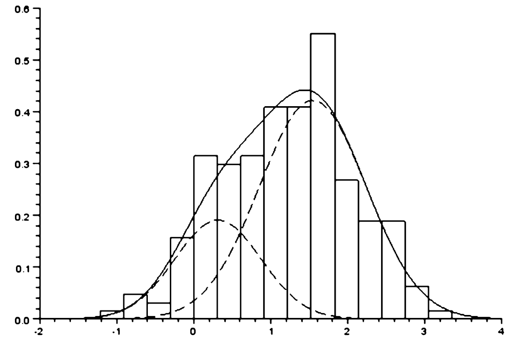
Discussion

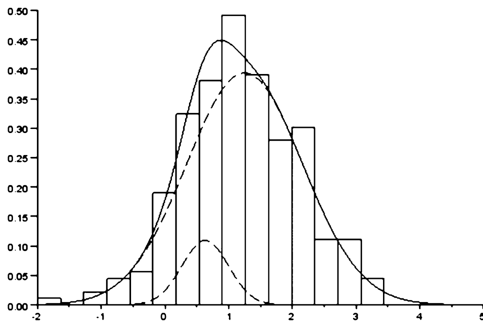
Intercellular cytoplasmic connections have been identified in multicellular eukaryotic organisms belonging to the four classical kingdoms. In the case of animals, gap junctions provide the cytoplasmic contact. They mediate interchange of molecules < 1000 Da. Plasmodesmata in plants provide intercellular transport of, for instance, photoassimilates, mRNA and proteins. The pore sizes in septa of fungi are even larger. They allow inter-compartmental streaming of macromolecules and organelles. Gap junctions and plasmodesmata can regulate their pore size and in this way function in developmental processes (Heinlein, 2002; Norris *et al*., 2008). Little is known about closure of fungal septa. So far, it is generally believed that septa are open in intact fungal hyphae. In this view, the mycelium would consist of a continuous cytoplasm. Recently, it was shown that septa of *Schizophyllum commune* can already be plugged in intact growing hyphae (van Peer *et al*., 2009). It was demonstrated that apical septa are open in intact vegetative hyphae of this basidiomycete. In contrast, only 50% and 10% of the second and third septa are open respectively. Septal plugging was shown to be reversible and to depend on environmental conditions (van Peer *et al*., 2009). A strain in which the *spc33* gene was inactivated did not form septal pore caps and, as a result, septal plugging was abolished (van Peer *et al*., 2010). In this study it was shown that septa of intact hyphae of the ascomycete *A. oryzae* can also be reversibly plugged. In this case, Woronin bodies are essential for closure of septa. These results refute the general view that a fungal mycelium consists of a continuous cytoplasm. It should be noted that it is not yet clear how general these results apply to other species of ascomycetes and basidiomycetes. We do demonstrate that septal closure results in heterogeneity of cytoplasmic composition of neighbouring hyphae within a mycelium of *A. oryzae*.

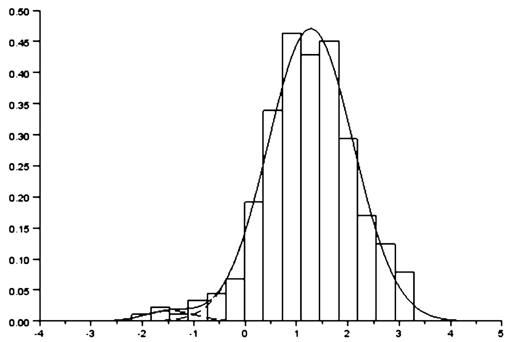
Using laser dissection it was shown that about 60% of the first three septa of hyphae at the periphery of an *A. oryzae* colony are open. The plugging state of the first septum did not correlate with that of the second or the third septum. The plugging state also did not depend on the environmental conditions. Septa were always closed when a GFP-tagged Woronin body had been localized at the septal pore. Conversely, septa were always open when a Woronin body was absent. This strongly indicated a role for Woronin bodies in plugging septa of intact vegetative hyphae. Indeed, all septa were open, irrespective of the growth conditions, in a Δ*Aohex1* strain that cannot form these organelles. Live cell imaging showed that Woronin body localization near septa was dynamic in intact vegetative hyphae. Taken together, these results imply that closure of septa in *A. oryzae* is a reversible process. In contrast to *S. commune*, there seems not to

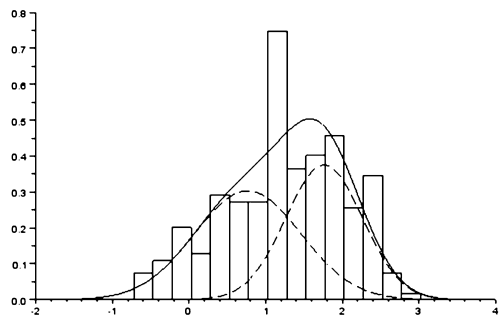
13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

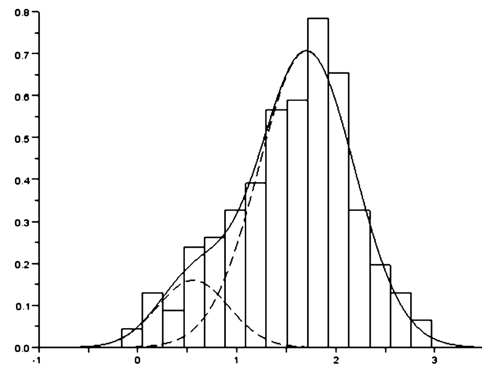


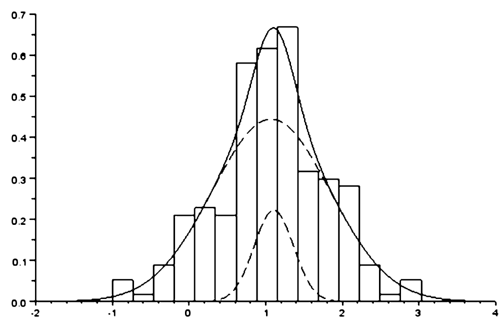


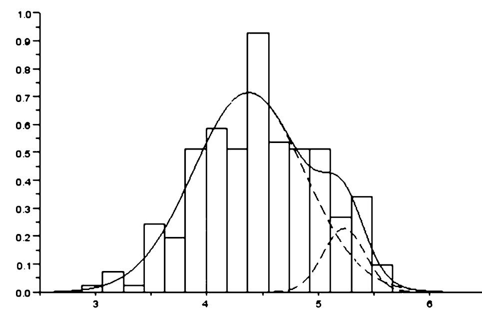












13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Fig. 4. Fluorescence intensity distributions resulting from *glaA*-driven GFP expression in *A. oryzae* strains with (A and B) and without Woronin bodies (Δ*Aohex1*) (C and D) and resulting from *aguA*-driven GFP expression in *A. oryzae* strains with (E and F) and without Woronin bodies (Δ*Aohex1*) (G and H). Data (± 200 hyphae for each strain) were fitted assuming the existence of a bimodal fluorescence distribution between the hyphae at the periphery of the mycelium after log transformation.

be a mechanism directing septal closure in intact vegetative hyphae. It seems to be a stochastic driven process.

The general accepted view that the cytoplasm is continuous in a fungal mycelium due to the porosity of septa (Jennings *et al*., 1974; Jennings, 1987) is in conflict with hyphal heterogeneity as has been observed in *A. niger* (Wösten *et al*., 1991; Vinck *et al*., 2005; 2011; Levin *et al*., 2007a,b; Etxebeste *et al*., 2009; de Bekker *et al*., 2011a) and *A. oryzae* (Maruyama *et al*., 2006). Septal plugging in intact hyphae, however, explains why hyphae can be heterogeneous with respect to RNA and protein composition. The fact that each septum has a chance of 40% to be closed implies that only in about 5% of the cases the cytoplasm of two hyphae is in physical contact when they are separated by six septa. Absence of Woronin bodies would result in a continuous cytoplasm and consequently hyphal heterogeneity would be abolished. This hypothesis was tested. It was shown that GFP distribution was heterogeneous between neighbouring hyphae of a wild-type *A. oryzae* when the reporter was expressed from the *A. niger glaA* and *aguA* promoters (Fig. 5A). Two populations of hyphae were distinguished: those with a high and those with a low GFP fluorescence. In contrast, populations of hyphae with low and high GFP content could not be shown to exist in the Δ*Aohex1* mutant (Fig. 5B). From this it is concluded that hyphal heterogeneity is abolished in strains that do not form Woronin bodies. We propose that heterogeneous gene expression still occurs in the Δ*Aohex1* mutant. However, since all septa are open, cytoplasmic streaming evenly distributes gene products between neighbouring hyphae.

So far, it is not clear why colonies send out exploring hyphae that are heterogeneous with respect to transcriptional and translational activity. Possibly, this increases the chance that (some of the) hyphae survive when a colony is exposed to stress conditions like the presence of antibiotics, reactive oxygen species or high temperature. Plugging of septa by Woronin bodies would maintain diversity of RNA and protein composition between the hyphae, and thereby would promote survival of the mycelium. In agreement with this, the *A. niger* Δ*Anhex1* strain died when exposed for 3 days to 45°C, whereas the strain with Woronin bodies survived (Bleichrodt, 2012). This effect could not be explained by more excessive bleeding (data not shown).

Experimental procedures

Strains and growth conditions

Strains used in this study are listed in Table 3. RIB40 was used as wild-type strain (Machida *et al*., 2005). Strain NSRKu70-1-1A (Escaño *et al*., 2009) is a derivative of NSRKu70-1-1 (Δ*ku70 niaD⁻ sC⁻ adeA⁻*; Escaño *et al*., 2009) expressing the *adeA* selection marker gene. NSRKu70-1-1AS (Tanabe *et al*., 2011) is a derivative of NSRKu70-1-1A, which has been transformed with the *sC* selection marker gene. NS4 (*niaD⁻ sC⁻*) (Yamada *et al*., 1997) is the parental strain of NSR13 (*niaD⁻ sC⁻ adeA⁻*; Jin *et al*., 2004). Inactivation of *Aohex1* in strain NSRKu70-1-1A, or in NSR13, resulted in strains NSRK-ΔHx5 and NSR-ΔHx11 respectively. Strain 11NSR-NAGHs expresses EGFP–AoHex1 under control of the *amyB* promoter in strain NSR-ΔHx11. Strains RB#140.1, RB#140.2, RB#154.3 and RB#154.4 are derivatives of *A. oryzae* NSRKu70-1-1AS (Δ*ku70*). The former two express sGFP under the control of the *A. niger glaA* promoter, while the latter two express sGFP under the control of the *A. niger aguA* promoter. Similarly, RB#141.3 and RB#141.4 express sGFP under the control of the *A. niger glaA* promoter, while RB#156.2 and RB#156.4 express sGFP under the control of

Table 2. 95% CI of the mean assuming a bimodal fluorescence distribution of *glaA*- or *aguA*-driven GFP expression after log transformation in hyphae of strains with and without Woronin bodies.

Strains	Background	Promoter used for GFP expression	*n*	CI (μ_1)	CI (μ_2)	CI (pf_1)
RB#140.1	Δ*ku70*	*glaA*	203	−0.10, 1.18	1.43, 2.30	0.078, 0.892
RB#140.2	Δ*ku70*	*glaA*	209	−0.18, 1.19	1.23, 2.71	0.041, 0.955
RB#141.3	Δ*ku70* Δ*Aohex1*	*glaA*	247	−2.00, 1.27	0.65, 3.04	0.005, 0.972
RB#141.4	Δ*ku70* Δ*Aohex1*	*glaA*	242	−2.21, 1.31	1.01, 2.73	0.007, 0.942
RB#154.3	Δ*ku70*	*aguA*	220	−0.50, 1.21	1.28, 2.35	0.051, 0.940
RB#154.4	Δ*ku70*	*aguA*	220	0.10, 1.49	1.58, 2.00	0.034, 0.819
RB#156.2	Δ*ku70* Δ*Aohex1*	*aguA*	212	−0.86, 1.18	0.97, 2.81	0.017, 0.974
RB#156.4	Δ*ku70* Δ*Aohex1*	*aguA*	220	−0.02, 1.64	1.61, 2.98	0.024, 0.976

n = sample size; CI (μ_1) and CI (μ_2) represent the confidence intervals of the lower and upper limits of populations 1 and 2 respectively. CI (pf_1) represents the confidence interval of the lower and upper limits of the participation fraction of population 1.

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

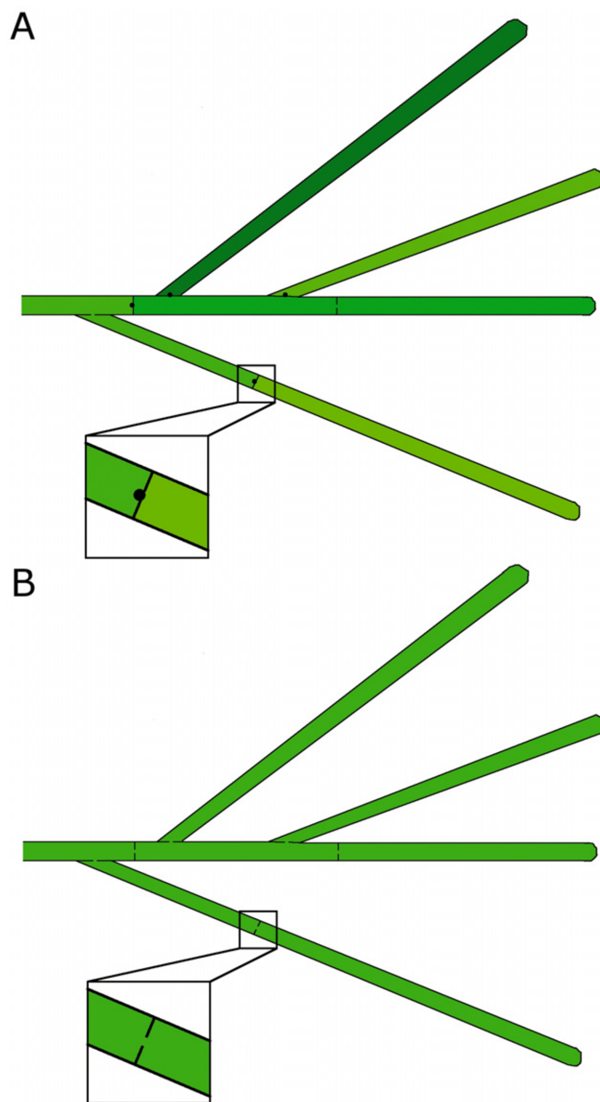


Fig. 5. Schematic representation showing the effect of septal plugging on hyphal heterogeneity. Wild type (A) shows normal septal plugging levels that maintain the differences in RNA and/or protein levels between neighbouring hyphae. Hyphae are shown with different gene expression levels (indicated by differences in green colour). Black dots indicate Woronin bodies that close septa (magnified in the inlay). The *hex1* mutant (B) shows homogeneous levels of gene products. Although there is heterogeneous gene transcription between neighbouring hyphae, these levels cannot be maintained. Since all septa are open (magnified in the inlay) cytoplasmic streaming distributes gene products between neighbouring hyphae.

the *A. niger aguA* promoter, but these strains are derivatives of *A. oryzae* NSRK-ΔHx5 (Δ*ku70* Δ*Aohex1*).

To obtain spores for inoculation, *A. oryzae* was grown on 3.7% PDA (potato dextrose agar, Sigma Aldrich, http://www.sigmaaldrich.com). Spores were harvested in 0.9% NaCl (w/v) containing 0.05% (v/v) Tween-20 and diluted to a final concentration of 5×10^5 spores ml^{-1}.

For microscopy, *A. oryzae* was grown in glass bottom dishes (MatTek, http://www.glass-bottom-dishes.com, P35G-1.5-20-C) essentially as described by van Peer *et al.* (2009). Strains with nitrate prototrophy were grown on CD + Met medium (0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002%, $FeSO_4.7H_2O$, 1% glucose, 0.0015% methionine, pH 5.5; Maruyama *et al.*, 2010), while strains with nitrate auxotrophy (*niaD*⁻) were grown on M medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002% $FeSO_4.7H_2O$, 1% glucose, pH 5.5; Ohneda *et al.*, 2005). The glass bottom dishes were filled with 30 µl of CD + Met medium or M medium containing 1% agarose. To this end, the glass bottom dishes and the agar medium were pre-warmed at 50°C. Spores (250 spores in 0.5 µl) were placed in the middle of an 18 mm cover glass and placed upside down on the non-solidified agarose medium. This resulted in a 118 µm thin agarose layer. After solidifying the agarose medium, 2 ml of liquid medium was added on top of the culture.

For the septal plugging experiments, cultures were grown for 2 days at 30°C under water saturating conditions, after which they were either or not exposed to stress conditions. Temperature stress was imposed by incubation at 4°C or 45°C for 30 min. For pH stress, the liquid medium was replaced by CD + Met medium with a pH of 2.0 or 11.0 and subsequent incubation for 1.5 h. To this end, the pH was adjusted with HCl or NaOH respectively.

Starvation stress was imposed from $t = 0$ onwards during 2 days of growth. For carbon starvation, solid medium contained 0.2% glucose and liquid medium contained no glucose. For nitrogen starvation solid medium contained nitrate/ammonium (see above), but liquid medium did not contain a nitrogen source.

Inactivation of the Aohex1 gene

The 1.9 kb upstream flanking region of the *Aohex1* open reading frame was amplified with primers aB4F and aB1R (Table S1) and using RIB40 genomic DNA as a template. The fragment was inserted into pDONR™ P4-P1R (Invitrogen, http://www.invitrogen.com) by BP recombination reaction generating the 5′ entry clone plasmid, pg5′Ahx1. The 1.6 kb downstream flanking region of *Aohex1* was amplified with primers aB2F and aB3R (Table S1) and inserted into pDONR™ P2R-P3 (Invitrogen) by BP recombination reaction generating the 3′ entry clone plasmid, pg3′Ahx1. The 5′ and 3′ entry clones together with the centre entry clone pgEsC (containing the *sC* marker gene) were subjected with LR clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pgΔdAoHex1. The *Aohex1* gene deletion fragment (~ 6.5 kb) was amplified by PCR using the plasmid pgΔdAoHex1 as template and primers aB4F and aB3R. Strain NSRKu70-1-1A (Escaño *et al.*, 2009) was transformed with the deletion fragment as described (Ohneda *et al.*, 2005). Transformants with the *sC*⁺ phenotype were selected on M medium containing 1.5% agar and 2% glucose. Disruption of the *Aohex1* gene in strain NSRK-ΔHx5 was confirmed by Southern blotting using restriction enzymes BamHI and EcoT221. Plasmid pgEsC containing the *sC* selection marker gene was introduced into *A. oryzae* strain NSRKu70-1-1A generating the control strain NSRKu70-1-1AS.

The *Aohex1* deletion vector pgDAHx1 was also constructed using the Multisite Gateway cloning system (Invitrogen)

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Table 3. Strains used in this study.

Strain	Parental strain	Genotype	Auxotrophy	Reporter plasmid	Reference
RIB40		Wild type			Machida *et al.* (2005)
NSR13	NS4		*niaD⁻ sC⁻ adeA⁻*		Jin *et al.* (2004)
NSRKu70-1-1A	NSRKu70-1-1	*Δku70*	*niaD⁻ sC⁻*		Escaño *et al.* (2009)
NSRKu70-1-1AS	NSRKu70-1-1A	*Δku70*	*niaD⁻*		Tanabe *et al.* (2011)
NSRK-ΔHx5	NSRKu70-1-1A	*Δku70 ΔAohex1*	*niaD⁻*		This study
NSR-ΔHx11	NSR13	*ΔAohex1*	*niaD⁻ sC⁻*		This study
11NSR-NAGHs	NSR-ΔHx11	*ΔAohex1*	*sC⁻*	PUNAGHs	This study
RB#140.1	NSRKu70-1-1AS	*Δku70*		pAN52-10S65TGFPn/s	This study
RB#140.2	NSRKu70-1-1AS	*Δku70*		pAN52-10S65TGFPn/s	This study
RB#141.3	NSRK-ΔHx5	*Δku70 ΔAohex1*		pAN52-10S65TGFPn/s	This study
RB#141.4	NSRK-ΔHx5	*Δku70 ΔAohex1*		pAN52-10S65TGFPn/s	This study
RB#154.3	NSRKu70-1-1AS	*Δku70*		P*aguA*_sGFP+	This study
RB#154.4	NSRKu70-1-1AS	*Δku70*		P*aguA*_sGFP+	This study
RB#156.2	NSRK-ΔHx5	*Δku70 ΔAohex1*		P*aguA*_sGFP+	This study
RB#156.4	NSRK-ΔHx5	*Δku70 ΔAohex1*		P*aguA*_sGFP+	This study

(Mabashi *et al.*, 2006). The upstream (2.0 kb) and downstream (2.0 kb) regions of the *Aohex1* gene were amplified by PCR using the primer combinations 5-hex1-F and 5-hex1-R, and 3-hex1-F and 3-hex1-R respectively (Table S1). The amplified upstream and downstream regions of *Aohex1* were introduced into pDNORP4-P1R and pDONRP2R-P3, respectively, with the Gateway BP clonase reaction. The resulting plasmids were subjected to Gateway LR clonase reaction together with the centre entry clone plasmid containing the *A. oryzae adeA* gene as a selection marker (Jin *et al.*, 2007) and the destination vector pDESTR4-R3 (Invitrogen). The resulting plasmid pgDAHx1 was used as a template to amplify the deletion cassette by PCR with the primers 5-hex1-F and 3-hex1-R. The amplified deletion fragment was introduced into *A. oryzae* NSR13 according to Maruyama and Kitamoto (2011). A representative transformant was selected in M medium for *adeA* prototrophy and named NSR-ΔHx11. Disruption of the *Aohex1* gene was confirmed by Southern analysis. For this, genomic DNA was digested with BamHI and NcoI and the 2.0 kb fragment of the *Aohex1* upstream region was used as a probe.

Expression of EGFP–AoHex1

The expression vector pUNAGHs (Juvvadi *et al.*, 2007) encompassing the fusion of *EGFP* and *Aohex1* under the control of the *amyB* promoter was introduced into NSR-ΔHx11 using *niaD* as a selection marker according to Maruyama and Kitamoto (2011). Strain 11NSR-NAGHs (Table 3) showed fluorescence representative for the transformants obtained.

Expression of sGFP *under control of the glaA and aguA promoters*

Aspergillus oryzae strains NSRKu70-1-1AS and NSRK-ΔHx5 were co-transformed with pAN52-10S65TGFPn/s (Siedenberg *et al.*, 1999) and pNR10 (Yoon *et al.*, 2010) or with P*aguA*_sGFP+ (Vinck *et al.*, 2011) and pNR10 as described (Punt and van den Hondel, 1992; de Bekker *et al.*, 2009). These plasmids contain *sGFP* (S65T) under the regulation of the *glaA* (pAN52-10S65TGFPn/s) or *aguA* (P*aguA*_sGFP+) promoter of *A. niger* and *niaD* (pNR10) under the control of the *amyB* promoter of *A. oryzae*. Nitrate prototrophic (*niaD⁺*) strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5% agar; de Bekker *et al.*, 2009).

Fluorescence microscopy

The reporter protein EGFP–AoHex1 was monitored over periods of up to 16 h on a Zeiss CLSM (Zeiss LSM 5 PASCAL; Zeiss, http://www.zeiss.com) using a 488 nm laser and a LP505 filter. For live cell imaging, images were taken in the Z-plane in three slices (slice thickness 0.4 μm) every 15 min using a Plan-Neofluor 25×/0.8 Imm Corr objective. The pixel time was 2.51 μs, the laser power 0.125 mW and the pinhole 136 μm. Images were taken with a resolution of 512 × 512 pixels and exported as tif files using the Zeiss LSM Image Browser v4.2 (http://www.zeiss.co.jp). Composition and layout of exported images was made with GIMP v2.6 (http://www.gimp.org/).

Heterogenic expression of sGFP *under the control of the glaA and aguA promoters*

Aspergillus oryzae was grown as a sandwiched culture (de Bekker *et al.*, 2011a) on minimal medium [0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.2 ml l^{-1} Vishniac (per litre: 10 g of EDTA, 4.4 g of $ZnSO_4$, 1.01 g of $MnCl_2$, 0.32 g of $CoCl_2$, 0.315 g of $CuSO_4$, 0.22 g of ammonium heptamolybdate, 1.47 g of $CaCl_2$ and 1.0 g of $FeSO_4$; Vishniac and Santer, 1957), pH 6.0; de Vries and Visser 1999] containing 3% agar and 200 mM xylose (*glaA* repressing) or 50 mM glucose (*aguA* repressing). To this end, 2 μl containing 1000 spores was spotted at the centre of a polycarbonate membrane (76 mm; Profiltra, http://www.profiltra.nl) that was placed on top of the solidified medium. After 24 h a Lumox membrane (20 × 20 mm, manually cut; Greiner Bio-One, http://www.greinerbioone.com) was placed on top of the culture with the hydrophobic side facing the colony. After 42 h of growth, the sandwiched colony was transferred for 6 h to minimal medium plates containing 25 mM maltose (*glaA* inducing) for strains RB#140.1, RB#140.2, RB#141.3 and RB#141.4. For strains

RB#154.3, RB#154.4, RB#156.2 and RB#156.4 the sandwiched colony was transferred for 6 h to minimal medium plates containing 25 mM xylose (*aguA* inducing). The Lumox membrane was removed and a piece of the polycarbonate membrane (approximately 10 × 10 mm) carrying the colony was cut and placed upside down in a glass bottom microscopy dish (MatTek, P35G-1.5-20-C) on a 20 μl drop of minimal medium. GFP fluorescence was monitored on the Zeiss LSM 5 system equipped with a Plan-Neofluar 16×/0.5 oil immersion objective. GFP was excited with a 488 nm laser and images were captured as a Z-stack of optical slices (pinhole 1–2 airy units; optimal interval 2.02 mm; 4× line average; 8 bit scan depth). Maximum intensity projections of the Z-sections (1024 × 1024 pixels) were used for further analysis. The fluorescence intensity was quantified by measuring the mean pixel value of hyphae using a macro in the KS400 software (Version 3.0; Carl Zeiss Vision, http://www.zeiss.de). Sections of 50–100 μm of leading hyphae were selected by hand and fluorescence was automatically quantified as the sum grey value per hypha with the background value from an equivalent area subtracted (Vinck *et al*., 2005). Signals were normalized with a custom Python script by dividing single hyphal fluorescence by the total fluorescence of all selected hyphae per image. To examine whether hyphal fluorescence followed a bimodal distribution, the normalized data were log transformed and subsequently modelled using five parameters (P, μ_1, σ_1, μ_2 and σ_2) as described (Vinck *et al*., 2005). The 95% confidence intervals (CI) of the parameters were estimated by bootstrapping (1000 replicates). Customs scripts in the Scilab programming language were used to fit the data (http://web.science.uu.nl/microbiology/images/fung/fittools.zip; http://web.science.uu.nl/microbiology/images/fung/manual%20fittools.pdf).

Analysis of plugging

Compartments were ruptured by laser dissection using the laser pressure catapulting function (LPC) of the P.A.L.M. laser dissecting microscope (Zeiss, http://www.zeiss.com). To this end, 60–70% of the power of the pulsed UV laser was used. Each experiment was carried out *in duplo* using 20 hyphae in each experiment. ANOVA analysis was used with Bonferroni *post hoc* correction when multiple comparisons were made between treatments. ANOVA analysis was used with Dunnet's *post hoc* correction when differences in septal plugging in a strain were assessed between a control condition and stress conditions. In all cases, a difference was assumed significant when $P < 0.05$.

Acknowledgements

This work was financed by the Netherlands Organization for Scientific Research (NWO) and by a Grant-in-Aid for Young Scientist from the Japan Society for the Promotion of Science (JSPS).

References

de Bekker, C., Wiebenga, A., Aguilar, G., and Wösten, H.A.B. (2009) An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*. *J Microbiol Methods* **76:** 305–306.

de Bekker, C., van Veluw, G.J., Vinck, A., Wiebenga, L.A., and Wösten, H.A.B. (2011a) Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Appl Environ Microbiol* **77:** 1263–1267.

de Bekker, C., Bruning, O., Jonker, M.J., Breit, T.M., and Wösten, H.A.B. (2011b) Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biol* **12:** R71.

Bleichrodt, R. (2012) Cytoplasmic streaming in *Aspergillus*. Thesis, University of Utrecht.

Collinge, A.J., and Markham, P. (1985) Woronin bodies rapidly plug septal pores of severed *Penicillium chrysogenum* hyphae. *Exp Mycol* **9:** 80–85.

Dhavale, T.J., and Jedd, G. (2007) The fungal Woronin body. In *The Mycota, Vol 8 – Biology of the Fungal Cell*. Howard, R.J., and Gow, N.A.R. (eds). Berlin: Springer-Verlag, pp. 87–96.

Escaño, C.S., Juvvadi, P.R., Jin, F.J., Takahashi, T., Koyama, Y., Yamashita, S., *et al*. (2009) Disruption of the *Aopex11-1* gene involved in peroxisome proliferation leads to impaired Woronin body formation in *Aspergillus oryzae*. *Eukaryot Cell* **8:** 296–305.

Etxebeste, O., Herrero-Garcia, E., Araújo-Bazán, L., Rodriguez-Urra, A.B., Garzia, A., Ugalde, U., and Espeso, E.A. (2009) The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. *Mol Microbiol* **73:** 775–789.

Gull, K. (1978) Form and function of septa in filamentous fungi. In *The Filamentous Fungi*, Vol. 3. Smith, J.E., and Berry, D.R. (eds). London: Arnold, pp. 78–93.

Heinlein, M. (2002) Plasmodesmata: dynamic regulation and role in macromolecular cell-to-cell signalling. *Curr Opin Plant Biol* **5:** 543–552.

Hoch, H.C., and Maxwell, D.P. (1974) Proteinaceous hexagonal inclusions in hyphae of *Whetzelinia sclerotiorum* and *Neurospora crassa*. *Can J Microbiol* **20:** 1029–1035.

Jedd, G. (2011) Fungal evo-devo: organelles and multicellular complexity. *Trends Cell Biol* **21:** 12–19.

Jedd, G., and Chua, N.H. (2000) A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. *Nat Cell Biol* **2:** 226–231.

Jennings, D.H. (1987) Translocation of solutes in fungi. *Biol Rev* **62:** 215–243.

Jennings, D.H., Thornton, J.D., Galpin, M.F., and Coggings, C.R. (1974) Translocation in fungi. *Symp Soc Exp Biol* **28:** 139–156.

Jin, F.J., Maruyama, J., Juvvadi, P.R., Arioka, M., and Kitamoto, K. (2004) Adenine auxotrophic mutants of *Aspergillus oryzae*: development of a novel transformation system with triple auxotrophic hosts. *Biosci Biotechnol Biochem* **68:** 656–662.

Jin, F.J., Watanabe, T., Juvvadi, P.R., Maruyama, J., Arioka, M., and Kitamoto, K. (2007) Double disruption of the proteinase genes, *tppA* and *pepE*, increases the production level of human lysozyme by *Aspergillus oryzae*. *Appl Microbiol Biotechnol* **76:** 1059–1068.

Juvvadi, P.R., Maruyama, J., and Kitamoto, K. (2007) Phosphorylation of the *Aspergillus oryzae* Woronin body protein, AoHex1, by protein kinase C: evidence for its role in the multimerization and proper localization of the Woronin body protein. *Biochem J* **405:** 533–540.

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Kaech, S.M., and Wherry, E.J. (2007) Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27:** 393–405.

Kasuga, T., and Glass, N.L. (2008) Dissecting colony development of *Neurospora crassa* using mRNA profiling and comparative genomics approaches. *Eukaryot Cell* **7:** 1549–1564.

Levin, A.M., de Vries, R.P., Conesa, A., de Bekker, C., Talon, M., Menke, H.H., *et al*. (2007a) Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryot Cell* **6:** 2311–2322.

Levin, A.M., de Vries, R.P., and Wösten, H.A.B. (2007b) Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *J Microbiol Methods* **69:** 399–401.

Lew, R.R. (2005) Mass flow and pressure-driven hyphal extension in *Neurospora crassa*. *Microbiology* **151:** 2685–2692.

Liu, F., Ng, S.K., Lu, Y., Low, W., Lai, J., and Jedd, G. (2008) Making two organelles from one: Woronin body biogenesis by peroxisomal protein sorting. *J Cell Biol* **180:** 325–339.

Mabashi, Y., Kikuma, T., Maruyama, J., Arioka, M., and Kitamoto, K. (2006) Development of a versatile expression plasmid construction system for *Aspergillus oryzae* and its application to visualization of mitochondria. *Biosci Biotechnol Biochem* **70:** 1882–1889.

Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., *et al*. (2005) Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438:** 1157–1161.

Managadze, D., Würtz, C., Sichting, M., Niehaus, G., Veenhuis, M., and Rottensteiner, H. (2007) The peroxin PEX14 of *Neurospora crassa* is essential for the biogenesis of both glyoxysomes and Woronin bodies. *Traffic* **8:** 687–701.

Managadze, D., Würtz, C., Wiese, S., Meyer, H.E., Niehaus, G., Erdmann, R., *et al*. (2010) A proteomic approach towards the identification of the matrix protein content of the two types of microbodies in *Neurospora crassa*. *Proteomics* **10:** 3222–3234.

Maruyama, J., and Kitamoto, K. (2011) Molecular biology of the Koji molds. *Methods Mol Biol* **765:** 447–456.

Maruyama, J., Juvvadi, P.R., Ishi, K., and Kitamoto, K. (2005) Three-dimensional image analysis of plugging at the septal pore by Woronin body during hypotonic shock inducing hyphal tip bursting in the filamentous fungus *Aspergillus oryzae*. *Biochem Biophys Res Commun* **331:** 1081–1088.

Maruyama, J., Kikuchi, S., and Kitamoto, K. (2006) Differential distribution of endoplasmic reticulum network as visualized by the BipA-EGFP fusion protein in hyphal compartments across the septum of the filamentous fungus, *Aspergillus oryzae*. *Fungal Genet Biol* **43:** 642–654.

Maruyama, J., Escaño, C.S., and Kitamoto, K. (2010) AoSO protein accumulates at the septal pore in response to various stresses in the filamentous fungus *Aspergillus oryzae*. *Biochem Biophys Res Commun* **391:** 868–873.

Masai, K., Maruyama, J., Sakamoto, K., Nakajima, H., Akita, O., and Kitamoto, K. (2006) Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. *Appl Microbiol Biotechnol* **71:** 881–891.

Momany, M., Richardson, E.A., Van Sickle, C., and Jedd, G. (2002) Mapping Woronin body position in *Aspergillus nidulans*. *Mycologia* **94:** 260–266.

Moore, R.T., and McAlear, J.H. (1962) Fine structures of mycota. Observations on septa of ascomycetes and basidiomycetes. *Am J Bot* **49:** 86–94.

Moukha, S.M., Wösten, H.A.B., Asther, M., and Wessels, J.G.H. (1993a) *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *J Gen Microbiol* **139:** 969–978.

Moukha, S.M., Wösten, H.A.B., Mylius, E.J., Asther, M., and Wessels, J.G.H. (1993b) Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. *J Bacteriol* **175:** 3672–3678.

Ng, S.K., Liu, F., Lai, J., Low, W., and Jedd, G. (2009) A tether for Woronin body inheritance is associated with evolutionary variation in organelle positioning. *PLoS Genet* **5:** e1000521.

Nobile, C.J., and Mitchell, A.P. (2007) Microbial biofilms: e pluribus unum. *Curr Biol* **17:** 349–353.

Norris, R.P., Freudzon, M., Mehlmann, L.M., Cowan, A.E., Simon, A.M., Paul, D.L., *et al*. (2008) Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development* **135:** 3229–3238.

Ohneda, M., Arioka, M., and Kitamoto, K. (2005) Isolation and characterization of *Aspergillus oryzae* vacuolar protein sorting mutants. *Appl Environ Microbiol* **71:** 4856–4861.

van Peer, A.F., Müller, W.H., Boekhout, T., Lugones, L.G., and Wösten, H.A.B. (2009) Cytoplasmic continuity revisited: closure of septa of the filamentous fungus *Schizophyllum commune* in response to environmental conditions. *PLoS ONE* **4:** e5977.

van Peer, A.F., Wang, F., van Driel, K.G., de Jong, J.F., van Donselaar, E.G., Müller, W.H., *et al*. (2010) The septal pore cap is an organelle that functions in vegetative growth and mushroom formation of the wood-rot fungus *Schizophyllum commune*. *Environ Microbiol* **12:** 833–844.

Punt, P.J., and van den Hondel, C.A.M.J.J. (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods Enzymol* **216:** 447–457.

Shatkin, A.J., and Tatum, E.L. (1959) Electron microscopy of *Neurospora crassa* mycelia. *J Biophys Biochem Cytol* **6:** 423–426.

Siedenberg, D., Mestric, S., Ganzlin, M., Schmidt, M., Punt, P.J., van den Hondel, C.A.M.J.J., and Rinas, U. (1999) *glaA* promoter controlled production of a mutant green fluorescent protein (S65T) by recombinant *Aspergillus niger* during growth on defined medium in batch and fed-batch cultures. *Biotechnol Prog* **15:** 43–50.

Soundararajan, S., Jedd, G., Li, X., Ramos-Pamploña, M., Chua, N.H., and Naqvi, N.I. (2004) Woronin body function in *Magnaporthe grisea* is essential for efficient pathogenesis and for survival during nitrogen starvation stress. *Plant Cell* **16:** 1564–1574.

Tanabe, Y., Maruyama, J., Yamaoka, S., Yahagi, D., Matsuo,

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

I., Tsutsumi, N., and Kitamoto, K. (2011) Peroxisomes are involved in biotin biosynthesis in *Aspergillus* and *Arabidopsis*. *J Biol Chem* **286:** 30455–30461.

Teertstra, W.R., Lugones, L.G., and Wösten, H.A.B. (2004) *In situ* hybridisation in filamentous fungi using peptide nucleic acid probes. *Fungal Genet Biol* **41:** 1099–1103.

Tenney, K., Hunt, I., Sweigard, J., Pounder, J.I., McClain, C., Bowman, E.J., and Bowman, B.J. (2000) *Hex-1*, a gene unique to filamentous fungi, encodes the major protein of the Woronin body and functions as a plug for septal pores. *Fungal Genet Biol* **31:** 205–217.

Trinci, A.P., and Collinge, A.J. (1974) Occlusion of the septal pores of damaged hyphae of *Neurospora crassa* by hexagonal crystals. *Protoplasma* **80:** 57–67.

Veening, J., Smits, W.K., and Kuipers, O.P. (2008) Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* **62:** 193–210.

Vinck, A., Terlou, M., Pestman, W.R., Martens, E.P., Ram, A.F., van den Hondel, C.A.M.J.J., and Wösten, H.A.B. (2005) Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Mol Microbiol* **58:** 693–699.

Vinck, A., de Bekker, C., Ossin, A., Ohm, R.A., de Vries, R.P., and Wösten, H.A.B. (2011) Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environ Microbiol* **13:** 216–225.

Vishniac, W., and Santer, M. (1957) The thiobacilli. *Bacteriol Rev* **21:** 195–213.

de Vries, R.P., and Visser, J. (1999) Regulation of the feruloyl esterase (*faeA*) gene from *Aspergillus niger*. *Appl Environ Microbiol* **65:** 5500–5503.

Wösten, H.A.B., Moukha, S.M., Sietsma, J.H., and Wessels, J.G.H. (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. *J Gen Microbiol* **137:** 2017–2023.

Yamada, O., Lee, B.R., and Gomi, K. (1997) Transformation system for *Aspergillus oryzae* with double auxotrophic mutations, *niaD* and *sC*. *Biosci Biotechnol Biochem* **61:** 1367–1369.

Yoon, J., Aishan, T., Maruyama, J., and Kitamoto, K. (2010) Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. *Appl Environ Microbiol* **76:** 5718–5727.

Supporting information

Additional supporting information may be found in the online version of this article.

13652958, 2012, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mmi.12077 by Utrecht University Library, Wiley Online Library on [07/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License