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A plastic vegetative growth threshold governs reproductive capacity in *Aspergillus nidulans*

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Abstract

Threshold-limited ontogenic phases separating somatic growth from reproduction are a common feature of cellular life. Long recognized for flowering plants and animals, this life-history component may also be prevalent among multicellular fungi. We establish the environmental and genetic basis of developmental competence, the capacity to respond to induction of asexual development, in the model filamentous saprotroph *Aspergillus nidulans*. Density and pH are critical parameters for competence timing, and we identify five genes with heterochronic effects through genetic screens and candidate mutagenesis, including the conserved GTPase RasB and ambient pH sensor PalH. Inheritance of competence timing is quantitative, semi-dominant, transgressive, and extremely variable among progeny. Transcriptional profiling over competence acquisition demonstrates substantial activity in metabolic and signaling networks, highly concordant across species, and a wave of gene expression around centromeres indicative of chromatin remodeling. Competence, likely determined by species-specific endogenous hormones and metabolic capacity, governs much of biology associated with the mature fungal form – asexual and sexual reproduction, secondary metabolism, and, in some species, pathogenesis – and provides a new model for nutrient-limited life-history phases and their elaboration from unicellular origins.

Introduction

Discrete states oriented toward growth or reproduction are fundamental to ontogenesis (Moran (1994), Istock and Istock (1967); Wilbur (1980)). In unicellular microbes they may be tightly integrated or even synonymous with the cell cycle (Johnston *et al.* (1977); Turner *et al.* (2012)), while in multicellular organisms intercellular signaling reflecting nutritional state is required to coordinate development. Plants pass through one or more vegetative phase changes before acquiring the capacity to flower (Poethig (2003); Poethig (2010)), many invertebrates attain the adult form through an intermediate juvenile stage (or stages) (Bishop *et al.* (2006); Truman and Riddiford (1999)), and mammals reach sexual maturity (Gluckman and Hanson (2006); Ahmed *et al.* (2009); Young (1976); Zacharias and Zacharias (1969)). All such examples involve interaction between the unfolding developmental program and nutrient accumulation, mediated by hormones.

Several genera of fungi, sister kingdom to animals within the Opistokhont supergroup, also require a minimum period of growth before acquisition of reproductive competence (Axelrod (1972); Noble and Andrianopoulos (2013)). While development of dormant, stress-resistant spores via asexual conidiation can be rapidly induced by exposure of a mature colony to an air interface and light, induction is without effect before a critical period of vegetative growth.

In contrast to plants and animals, competence in filamentous fungi is not associated with obvious morphological differentiation and, perhaps consequently, has received little attention since description in *Trichoderma* (Gressel *et al.* (1967)), *Penicillium* (Morton *et al.* (1958); Hadley and Harrold (1958)) and *Aspergillus* (Axelrod *et al.* (1973)) species (exceptions include Roncal and Ugalde (2003); Gravelat *et al.* (2008); Sheppard (2005); Ruger-Herreros *et al.* (2011); Adams *et al.* (1998); Nahlik *et al.* (2010)). Early work in *A. nidulans*

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showed competence acquisition is not simply a consequence of external nutrient limitation (Pastushok *et al.* (1976)), unlike analogous transitions in Dictyostelid amoebae and bacteria (Marin (1976); Bonner (2003)). Rapid, concerted changes in metabolic enzyme inducibility (Gealt and Axelrod (1974)), intracellular iron availability (Hall and Axelrod (1978)) and nutrient uptake rate were described, suggesting competence represents a transition between somewhat discrete organismal states (Kurtz and Champe (1979); Kurtz (1980)). More recently, competence for one species, *P. cyclopium*, was shown to be under control of a diterpenoid hormone (Roncal *et al.* (2002)) constitutively produced via the highly conserved mevalonate pathway, which produces diverse signaling compounds, such as steroids and insect juvenile hormones, through hydroxymethylglutaryl-CoA (Keller *et al.* (2005)).

While the genetic basis for synthesis and sensing of this cue remain unknown, regulation of growth and development by extracellular signals is known to be common in fungi, controlling spore germination inhibition, mating type recognition, negative autotropism, and metabolic influence on alternative reproductive strategies (Yu and Keller (2005); Vining (1990); Fox and Howlett (2008)). In the Aspergilli, much is also known of the molecular genetics of development, and of metabolism, hyphal extension, and mitosis during growth (Goldman and Osmani (2007)), but relatively little of organism level integration.

For *A. nidulans*, reproduction involves some combination of rapid vegetative growth, producing clonal nuclei distributed throughout the mycelium; asexual development, producing spores by the millions through iterated mitosis of stem-cell like phialides; parasexual development, resulting in chromosomal reassortment with rare recombination; and, given sufficient nutrients, sexual development within durable fruiting bodies (Figure 1A) (Pontecorvo (1952); Todd *et al.* (2007a); Champe *et al.* (1994); Etxebeste *et al.* (2010); Han (2009)). Differentiation is a response to conditions unfavorable to growth, such as the substrate-air interface. Asexual and sexual reproduction are reciprocally regulated by light during growth on solid substrate (Mooney and Yager (1990); Rodriguez-Romero *et al.* (2010)), but both are repressed indefinitely given sufficient nutrients in homogenous liquid culture. Upon establishment of a spatially patterned microenvironment during solid culture, developmental commitment is established in the colony interior through well-characterized regulatory networks. Upstream developmental activators (UDAs) – fungal specific transcription factors, conserved signal transduction components such as heterotrimeric G-proteins, and factors involved in production and perception of secreted signals – channel environmental cues into the late acting central developmental pathway (CDP), a positive feedback, morphogenetic network comprising transcription factors such as bristle (*brlA*) and abacus (*abaA*), which orchestrates conidiophore and asexual spore production (Clutterbuck (1969); Adams *et al.* (1988); Aguirre (1993); Sewall *et al.* (1990); Prade and Timberlake (1993); Andrianopoulos and Timberlake (1994)). Sexual development may follow, days later, and may be subject to a second competence threshold (Champe *et al.* (1981)).

Early work on asexual developmental competence in *A. nidulans* yielded some genetic, as well as biochemical, insight. Precocious mutants were generated (Axelrod *et al.* (1973); Yager and Kurtz (1982); Butnick *et al.* (1984)), and cloning of temperature sensitive aconidial (*aco*) competence mutants identified subunits four and seven of the COP9 signalosome (CSN), with a third mapped to a small interval containing subunit five (Adams *et al.* (1992); Sutton (1999)). The CSN integrates nutrient sensing with the cell cycle in *S. cerevisiae* (Zemla *et al.* (2013)), and hormone sensing through regulation of proteasome activity in plants (Hind *et al.* (2011); Browse (2009)), but direct molecular connections to competence are lacking in *A. nidulans*. No genes underlying precocious competence have been identified and, more broadly, little is known of the role of environment, non-morphological aspects of differentiation such as polarization and transcriptional regulation, or the heritability of competence timing phenotypes.

Results

Environmental dependence of competence timing

We surveyed environmental and metabolic dependence of competence in a wild-type strain (WIM126) and two related, genetically undefined precocious strains. WIM27 (hereafter *prcA*) was isolated in a UV mutagenesis screen (Axelrod *et al.* (1973)) and WIM28 (hereafter *prcA; prcB*), with an enhanced phenotype, was generated by further mutagenesis of WIM27 (Kurtz and Champe (1979)).

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Total time to conidiation consists of two components: competence, from breaking of spore dormancy to acquisition of reproductive capacity; and maturation, from induction of development to production of the conidiophore structure (Fig. 1A-B). Minimum total time to conidiation can be measured by growing single-spore derived colonies under inducing conditions (e.g. on the surface of an agar plate, under light). Maturation time can then be inferred by growing under non-inducing conditions (e.g. an agar plate overlaid with liquid medium) past this point, inducing development (e.g. by decanting the liquid overlay), and monitoring the time until conidiophore emergence.

Varying temperature, organism density, pH, primary metabolism and osmotic pressure, we found all but the last of these treatments altered competence timing significantly (Fig. 2A-D). We also found that competence and maturation often varied independently. As expected, competence was most rapid on primary carbon sources supporting rapid growth, such as glucose and fructose, and slowest on poorer, non-repressing sources such as glycerol and lactose (unpublished observations). On glucose medium, wild-type time to conidiation at 37° was 24.2 hours (± 0.07 hours standard error), including a maturation time of 4.1 ± 0.06 hours. Consistent with initial reports, *prcA* produced conidia around 2 hours earlier (22.6 ± 0.09), with *prcA; prcB* 1.5 hours earlier again (21.2 ± 0.05; Fig. 2A), and maturation timing was not significantly different to wild-type (p = 0.560 for *prcA*, p = 0.148 for *prcA; prcB*). The precocious phenotype was temperature dependent: the margin between wild-type and mutants, as a proportion of total time to competence, fell progressively with temperature such that the mutants were not significantly different to wild-type at 25°. For temperature, competence was most rapid within the optimal growth range (37-42°), as expected for metabolic dependence, while maturation was only slightly accelerated (ANOVA strain effect $p < 2\text{x}10^{-8}$, temperature effect $p < 10^{-50}$ for competence, 0.296 and 0.029 for maturation; Fig. 2A).

Competence timing varied inversely with organism density for all strains, while maturation again remained relatively constant (strain effect $p < 10^{-13}$, density effect $p < 10^{-70}$ for competence, *prcA* p = 0.333 and *prcA; prcB* p = 0.096 for maturation; Fig. 2B). Effect size of the precocious phenotype was similar across the density range. Timing was identical for growth at an air interface on solid culture and for stationary liquid culture (unpublished observations), suggesting competence is not highly sensitive to colonial spatial organization or oxygen availability.

Buffering medium across a pH range significantly altered both competence and maturation timing in all strains, with effects dependent on both pH and genotype. Under acidic or neutral buffering (pH 7), the general effect on competence was to accelerate wild-type to similar, or even earlier, timing as the mutants (Fig. 2C). While wild-type at pH 6 was not significantly different to *prcA; prcB* on unbuffered medium, the mutant lines remained precocious under alkaline conditions, suggesting an acidity-mimicking alteration in ion homeostasis may underlie the precocious phenotype. Buffering at any pH removed entirely the phenotypic enhancement seen in *prcA; prcB* relative to *prcA*, however, further suggesting this strain carries a pH-sensitive mutation (or mutations), which can be phenocopied by external buffering. Variation in competence timing was driven largely by maturation, which varied near inversely across the pH range for wild-type (linear regression coefficient = -0.63, p = 0.0006), while the response was reduced for *prcA; prcB* (coefficient = -0.183, p = 0.0091) and not significant for *prcA* (coefficient = -0.11, p = 0.3333; Fig. 2D).

Transcriptional profiling of competence acquisition

We profiled transcription during competence acquisition by microarray, first sampling wild-type mycelium hourly over a 5-hour spanning window (centered on competence at 14.5 hours for moderate density liquid culture) (Supplementary Fig. 1). At a false discovery rate of 5%, almost a quarter of the transcriptome was significantly differentially expressed at any single time-point. The global trajectory of gene expression over the window, relative to the earliest sample of 12 hours, was predominantly linear, with 1493 genes upregulated and 965 downregulated at 16 hours. This suggests competence is a transition between states, rather than a transient signalling event. The data were strongly correlated with published gene expression data from early vegetative growth of varying age in *A. nidulans* (Breakspear and Momany (2007)) and *A. fumigatus* (Sheppard (2005)), but not with germination in *A. nidulans* (Breakspear and Momany (2007)) or *A. niger* (Leeuwen *et al.* (2013)), showing these developmental landmarks are largely distinct (Fig. 3A). Overall

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concordance in expression direction between *A. nidulans* and *A. fumigatus* after competence acquisition was 74% (see Methods), representing almost one third of orthologous genes (Supplementary Fig. 2).

To test for candidate gene expression differences that cause early competence acquisition, we next sampled the precocious mutant *prcA* over the window, testing for expression patterns best explained by (1) time, (2) genotype, (3) additive effects of both time and genotype, or (4) additive effects as well as interaction between these factors (Figure 3B). The mutant attains competence at 12.5-13 hours under the moderate density liquid culture conditions used. Functional analysis (Gene Ontology, Gene Set Enrichment Analysis, KEGG pathways) revealed highly significant activity: genes related to translation and ribosomal biogenesis (translation initiation and elongation, tRNA modification, RNA processing and transport) were progressively downregulated at and beyond competence acquisition, while transcripts annotated with signal transduction, polarized growth, endocytosis, plasma membrane modification, and vacuole and metal ion homeostasis functions increased in abundance (Fig. 3B, models 3 and 4). Dynamic regulation of metabolic genes was also apparent, spanning gluconeogenesis, ergosterol biosynthesis, the GABA shunt and central acetyl-CoA pathways, suggesting a shift from purely glycolytic primary metabolism, which may be maximal during early, pre-competence growth (Kurtz and Champe (1979); Kurtz (1980)). Accordingly, expression of the central energy homeostasis regulator pyruvate dehydrogenase kinase (PDK) increased steadily throughout the time course.

Gene expression during competence acquisition was also clearly altered at the level of chromosome structure or subnuclear localisation. First, competence acquisition was associated with establishment (or exaggeration) of a sinusoidal pattern in mean expression symmetrical around centromeres. This was accelerated in the precocious mutant, and marked in *A. fumigatus* (Fig. 3C). Second, expression from a number of verified and potential coregulated gene clusters was often altered in the precocious mutant, so that correlation with wild-type was often minimal at telomeres (Supplementary Fig. 3).

Identification and genetic analysis of precocious mutants

Although a number of precocious competence mutants have been generated, underlying genes and the heritability of competence timing phenotypes are unknown. We undertook genetic and parasexual analysis of three undefined precocious mutant strains, two from the original UV mutagenesis screens (*prcA* and *prcA; prcB*) and a third (*prcC*) from a chemical mutagenesis screen (see Methods).

Mutants were first crossed to auxotrophic lines to introgress selectable markers, allowing diploid creation by complementation with a mapping strain marked on each of the eight chromosomes. Parasexual analysis, whereby haploids are regenerated with segregation of whole chromosomes, implicated loci of major effect on chromosome I for *prcB* and chromosome III for *prcA*, while results for *prcC* were ambiguous. Precocious competence timing in heterozygous diploids was, in all cases, dominant or semi-dominant to wild-type, however unambiguous designation was hampered by instability of the precocious phenotype through passaging and storage of spores. We note that instability of multiple precocious mutants was seen during Axelrod's initial mutagenesis screens (Axelrod *et al.* (1973)), and we also observed this for two additional strains generated by repeating the screen.

Testing against a battery of environmental and chemical treatments revealed multiple phenotypes cosegregating with *prcB* in sexual crosses, including molybdate sensitivity and an inability to elicit contact inhibition from neighboring wild-type colonies (unpublished observations). Selection for restoration of molybdate tolerance by transformation with a genomic DNA library identified multiple unique inserts from a single locus on chromosome I that remediated both molybdate sensitivity and early competence acquisition, minimally spanning two genes (AN6885, AN6886). In parallel, we sequenced the genome of a *prcA; prcB* strain to 35X coverage (see Methods), detecting an early frameshift in AN6886, encoding the ambient pH sensor PalH. Molybdate sensitivity is a known phenotype of acidity-mimicking mutations, including palH loss of function mutations (Calcagno-Pizarelli *et al.* (2007); Negrete-Urtasun *et al.* (1999)). The frameshift cosegregated with molybdate sensitivity and the above phenotypes in eight F_1 progeny by Sanger sequencing, and we conclude that *prcB* is a null allele of *palH*.

For *prcA* and *prcC*, pooled genomes for wild-type and precocious F_1 backcross progeny were sequenced to a

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depth of approximately 160X each. For *prcC* we detected a frameshift in the *nsdD* gene, which encodes a GATA zinc finger transcription factor required for sexual development. A *nsdD*Δ deletion mutant showed precocious competence acquisition, consistent with a prior report of early conidiation for loss of function mutants (Han *et al.* (2001)).

From *prcA* and wild-type F_1 pools, each comprising 12 haploid genomes, we detected 2153 single nucleotide variants (SNVs) and small insertion/deletions after filtering (see Methods). Most (1450) were fixed in both pools relative to the reference FGSCA4. Of 406 variants segregating and variable in frequency across pools (Supplementary Fig. 4), two SNVs were fixed in the precocious pools and at low frequency (1/12) in wild-type. Both were non-synonymous coding mutations with evidence for local linkage disequilibrium (Supplementary Fig. 5), located on chromosomes I and III. Single gene deletion mutants failed to alter developmental timing, however, and further parasexual analysis suggested the presence of chromosomal abnormalities (see Supplementary Data). Without resolution of the genetic basis of precocious competence in *prcA*, the possibility remained that phenotypic instability and quantitative inheritance seen in crosses of the progenitor (WIM27) and derived strains might be due to structural variation or multiple mutations.

A mutational survey of candidate genes

We conducted candidate mutagenesis to study heritability for defined mutations in a standard genetic background, and broaden understanding of genetic influences on competence timing. First, we generated gain (G16V) and loss (T21N) of function point mutations altering activity of the *ras* family GTPase RasB, since activity of a related protein, RasA, has been shown to alter the timing of developmental transitions (although effects on competence were not shown; Som and Kolaparthi (1994)). In a *rasB*Δ deletion background, modulation of RasB activity also showed pleiotropic effects on growth and development. GTPase activity was anticorrelated with germination and conidiation ($p < 10^{-4}$ and $p < 10^{-18}$ by linear model, mutant genotypes coded as -1/1) while maturation rate was accelerated slightly by both alleles (ANOVA strain effect $p < 10^{-4}$; Fig. 5A). The net effects on competence were strong in both directions: -1.63 ± 0.1 hours for $RasB^{T21N}$ and 1.68 ± 0.18 hours for $RasB^{G16V}$, Fig. 5B).

We additionally generated and analyzed 70 targeted deletion or disruption mutants (see Methods), selected on criteria of differential expression over competence acquisition, predicted gene function (predominantly transcription factors) and evolutionary conservation. Null mutants of two genes showed significantly earlier competence acquisition: homeodomain transcription factor *ahpA* (21.84 ± 0.165 hours), and C_2H_2 transcription factor *msnA* (22.2 ± 0.35 hours; see Supplementary Data). Backcrossing of both mutant strains showed transgressive, continuous inheritance of competence timing, as seen for *prcA* strains (Fig. 6B). In both cases, precocious competence was partially dominant in the F_1 – for *ahpA*Δ, no progeny of wild-type timing were seen – and additionally dependent on F_1 genotype ($p = 5.2\text{x}10^{-6}$ for *ahpA*Δ, $1.3\text{x}10^{-4}$ for *msnA*Δ, two-sided Wilcoxon rank sum test). Quantitative and highly variable heritability of competence timing is therefore inherent to the precocious phenotype, not an artifact of background variation segregating in *prcA* strains.

Discussion

We have outlined the environmental, genetic, and transcriptional basis of a reproductive maturity life-history threshold in *A. nidulans*, conserved throughout filamentous Ascomycetes at least. Competence varies with temperature, pH and quality of carbon source, consistent with an interaction between primary metabolism and a density-dependent signal. We have focused on asexual development, as the earliest manifestation of competence and by far the better studied reproductive mode. An additional, much later threshold for competence to respond to sexual development has also been demonstrated using a temperature sensitive allele of the pivotal regulator *velvetA* (Champe *et al.* (1981)).

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Transcriptional and colonial differentiation during development

Molecular genetic studies have shown the expression of many genes changes, in quantity or localization, during early vegetative growth (Garzia *et al.* (2009); Etxebeste *et al.* (2009); Etxebeste *et al.* (2008); Miller *et al.* (1991); Kwon *et al.* (2010); Soid-Raggi *et al.* (2006); Harispe *et al.* (2008); Kawasaki and Aguirre (2001); Tsitsigiannis (2004)), and biochemical and cell biological studies have demonstrated some broad effects of age on cellular state (Gealt and Axelrod (1974); Hall and Axelrod (1977)). Competence timing has rarely been determined in these studies, however, and so the relationship between developmental and chronological age has been confounded. We find that while differentiation during competence acquisition is not apparent at the level of morphology or growth kinetics, transcriptional differentiation is extensive and highly concordant across the two Aspergilli examined.

While multiple physiological correlates of competence acquisition show non-linear, switch-like dynamics, we observed a predominantly monotonic transition in gene expression over the 4-hour window suggesting a reduction in biosynthetic activity and an increase in signaling, development and secondary metabolic gene networks. Altered asymmetry at the level of the colony might be a trivial explanation of some of the broad patterns of gene expression observed. Kinetics and morphology of early filamentous growth – germination, nuclear division, septation and branching – have been studied extensively (Fiddy *et al.* (1976); Dynesen and Nielsen (2003); Roper *et al.* (2011); Rittenour *et al.* (2009); Trinci (1974)), but over a wide range of conditions and rarely with regard to competence state, complicating inference. Dynesen and Nielsen monitored the hyphal growth unit (the ratio between total hyphal length and the number of hyphal tips) in low-density stationary cultures at 30° and found it approaches a constant value soon after hyphae possess 8-10 tips (around 14 hours in rich medium) (Dynesen and Nielsen (2003)). Based on the range of growth conditions examined here, this would be well before competence acquisition, ruling out a change in apical to subapical compartment ratio. Asymmetry in metabolic and transcriptional activity within the colony, for which there is abundant evidence at diverse stages of vegetative growth, may contribute to, or even drive, the observed patterns, however.

Documented biochemical changes in hyphal differentiation from unpatterned to polar growth include establishment of apical-high gradients of reactive oxygen species, Ca^{2+} and protons (Robson *et al.* (1996); Rodriguez-Urra *et al.* (2012); Roncal *et al.* (1993); Semighini and Harris (2008)). It seems likely, upon case-by-case examination of experimental conditions that these aspects of hyphal differentiation precede competence acquisition. Importantly, however, the FlbB/FlbE regulatory complex appears to assemble apically at or shortly after competence acquisition, linking developmental induction capacity with the morphogenetic, positive feedback, central development pathway (Clutterbuck (1969); Adams *et al.* (1988); Aguirre (1993); Sewall *et al.* (1990)). It remains to establish the precise timing of these and other hyphal differentiation events with respect to density and competence, and the potential for spatial regulation of transcription.

Functional analysis of gene expression over the competence acquisition window supports early biochemical studies showing a progressive decrease in rate of transport of glucose and other metabolites (Kurtz and Champe (1979); Kurtz (1980)), and a sharp change in iron availability or oxidative state (Hall and Axelrod (1978)). Strong enrichments were seen among upregulated genes for primary metabolism and other iron intensive pathways such as ergosterol biosynthesis, associated with an oxidative stress response. Downregulated activity was mainly related to ribosome biogenesis and translation, and appeared to follow the stress response. Correlation between mRNA and protein abundance is generally good, where it has been tested, in fungi (r 0.4-0.7) (Lee *et al.* (2011); Vogel and Marcotte (2012); Vogel *et al.* (2011)), but may be weaker for downregulated genes and for specific classes such as ribosomal biogenesis, since these proteins may be relatively stable. Such systemic changes are apparently without consequence for growth rate, which is exponential for several hours before and after competence acquisition (Hall and Axelrod (1978)). In all, the data support the view that the precompetent growth phase is dedicated principally to rapid biomass accumulation, and that competence acquisition elicits a significant stress response, with redistribution of reducing power to secondary metabolic pathways and alterations to intracellular, and perhaps extracellular, transport.

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Genetic and epigenetic basis of competence timing

Instability of multiple precocious mutants was seen during Axelrod's initial mutagenesis screens (Axelrod *et al.* (1973)), and we again observed this for two additional strains generated by repeating the screen. This suggested the possibility some precocious strains might not be true genetic mutants, with variability in competence timing allowing selection of transiently heritable epigenetic mutants. Alternatively, if competence is genetically robust, the effect of mutational perturbation might be compensated over time.

Genetic analysis of *prcA* and *prcA; prcB* – where precocious competence has been stably maintained almost 40 years since isolation – showed competence timing was inherited as a continuous quantitative trait with variable stability among progeny. F_1 progeny scored as precocious immediately after isolation often reverted to wild-type timing after storage (wild-type spores are stable for months in water at 4°), while others remained stably precocious under identical conditions. This suggested only a subset of initially precocious progeny might carry underlying genetic variation, and that biochemical activity in resting spores might reset competence timing. Less ambiguously, progeny of defined precocious mutants *ahpA*Δ and *msnA*Δ also showed continuous, transgressive variation in competence timing, with the F_1 genotypic classes overlapping in phenotypic values. This demonstrates that timing is a considerably plastic trait, at least as assayed here over a single generation. Monitoring over additional generations is required to additionally address the possibility of the robustness of wild-type timing to genetic perturbation.

Partial dominance in the F_1 suggests shared cellular environment during meiosis exerts an effect on ascospore composition, which is maintained through vegetative growth and transmitted to the asexually derived conidia used for assay. The long-term stability of this transgenerational inheritance is unknown, and many other unanswered questions remain, not least the basis of variable penetrance. Small RNAs have been implicated in phenotypic plasticity in other species (e.g. Calo *et al.* (2014)) and, as in analogous life-history transitions in other systems, parental nutrient deposition (e.g. linolenic fatty acids in asexual spores (Vanetten and Gottlieb (1965); Evans and Gealt (1985))) may influence competence timing and the observed mode of inheritance.

Evolution of reproductive competence and complex life cycles

Differentiation is typically considered as the generation of discrete cell types, and life-history stages have historically been delineated as simple (direct) or complex (indirect) (Moran (1994); Wiegmann *et al.* (2009)). One definition of this complexity is an abrupt ontogenetic change in an individual's morphology, physiology, and behavior, usually associated with a change in habitat (Wilbur (1980)), while others have elevated ecological or morphological criteria (Istock and Istock (1967)). Clearly, a narrow definition is more broadly defendable, as physiological differentiation is likely to be pervasive, particularly in organisms of limited morphological diversity (Beadle *et al.* (1938); Stoodley *et al.* (2002); Agathocleous and Harris (2013)). Still, competence in filamentous fungi appears to be broadly analogous to nutrient-limited, hormone-mediated early life-history stages in animals and plants. Minimum time to competence occurs within temperature and pH ranges optimal for growth, on preferred carbon and nitrogen sources (according to hierarchical catabolite repression systems (Hynes and Kelly (1977); Wong *et al.* (2008))). Competence timing is density dependent, presumably as a response to resource colonization (the response to other species, or potentially even heterokaryon incompatible strains of the same species, would be expected to differ), and a diterpenoid hormone sufficient for both competence acquisition and induction of asexual development has been isolated from *P. cyclopium* (Roncal *et al.* (2002); Roncal and Ugalde (2003)).

Ultimately, development and primary metabolism must be temporally or spatially segregated activities (Adams *et al.* (1988); Adams and Timberlake (1990)). For filamentous fungi, this may be ensured by delayed reproductive capacity and the mycelial growth habit, with exploratory nutrient acquisition at the colony margins and a depleted interior committed to secondary metabolism and reproduction. Beyond this basic necessity, the precise timing of competence acquisition presumably reflects life-history optimization for colonization of ephemeral, patchy environments (Andrews (1992); Heaton *et al.* (2016)). A bang-bang strategy – complete allocation toward vegetative growth, followed by a resource-dependent switch to complete allocation toward spore production – has been predicted to be optimal for an asexual saprobe (Gilchrist *et al.* (2006)). Under this patch array model, fitness is maximized by efficient colonization of newly arising

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patches of fixed resource levels, which involves trade-offs between spore production schedule and quantity, as well as propagule quantity and quality (motility, durability). A genetically assimilated competence schedule should then reflect the species' present and historical ecology, and it's level of predictability, particularly for critical factors such as patch size, quality and density. Variance in patch size and distribution (Gilchrist *et al.* (2006)), as well as advantages accruing from later, more costly sexual development, may explain the apparent superiority of a mixed strategy of continued vegetative growth, along with environmentally induced asexual development, over the finality of a post-competence reproductive bang. Diurnal cycles of light, temperature and air movement, of obvious relevance to patch availability and spore dispersal, may also contribute to the observed set point of competence + maturation at 24 hours, although it remains to see if this holds outside laboratory conditions, and for species other than *A. nidulans*.

The existence of a threshold-limited life-history trait in diverse fungi of relatively simple morphological complexity, coupled with increasingly dense phylogenetic sampling and genomic resources in a number of clades, offers a number of interesting opportunities. For one, our understanding of the underlying metabolic basis of more familiar adult fungal activities would gain from studying how regulatory controls are imposed at the colony level on a precompetent state committed solely to rapid cell division and hyphal growth. More broadly, further study of competence timing, and its variation within and across species, would allow testable inferences of fungal ecology and life-history theory. Lastly, although nutrient limited thresholds are ubiquitous in complex life cycles, their bioenergetic basis is in most cases poorly understood. Comparative study of the genetic basis of competence across fungal phylogeny would yield insight into the integration of conserved growth and divergent developmental networks, and of the evolution of complex life cycles from simple beginnings.

Materials and Methods

Strains and culture conditions

Strains used during this study are shown in Supplementary Table 1. All are derived from the reference Glasgow isolate FGSCA4 (Arnaud *et al.* (2009)). Minimal medium (ANM + 10 mM ($(NH_4)_2$T), carbon free (CF), complete medium (CM) and basic growth conditions, handling, genetic and parasexual analysis were as described (Todd *et al.* (2007b); Todd *et al.* (2007a)). Yeast extract glucose (YEG) medium contained 2% glucose and 0.5% yeast extract. Alternative carbon sources were added to CF medium to 1% w/v. Assays to detect molybdate sensitivity were conducted as described (Arst *et al.* (1970)). Buffering of 2% high-grade agar minimal medium used citrate/sodium citrate (pH 4-6) (Gomori (1946)) and phosphate/citrate (pH 6-8) (Pearse (1991)) buffers at a final concentration of 10 mM.

Competence timing assays

All assays were performed at low density on solid culture (<=100 colonies per 90 mm diameter plate), using spore dilutions quantified by hemocytometer and stored at 4° in H_20 for up to one month. Testing of specific pH, metabolic substrates or chemical agents was on minimal medium, all other assays used unbuffered complete medium.

Competence acquisition is the total time to conidiation, less maturation (time from induction to visible production of conidiophore vesicles). Conidiation was defined as the mean time, from inoculation, at which 50% of at least 30 isolated colonies had produced a conidiophore vesicle, for at least three replicate plates. Maturation time was defined identically except time zero was induction of competent colonies, achieved by decanting a 15 ml liquid medium overlay. Plates were incubated at 37° under constant fluorescent white light (1 m from polycarbonate shielded F36W/840 triphosphor fluorescent white lighting, Sylvania Lamps).

Generation of gene deletion, disruption and transgenic strains

Transformation strains carried an *nkuA* deletion to maximize the frequency of homologous integration (Nayak (2005)). For gene disruption, PCR primers targeted to the 5' coding region of genes were designed

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with an amplicon size of 400-600 bp. Products were cloned (plasmid Af pyro Pst H3 EI SK+ (*Ssp*I)) for transformation of strain TN02A25, selecting for pyridoxine prototrophy. Putative transformants were screened for the presence of the transforming plasmid by PCR against *A. fumigatus pyroA*. For genetic analysis of strains with a competence phenotype, the parental transformation strain was restored to riboflavin prototrophy to allow efficient backcrossing. Segregating auxotrophic markers, which do not alter competence timing on supplemented medium, were scored in crosses to confirm Mendelian segregation.

Deletion of *rasB* was by gene replacement in MUS51BARB12 with the *Neurospora crassa pyr-4* marker, site-directed mutagenesis was by inverse PCR and confirmed by Sanger sequencing. GTPase mutants were targeted to the *yA* locus in MUS51BARB12 and assayed in a *rasB*Δ background. For all other targeted gene deletions, cassettes based on the *A. fumigatus pyrG* selectable marker were obtained from the FGSC and amplified by PCR for transformation of strains PPU+ and MUS51BARB12. Gene replacement is highly efficient in *A. nidulans nkuA* strains (Nayak (2005)), and 5-100 transformants were typically obtained for each transformation. The AMA1 genomic library used for suppression of *prcB* molybdate sensivity has been described previously (Aleksenko and Clutterbuck (1997)).

Mutagenesis screens

A screen for precocious competence acquisition followed Axelrod (1973), with mutagenesis by treatment with 4-nitroquinoline oxide to 70% lethality. Generation of the precocious strain PCV9 (*prcC*) used a filtration enrichment screen based on an unpublished observation of interaction between competence state and *velvetA* (*veA*) genotype (Kennedy (1999)). As reported for a veA_1 mutant, ectopic expression of *brlA* drives conidiation in liquid culture (Adams *et al.* (1988)). This holds for veA_1 and veA^+ in the incompetent state, but not for competent veA^+. The ability to recover conidia from liquid cultured mycelium produced by *brlA* overexpression in a wild-type background therefore reflects competence state. A veA^+ strain carrying *brlA* under control of the inducible *alcA* promoter (pTA29, Adams *et al.* (1988)) was mutagenized as above, grown to competence in glucose minimal medium, then switched to 20 mM threonine to induce *brlA* expression for 12 hours. Production of conidia is expected from (1) mutants that have not yet acquired competence or are incapable of doing so, and (2) mutants that mimic veA_1 or otherwise suppress veA^+. PCV9 fell into the latter class, and was subsequently found to be precocious.

Genome sequencing

Genome sequencing for a single *prcA; prcB* strain (PABH13) was performed at the Allan Wilson Genome Centre (75 bp single end reads, Illumina Genome Analyser II). Sequencing of mutant and wild-type bulked segregant pools for *prcA* (12 F_1 progeny), *prcA; prcB* (12 F_1 progeny) and *prcC* (4 F_1 progeny) was by Macrogen (100 bp paired-end reads, Illumina HiSeq 2000). Sequence data are available from NCBI SRA (BioProject PRJNA319684).

For reference-based variant calling, reads were mapped by BWA mem (0.7.3) (Li and Durbin (2010)) with default settings to *A. nidulans* FGSC4 reference genome version s04-m05-r02 (AspGD) and processed with samtools (0.1.16) (Li *et al.* (2009)) and Picard (http://picard.sourceforge.net) utilities. Variants were called using the Genome Analysis Toolkit (GATK) HaplotypeCaller (2.6-4) for haploid samples or UnifiedGenotyper for pooled samples, and filtered by mapping quality (MQ < 40), depth (10 < DP < 2X mean coverage), strand bias (FS > 60), variant position relative to reads (ReadPosRankSum < -8), and variant quality/depth (QD < 2) (McKenna *et al.* (2010); Li (2011)). Remaining variants within regions masked by RepeatMasker (4.03) or clustered around centromeric gaps in the reference sequence were also excluded. For bulked sequencing, singleton calls across combined pools were removed. Functional predictions were generated by snpEff (3.0) (Cingolani *et al.* (2014)). Structural variation was analyzed with BreakDancer (1.3.6) (Chen *et al.* (2009)) and pindel (0.2.4) (Ye *et al.* (2009)), and by genome alignment after de novo assembly. Assembly used ABySS (1.3.4) (Simpson *et al.* (2009)) after Quake (0.3.2) read correction (Kelley *et al.* (2010)). Contigs were aligned to the reference with MUMmer (3.22) (Kurtz *et al.* (2004)) or by the UCSC chain/net pipeline (Kent *et al.* (2011)).

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Microarrays, data processing and analysis

Mycelium for wild-type and *prcA* microarrays spanning competence acquisition were from 145 mm diameter petri dishes containing 50 ml YEG inoculated with 10^5 conidia ml^{-1}, incubated at 37°. Between 10 and 50 plates were pooled per time point, harvested by vacuum filtration and immediately frozen in liquid nitrogen. Total RNA was prepared in TRIzol (Invitrogen) after disruption by 1 mm zirconia beads (Daintree Scientific) in a FastPrep FP120 (MPBio). Poly-A RNA was isolated on oligo-(dT) cellulose (Sigma), reverse transcribed using the Invitrogen Superscript Indirect Labeling Kit with random primers, and cDNA purified using Zymo DNA Clean & Concentrator columns according to Pathogen Functional Genomics Resource Center (PFGRC) protocol M007 (http://pfgrc.jcvi.org/index.php/microarray/). Samples were quantified by NanoDrop and competitively hybridized to version 2 whole genome glass slide microarrays provided by the PFGRC, according to protocol M008.

For wild-type a closed-loop circuit with a minimum of three dye-swapped competitive hybridizations for each time point was used, for a total of 16 hybridizations (see Supplementary Fig. 1). For *prcA*, two biological replicates as dye-swaps were hybridized per time point against wild-type, for a total of six hybridizations. Microarrays were scanned on an Axon GenePix 4100 or 4000B, ensuring channel ratios were in the range 0.95-1.05. Raw data were processed using the R package (http://www.r-project.org/) limma, with background correction (normexp offset = 50) and duplicateCorrelation for replicate probes, normalized to wild-type at 12 hours (Ritchie *et al.* (2007); Oshlack *et al.* (2007); Smyth (2004); Smyth and Speed (2003); Smyth *et al.* (2005)). Raw and processed data are available from NCBI GEO (GSE68352).

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Figure 1. *A. nidulans* reproductive cycles and the logic of developmental competence.
A. Three of the four reproductive cycles: asexual, sexual and hyphal growth, which may occur simultaneously in a single colony of sufficient age (adapted from Casselton and Zolan (2002)). In this work we focus on conidiation (asexual development), which is the first visible manifestation of developmental competence. Sexual development follows much later under conditions of sufficient nutrients and incubation in the dark. **B.** Defining competence in *A. nidulans*. Conidiophores per colony plotted by time since inoculation for five experiments varying the time of induction (black arrowheads) by exposure of single spore-derived colonies to air and light. Minimum time to conidiation (in blue) comprises the time required for competence acquisition (grey) and a constant maturation period for conidiophore production (red). Modified from Axelrod *et al.* (1973).

Figure 2. Environmental dependence of competence acquisition in wild-type and precocious mutants.
A. Competence acquisition is temperature dependent, and the precocious phenotype is temperature sensitive. Mutants are significantly accelerated relative to wild-type (WIM27 *prcA* $p = 2.25\text{x}10^{-7}$, WIM28 *prcA; prcB* $p = 1.27\text{x}10^{-13}$ by linear model for 30-42°), but not at 25° (*prcA* $p = 0.7$, *prcA; prcB* $p = 0.258$). Maturation rate is not significantly different to that of wild-type: 4.1 ± 0.06 hours (*prcA* $p = 0.927$, *prcA; prcB* $p = 0.121$ by linear model). **B.** Competence acquisition is accelerated at densities above 10^3 spores/plate (15.72 spores/cm^2). $p = 0.015$ at 10^3 spores/plate by linear model relative to 10 spores/plate. Maturation rate is not significantly different to wild-type (*prcA* $p = 0.56$, *prcA; prcB* $p = 0.148$ by linear model). **C, D.** Medium pH interacts with genotype and developmental stage to determine competence acquisition timing. Wild-type maturation rate is strongly pH dependent above pH 5. Maturation for the precocious mutants is reduced under all conditions, relative to unbuffered medium, and pH sensitivity is abolished (*prcA* $p = 0.333$) or dampened (*prcA; prcB* $p = 0.0091$). *prcA* and *prcA; prcB* show precocious competence for basic pH or unbuffered medium only, and *prcA; prcB* is significantly precocious relative to *prcA* only on unbuffered medium. Plotted data are mean ± standard error for at least three biological replicates per strain and condition.

Figure 3. Transcriptional profiling of competence acquisition and comparative analysis.
A. Hierarchical clustering of wild-type and *prcA* with *A. fumigatus* post-competence growth (12 vs. 8 h), *A. nidulans* during germination and early vegetative growth (3 h vs. spores, 5 h vs. 3 h), and *A. niger* during germination and early vegetative growth (2, 3 and 8 h vs. spores). Genes with missing data for more than two samples were excluded. Post-competence expression in wild-type *A. nidulans* is highly correlated with *prcA* (Spearmans $\rho = 0.59$ for wild-type 16 hours and *prcA* 16 hours), and with published data for *A. fumigatus* ($\rho = 0.61$ for wild-type 16 hours and *A. fumigatus* 12 hours) (Sheppard (2005)) and *A. nidulans* early vegetative growth ($\rho = 0.59$ for wild-type at 16 hours (here) and 5 hours from Breakspear and Momany (2007). **B.** Time and genotype-dependence of gene expression over competence acquisition. The distribution of correlation coefficients for each significantly differentially expressed gene is plotted for three of four linear models tested, where the model is preferred by Aikake Information Criterion (AIC; see Methods): time (model 1), genotype (m2; the smallest class, not shown), additive effects of time and genotype (m3) or additive and interaction effects of time and genotype (m4). Significantly enriched Gene Ontology (GO) terms among genes upregulated at competence are shown for each class, and for each significant GO hierarchy (central nodes in white). Node size represents the number of genes in each category, node color represents the significance of enrichment from white (no enrichment), through blue ($p < 0.05$ after multiple correction) and red (minimum of 1.672×10^{-5} for polarized growth under model 3) (Maere *et al.* (2005)). **C.** Epigenetic gene regulation in *A. nidulans* and *A. fumigatus* at competence acquisition. Gene expression relative to centromere position for wild-type and *prcA A. nidulans* (upper), and *A. fumigatus* (lower), with LOESS smoothing across all chromosomes. Competence acquisition is associated with establishment, or exaggeration, of a sinusoidal wave in expression symmetrical around the centromere, evident for *prcA* at 12 h (before competence at 12.5-13 h), and for wild-type *A. nidulans* after competence acquisition. Symmetry is reduced in *A. fumigatus* due to a more variable karyotype skewed toward submetacentric chromosomes.

Figure 4. Competence timing is inherited as a continuous trait for precocious mutants.
Conidiation timing (proportion of colonies conidiating after 22 hours growth) for a sample of F_1 progeny from crosses between *prcA* and wild-type (WIM27 x WIM126, left) and *prcA; prcB* and wild-type (WIM28 x WIM126, right), mean ± standard error from 2-3 biological replicates. Shaded bars indicate parental timing

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(green for wild-type).

Figure 5. Genetic analysis of defined precocious mutants
A. RasB activity modulates the timing of multiple developmental landmarks. For gain (G16V) and loss (T21N) of function GTPase mutations, activity is negatively correlated with germination and conidiation timing, while both alleles slightly accelerate maturation timing. All strain contrasts are significant for germination at 6 h ($p < 0.05$ by two-sided Wilcoxon rank sum test), maturation at 3.5 h ($p < 0.05$), and for conidiation ($p < 0.005$ for a linear model including all time points, strain effect $p < 10^{-15}$ by ANOVA). Data are mean percentages (± standard error) for at least three replicate experiments. **B.** Competence is significantly accelerated in $RasB^{T21N}$ and delayed in $RasB^{G16V}$ after accounting for differences in conidiation and maturation. Mean competence timing based on estimation of conidiation and maturation timing (50% colonies conidiating) by linear regression (± additive standard errors). **C.** Competence timing for the F_1 of backcrosses of precocious deletion mutants *ahpA*Δ and *msnA*Δ to their wild-type transformation strains. In both cases, F_1 timing is shifted toward that of the precocious parent, mean timing by genotype is significantly different ($p = 5.2\text{x}10^{-6}$ and $1.3\text{x}10^{-4}$, by Wilcoxon rank sum test), and progeny transgress the precocious parent in range. Data are mean ± standard error of 3 biological replicates.

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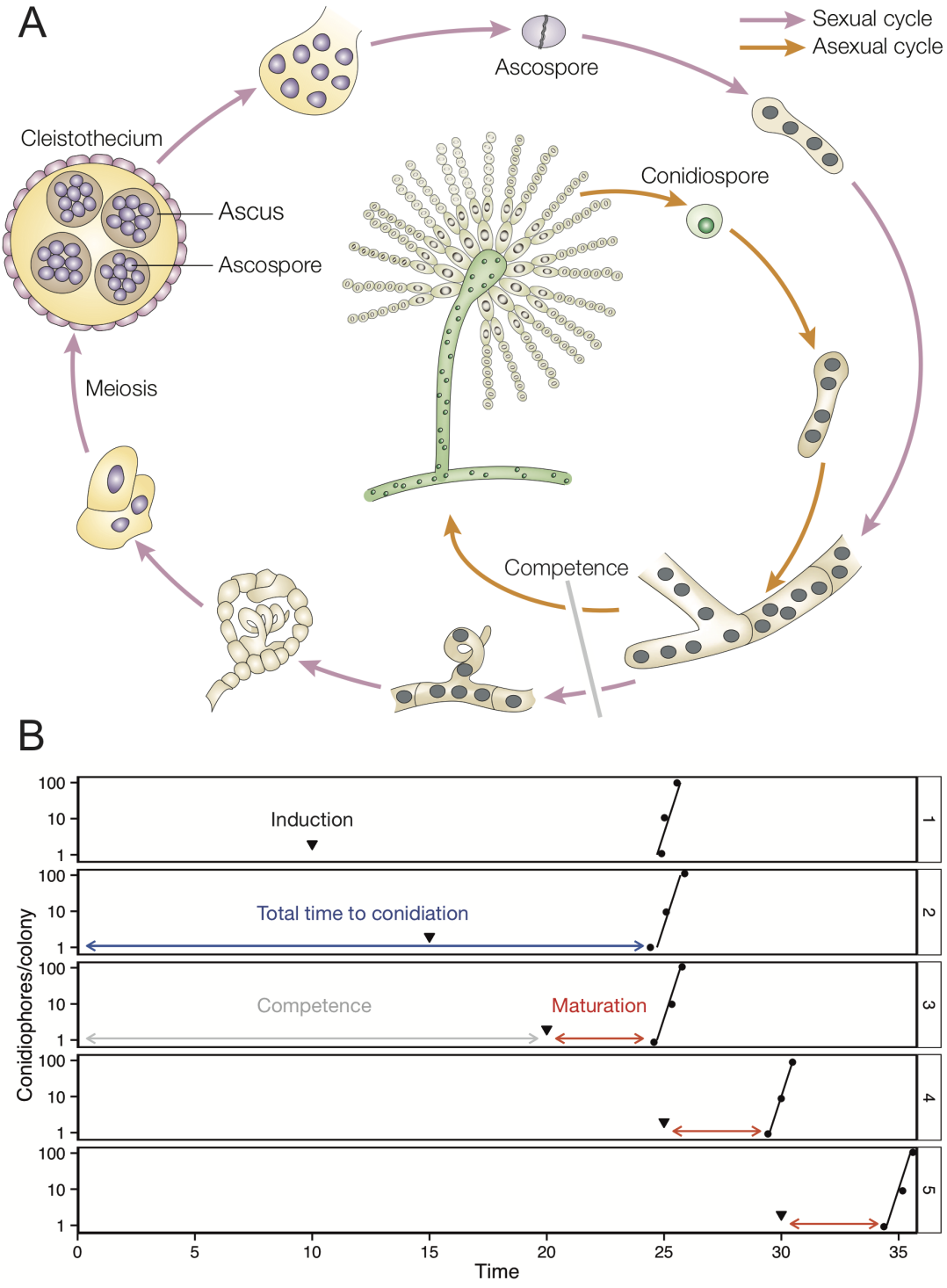

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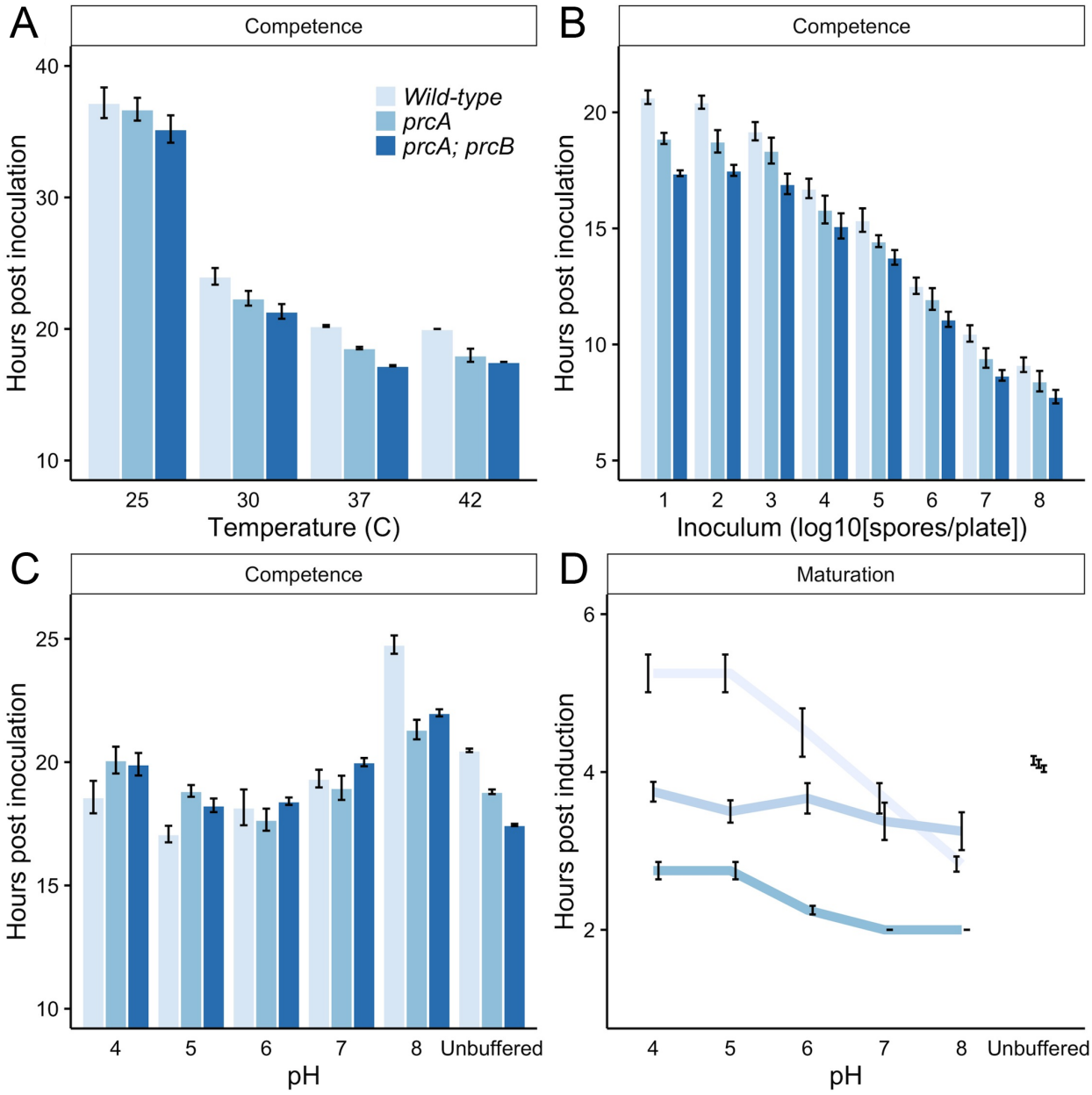

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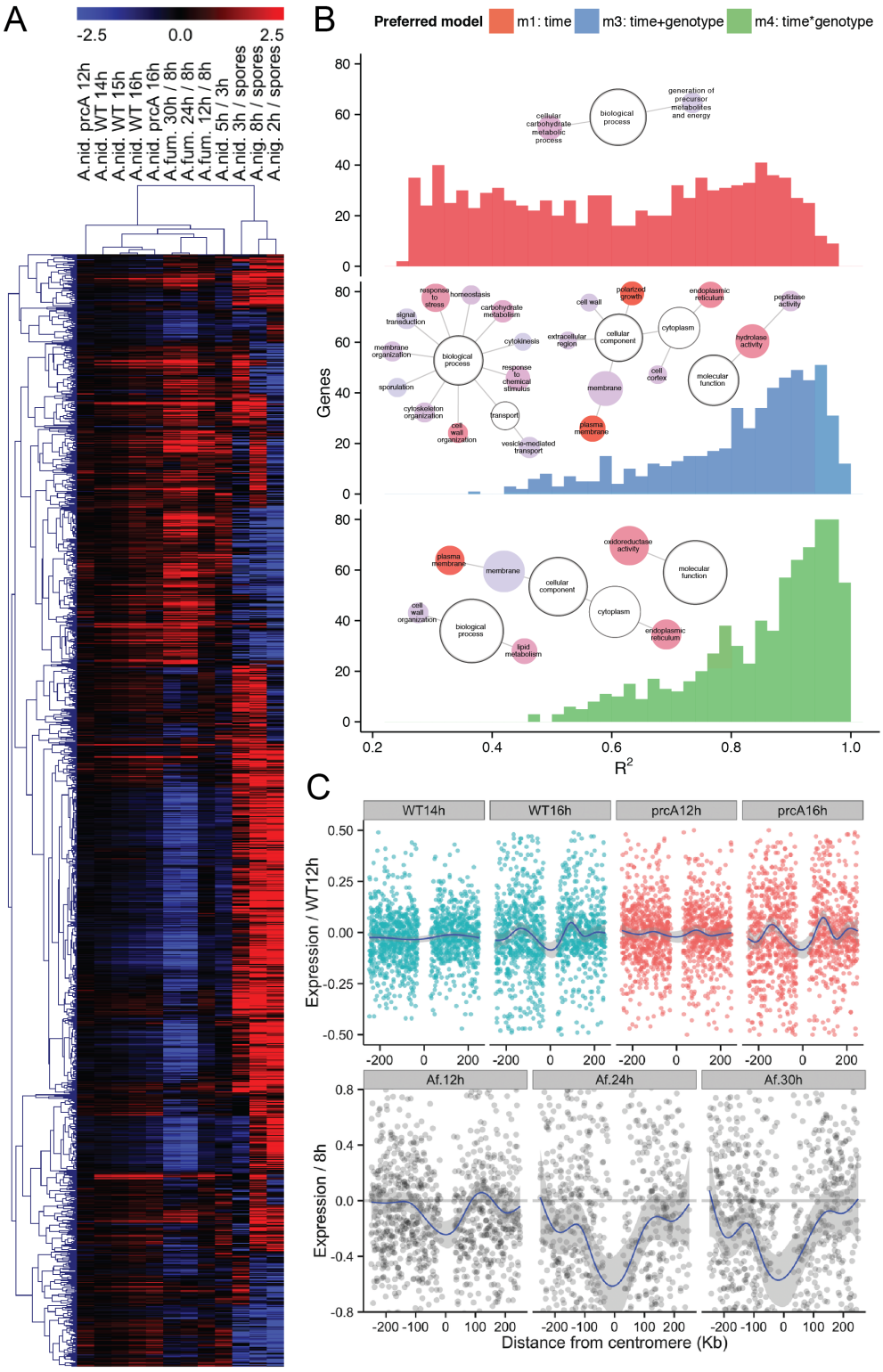

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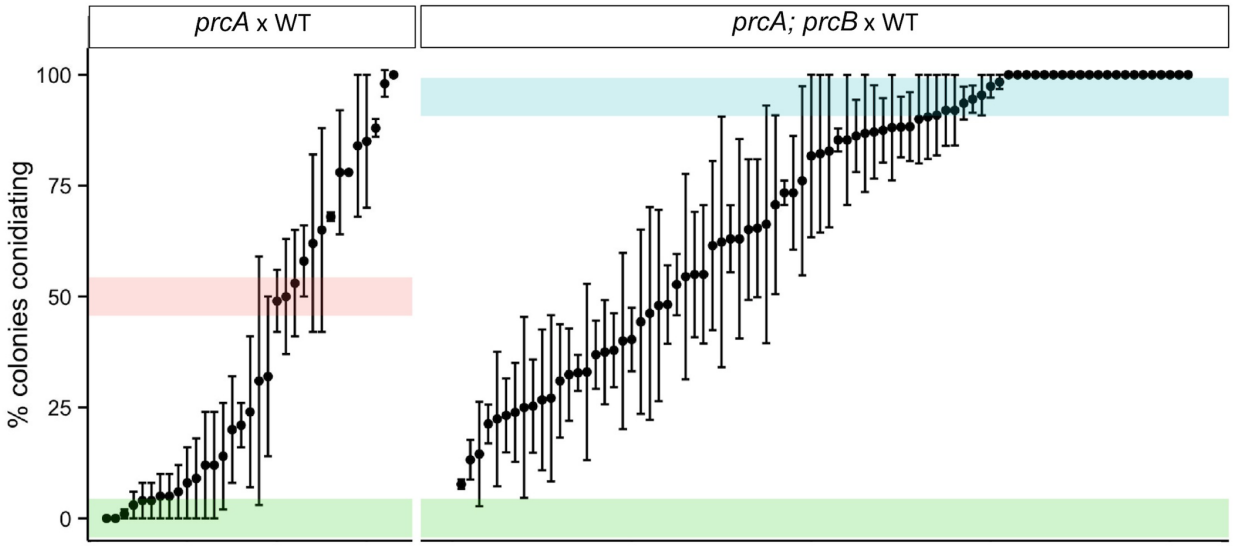

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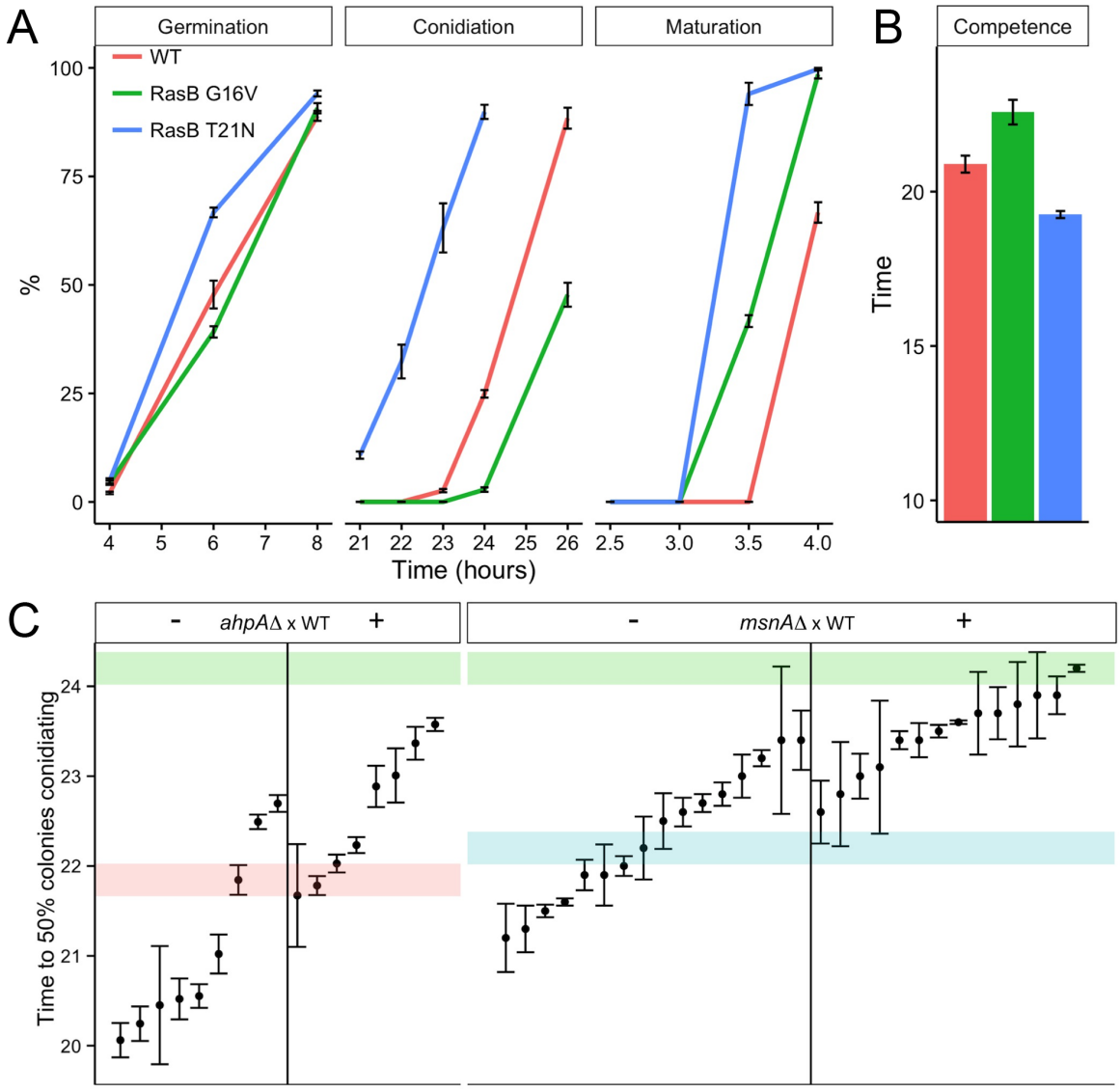

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