A Plastic Vegetative Growth Threshold Governs Reproductive Capacity in *Aspergillus nidulans*

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ABSTRACT Ontogenetic phases separating growth from reproduction are a common feature of cellular life. Long recognized for flowering plants and animals, early literature suggests this life-history component may also be prevalent among multicellular fungi. We establish the basis of developmental competence—the capacity to respond to induction of asexual development—in the filamentous saprotroph *Aspergillus nidulans*, describing environmental influences, including genotype-by-environment interactions among precocious mutants, gene expression associated with wild type and precocious competence acquisition, and the genetics of competence timing. Environmental effects are consistent with a threshold driven by metabolic rate and organism density, with pH playing a particularly strong role in determining competence timing. Gene expression diverges significantly over the competence window, despite a lack of overt morphological change, with differentiation in key metabolic, signaling, and cell trafficking processes. We identify five genes for which mutant alleles advance competence timing, including the conserved GTPase RasB (AN5832) and ambient pH sensor PalH (AN6886). In all cases examined, inheritance of competence timing is complex and non-Mendelian, with F₁ progeny showing highly variable transgressive timing and dominant parental effects with a weak contribution from progeny genotype. Competence provides a new model for nutrient-limited life-cycle phases, and their elaboration from unicellular origins. Further work is required to establish the hormonal and bioenergetic basis of the trait across fungi, and underlying mechanisms of variable inheritance.

KEYWORDS fungi; life-history; development; reproductive maturity; developmental competence

ORGANISMS must grow before they can reproduce. Discrete states oriented toward growth or reproduction are fundamental to ontogenesis, and important components of fitness (Istock 1967; Stearns 1976, 1992; Wilbur 1980; Roff 1993; Moran 1994). In unicellular microbes, the decision to grow or differentiate 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 coordinates development. Plants pass through one or more vegetative phase changes before acquiring the capacity to flower (Poethig

2003, 2010), many invertebrates attain the adult form through an intermediate juvenile stage (or stages) (Truman and Riddiford 1999; Bishop *et al.* 2006), and mammals reach sexual maturity (Zacharias and Wurtman 1969; Young 1976; Gluckman and Hanson 2006; Ahmed *et al.* 2009). 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 Opisthokont 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

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attention since description in *Trichoderma* (Gressel and Galun 1967), *Penicillium* (Hadley and Harrold 1958; Morton *et al.* 1958), and *Aspergillus* (Axelrod *et al.* 1973) species (exceptions include Adams *et al.* 1998; Roncal and Ugalde 2003; Sheppard 2005; Gravelat *et al.* 2008; Nahlik *et al.* 2010; Ruger-Herreros *et al.* 2011). Early work in *Aspergillus nidulans* showed competence acquisition is not simply a consequence of external nutrient limitation (Pastushok and Axelrod 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 found; suggesting competence represents a transition between somewhat discrete organismal states (Kurtz and Champe 1979; Kurtz 1980). More recently, competence for one species, *Penicillium cyclopium*, was shown to be under control of a diterpenoid hormone constitutively produced via the highly conserved mevalonate pathway (Roncal *et al.* 2002), 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 is unknown, regulation of growth and development by extracellular signals is known to be common in fungi, controlling processes such as spore germination inhibition, mating type recognition, negative autotropism, and metabolic influence on alternative reproductive strategies (Vining 1990; Yu and Keller 2005; 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, but relatively little of integration between these areas, which may be most relevant to competence.

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 in conidiophores; parasexual development, resulting in chromosomal reassortment with rare recombination; and, given sufficient nutrients, sexual development producing durable fruiting bodies (Figure 1A) (Pontecorvo *et al.* 1953; Champe *et al.* 1994; Todd *et al.* 2007b; Han 2009; Etxebeste *et al.* 2010). 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 homogeneous 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) are fungal-specific transcription factors, conserved signal transduction components such as heterotrimeric G proteins, and factors involved in production and perception of secreted signals. UDAs chan-

nel environmental cues into the late-acting central developmental pathway; a positive feedback, morphogenetic network comprising transcription factors such as bristle (*brlA*, AN0973) and abacus (*abaA*, AN5836), which orchestrates conidiophore and asexual spore production (Clutterbuck 1969; Adams *et al.* 1988; Sewall *et al.* 1990; Aguirre 1993; 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 *Saccharomyces cerevisiae* (Zemla *et al.* 2013), and hormone sensing through regulation of proteasome activity in plants (Browse 2009; Hind *et al.* 2011), 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, nonmorphological aspects of differentiation, or heritability. We aimed to address this by studying the effects of basic environmental variables on competence, profiling transcription in wild type and a precocious mutant to uncover the functional basis of accelerated competence acquisition, and identify genes with heterochronic effects through mutagenesis.

Materials and Methods

Strains and culture conditions

Strains used during this study are shown in Supplemental Material, Table S1. All are derived from the reference Glasgow isolate FGSCA4 (Arnaud *et al.* 2009). *Aspergillus* nitrogen medium [ANM + 10 mM (NH₄)₂Tartrate], carbon free (CF), complete medium (CM), basic growth conditions, handling, genetic, and parasexual analysis were as described (Todd *et al.* 2007a,b). 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 H₂O for up to 1 month. Testing of specific pH, metabolic substrates, or chemical agents was on minimal medium, all other assays used unbuffered CM (File S1, File S2, File S3, File S4, File S5, File S6, File S7, and File S8).

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) Schematic defining competence in *A. nidulans*, modified from an experiment by Axelrod *et al.* (1973). Conidiophores per colony plotted by time since inoculation for five experiments varying the time of induction (black ▼) by exposure of single spore-derived colonies to air and light. Minimum time to conidiation (in blue) comprises the time required for competence acquisition (gray) and a constant maturation period for conidiophore production (red).

Competence acquisition is the total time to conidiation, less maturation (time from induction of competence mycelium 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. For screening of new mutants, constancy of maturation was confirmed by measuring the lag to conidiation from induction of plates of varying age (in the range 2–6 hr postcompetence). 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 with an amplicon size of 400–600 bp. Products were cloned [plasmid Af pyro Pst H3 EI SK+ (*SspI*)] 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* (Afu5g08090) and by backcrossing, following the selectable marker. For genetic analysis of strains with a competence phenotype, the parental transformation strain was restored to riboflavin prototrophy by transformation with plasmid pPL3 to allow efficient backcrossing. Segregating auxotrophic markers, which do not alter competence timing on fully supplemented medium, were scored in crosses to confirm Mendelian segregation.

Deletion of *rasB* (AN5832) was by gene replacement in MUS51BARB12 with the *Neurospora crassa* *pyr-4* (NCU03488) marker, verified by Southern blot. Site-directed mutagenesis was by inverse PCR, confirmed by Sanger sequencing. GTPase mutants were targeted to the *yA* (AN6635) locus in MUS51BARB12

and assayed in a *rasBΔ* (AN5832) background. A screen for histone modification mutants used deletion mutants for *cclA* (AN9399, strain 12270), *rtfA* (AN4570, 12272), AN5894 (12273), AN0888 (12632), AN8211 (12633; generously provided by Joseph Strauss and Nancy Keller), *dmtA* (AN6638, 12209), and *kdmA* (AN1060, 12062; which we generated by gene replacement and verified by Southern blot). For all other targeted gene deletions, cassettes based on the *A. fumigatus* *pyrG* (Afu2g08360) selectable marker were obtained from the Fungal Genetics Stock Center and amplified by PCR for transformation of strains PPU+ and MUS51BARB12. Gene replacement is highly efficient in *A. nidulans* *nkuAΔ* (AN7753) strains (Nayak 2005), and 5–100 transformants were typically obtained for each transformation. The AMA1 genomic library used for suppression of *prcB* molybdate sensitivity has been described previously (Aleksenko and Clutterbuck 1997).

Mutagenesis screens

Screening for precocious competence acquisition followed Axelrod *et al.* (1973), with mutagenesis of A770 (strain 10076) by treatment with 4-nitroquinoline oxide to 70% lethality. Generation of the precocious strain PCV9 (*prcC*) used a novel filtration enrichment screen based on an unpublished observation of interaction between competence state and *velvetA* (*veA* AN1052) genotype (Kennedy 1999). As reported for a *veA₁* mutant, ectopic expression of *brlA* (AN0973) in liquid culture drives conidiation and blocks vegetative growth (Adams *et al.* 1988). This holds for *veA₁* 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.

Sibling strains, *veA₁* or wild-type *veA* but otherwise of identical known genotype (including the arginine auxotrophy marker *argB2*), were transformed with plasmid pTA29, carrying the *argB* (AN4409)-selectable marker and *brlA* under control of the *alcA* promoter (Adams *et al.* 1988), yielding strains 12097 and 12098. Growth arrest on *alcA* (AN8979) inducing solid medium (threonine or ethanol as carbon source) confirmed functional *brlA* expression.

Strain 12098 was mutagenized as above, grown to competence in glucose minimal medium, then immediately switched to 20 mM threonine to induce *brlA* expression for 12 hr. Production of conidia is expected from mutants that (1) have not yet acquired competence or are incapable of doing so (e.g., delayed or aconidial mutants), (2) prevent *veA*-mediated degradation of *brlA* transcript, or (3) mimic *veA₁* or otherwise suppress *veA*. Nine *veA₁*-like, seven aconidial, and six delayed unique mutants were obtained. Of the *veA₁*-like mutants with repressed sexual development, PCV9 (*prcC*) alone proved 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 Analyzer II). Sequencing

of mutant and wild-type bulked segregant pools for *prcA* (12 F₁ progeny), *prcA*; *prcB* (12 F₁ progeny) and *prcC* (four F₁ progeny) was by Macrogen (100 bp paired-end reads, Illumina HiSeq 2000).

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://broadinstitute.github.io/picard/> utilities to mark duplicates. Variants were called using the Genome Analysis Toolkit HaplotypeCaller (2.6-4) for haploid samples or UnifiedGenotyper for pooled samples, and filtered by mapping quality (MQ < 40), depth (10 < DP < 2 × mean coverage), strand bias (FS > 60), variant position within 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), and contigs were aligned to the reference with MUMmer (3.22) (Kurtz *et al.* 2004) and by the University of California Santa Cruz chain/net pipeline (Kent *et al.* 2011).

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 10⁵ 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, Carlsbad, CA) after disruption with 1-mm zirconia beads (Daintree Scientific) in a FastPrep FP120 (MPBio). Poly-A RNA was isolated on oligo-(dT) cellulose (Sigma Chemical, St. Louis, MO), reverse transcribed using the Invitrogen Superscript Indirect Labeling Kit with random primers, and complementary DNA purified using Zymo DNA Clean and 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 Figure S1). 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 Instruments (Foster City, CA) 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 hr (Smyth and Speed 2003; Smyth 2004; Smyth *et al.* 2005; Ritchie *et al.* 2007; Oshlack *et al.* 2007).

Data availability

All strains are available upon request. Sequence data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (PRJNA319684), raw and processed gene expression data are available from the NCBI Gene Expression Omnibus (GSE68352).

Results

Environmental dependence of competence timing

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 (Figure 1, A and B). Minimum total time to conidiation was measured by growing single spore-derived colonies under inducing conditions (the surface of an agar plate, under light). Maturation time was then inferred (and subtracted) by growing under noninducing conditions (an agar plate overlaid with liquid medium) past this point, inducing development (by decanting the liquid overlay), and monitoring the time until conidiophore emergence (Axelrod 1972; Axelrod *et al.* 1973).

To test the environmental dependence of competence and the basis of precocious mutant phenotypes, we surveyed basic conditions in a wild-type strain (WIM126) and two related, genetically undefined mutant strains. WIM27 (hereafter *prcA*) was isolated in a UV mutagenesis screen for early conidiation (Axelrod *et al.* 1973) and WIM28 (hereafter *prcA*; *prcB*), with an enhanced phenotype, was generated by further mutagenesis of WIM27 (Kurtz and Champe 1979).

Temperature, organism density, and pH altered competence timing significantly (Figure 2). We also found that competence and maturation often varied independently. On glucose medium, wild type time to conidiation at 37° was 24.2 hr (± 0.07 hr SE), including a maturation time of 4.1 ± 0.06 hr. Consistent with initial reports, *prcA* commenced conidiation around 2 hr earlier (22.6 ± 0.09), with *prcA*; *prcB* around 1.5 hr earlier again (21.2 ± 0.05 ; Figure 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 $P < 2 \times 10^{-8}$, temperature effect $P <$

10^{-50} for competence, against 0.296 and 0.029 for maturation; Figure 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; Figure 2B). The effect size of the precocious phenotype was similar across densities.

Buffering medium across a pH range significantly altered both competence and maturation timing for all strains, with effects dependent on both pH and genotype. Under acidic or neutral buffering (pH 7), competence of the wild type was generally accelerated to similar, or even earlier, timing to that of the mutants (Figure 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 , $P = 0.3333$; Figure 2D).

Transcriptional profiling of competence acquisition

We profiled transcription during competence acquisition by microarray, first sampling wild-type mycelium hourly over a 5 hr window centered on competence (14.5 hr under moderate density liquid culture; Figure S1). 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 hr, was predominantly linear, with 1493 genes upregulated and 965 downregulated at 16 hr. This suggests competence is a transition between states, rather than a transient signaling event. The data were strongly positively correlated with published gene expression data from early vegetative growth of varying age in *A. nidulans* (Spearman's $\rho = 0.59$) (Breakspear and Momany 2007), *A. fumigatus* ($\rho = 0.61$) (Sheppard 2005), and *A. niger* ($\rho = 0.51$) (van Leeuwen *et al.* 2013), but not with germination in *A. nidulans* ($\rho = -0.04$) (Breakspear and Momany 2007) or *A. niger* ($\rho = -0.54$) (van Leeuwen *et al.* 2013), showing these developmental landmarks are largely distinct (Figure 3A). Overall concordance in expression direction between *A. nidulans* and *A. fumigatus* after competence acquisition was 74% representing almost one third of orthologs (Figure S2).

To test for candidate expression differences causing early competence acquisition, we next sampled the precocious mutant *prcA* over the window (competence attained at

Figure 2 Environmental dependence of competence acquisition in wild type and precocious mutants. (A and B) Competence timing is shown, with additive errors from conidiation and maturation measurements, under conditions in which maturation is not strongly condition-dependent. (C and D) Competence and maturation is shown separately for pH, which influences both components. (A) Competence acquisition is temperature dependent, and the precocious phenotype is temperature sensitive. Mutants are significantly accelerated relative to wild type (WIM27 *prcA*, $P = 2.25 \times 10^{-7}$; WIM28 *prcA*; *prcB*, $P = 1.2 \times 10^{-13}$ 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 hr (*prcA*, $P = 0.927$; *prcA*; *prcB*, $P = 0.121$ by linear model). (B) Competence acquisition is accelerated at densities $>10^3$ spores/plate (15.72 spores/cm²), $P = 0.015$ at 10^3 spores/plate relative to 10 spores/plate. Maturation rate is not significantly different to wild type (*prcA*, $P = 0.56$; *prcA*; *prcB*, $P = 0.148$). (C and 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 $\pm$ SE for at least three biological replicates per strain and condition.

12.5–13 hr), testing by linear model for 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). Functional analysis of these groups [gene ontology (GO), Gene Set Enrichment Analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways] revealed highly significant activity: genes related to translation and ribosomal biogenesis (translation initiation and elongation, transfer RNA 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 (Figure 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, precompetent growth (Kurtz and Champe 1979; Kurtz 1980). Accordingly, expression of the central energy homeostasis regulator pyruvate dehydrogenase kinase (PDK, AN6207) increased steadily throughout the time course (effect of time across *A. nidulans* and *A. fumigatus*, $P = 0.0006$ by ANOVA; see Figure S3).

Gene expression during competence acquisition was also clearly altered at the level of chromosome structure or subnuclear localization. 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* (Figure 3C). Second, expression from a number of verified and potential core-regulated gene clusters was altered in the precocious mutant, such that correlation with wild type was often minimal at telomeres (Figure S4). These observations suggest that gross alterations in chromatin are associated with competence acquisition, however, a survey of deletion mutants for predicted histone modification factors, or application of the histone acetylation inhibitor Trichostatin A or DNA methylation inhibitor 5-azacytidine, failed to alter competence timing (see Materials and Methods).

Identification and genetic analysis of precocious mutants

Although a number of precocious competence mutants had been generated, underlying genes and the heritability of competence timing phenotypes were unknown. We undertook genetic and parasexual analysis of three undefined

Figure 3 Transcriptional profiling of competence acquisition and comparative analysis. (A) Hierarchical clustering of wild type and *prcA* with *A. fumigatus* postcompetence growth (12 vs. 8 hr), *A. nidulans* during germination and early vegetative growth (3 hr vs. spores, 5 vs. 3 hr), and *A. niger* during germination and early vegetative growth (2, 3, and 8 hr vs. spores). Postcompetence expression in wild-type *A. nidulans* is highly correlated with *prcA* ($p = 0.59$ for wild type 16 hr and *prcA* 16 hr), with published data for *A. fumigatus* ($p = 0.61$ for wild type 16 hr and *A. fumigatus* 12 hr) (Sheppard 2005), and *A. nidulans* early vegetative growth [$p = 0.59$ for wild type at 16 hr (here) and 5 hr from Breakspear and Momany 2007]. Genes with missing data for more than two samples were excluded. (B) Time and genotype dependence of gene expression over competence acquisition. The distribution of correlation coefficients for differentially expressed genes is plotted for three of four linear models tested, where the model is preferred by Akaike information criterion: time (model m1), 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 GO terms among genes upregulated at competence are shown for each class, for each GO hierarchy (central nodes in white). Node size represents the number of genes in each category, node color represents enrichment significance from white (no enrichment), through blue ($P < 0.05$ after multiple correction) and red (minimum of 1.672×10^{-5} for polarized growth under m3) (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* (top), and *A. fumigatus* (bottom), across all chromosomes (LOESS smooth). Competence acquisition is associated with establishment, or exaggeration, of a sinusoidal wave in expression sym-

metrical around the centromere, evident for *prcA* at 12 hr (before competence at 12.5–13 hr), 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. WT, wild type.

precocious mutant strains, two from the original UV mutagenesis screens (*prcA* and *prcA*; *prcB*) and a third (*prcC*) from a chemical mutagenesis screen (see *Materials and 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. Genetic analysis showed competence timing was in both cases inherited as a continuous quantitative trait (Figure 4). 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 semidominant 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 produce extracellular protease or to elicit contact inhibition from neighboring wild-type colonies. 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 35 $\times$ coverage (see *Materials and Methods*), and detected an early frameshift in AN6886, which encodes the ambient pH sensor PalH. Molybdate sensitivity is a known phenotype of acidity-mimicking mutations, including palH loss of function mutations (Negrete-Urtasun *et al.* 1999; Calcagno-Pizarelli *et al.* 2007). The frameshift cosegregated with molybdate sensitivity and the above phenotypes in eight F₁ 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 F₁ backcross progeny were sequenced to a depth of $\sim 160\times$ each. For *prcC*, we detected a premature stop codon (Y250*) in the *nsdD* (AN3152) gene [1 of 14 genes with mutations predicted to be highly deleterious by SnpEff unique to the bulk (Cingolani *et al.* 2014)], which encodes a GATA zinc finger transcription factor required for sexual development. A *nsdD* Δ deletion mutant showed precocious competence acquisition and a constitutive conidiation phenotype identical to that of the mutant, consistent with a prior report of early conidiation for loss of function mutants (Han *et al.* 2001).

From *prcA* and wild-type F₁ pools, each comprising 12 haploid genomes, we detected 2153 single nucleotide variants (SNVs) and small insertion/deletions after filtering (see *Materials and Methods*). Most (1450) were fixed in both pools relative to the reference FGSCA4. Of 406 variants segregating and variable in frequency across pools (Figure S5), 2 SNVs were fixed in the precocious pools and at low frequency (1/12) in wild type, located on chromosomes I and III. Both were nonsynonymous coding mutations with evidence for local linkage disequilibrium (Figure S6). Single gene deletion mutants failed to alter developmental timing, however, and

further parasexual analysis suggested the presence of chromosomal abnormalities. 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 (AN5832), since activity of a related protein, RasA (AN0182), has been shown to alter the timing of developmental transitions (although effects on competence were not shown, see 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 negatively correlated with germination and conidiation ($P < 10^{-4}$ and $P < 10^{-18}$ by linear model), while maturation rate was accelerated slightly by both alleles (ANOVA strain effect, $P < 10^{-4}$; Figure 5A). The net effects on competence were strong in both directions: -1.63 ± 0.1 hr for RasB^{T21N} and 1.68 ± 0.18 hr for RasB^{G16V} (Figure 5B).

We additionally generated and analyzed 70 targeted deletion or disruption mutants (see *Materials and Methods*), selected on criteria of significant differential expression over competence acquisition (up- or downregulated), predicted gene function (predominantly transcription factors) and evolutionary conservation (presence of orthologs across Ascomycetes at least). Null mutants of two genes, both weakly (less than twofold) upregulated with competence acquisition in *prcA*, showed significantly earlier conidiation and competence acquisition: homeodomain transcription factor *ahpA* (AN2020; 21.84 ± 0.165 hr), and C₂H₂ transcription factor *msnA* (AN1652; 22.2 ± 0.35 hr, see File S8). Backcrossing of both mutant strains showed transgressive, continuous inheritance of competence timing, as seen for *prcA* strains (Figure 5C). In both cases, precocious competence was partially dominant in the F₁—for *ahpA* Δ , no progeny of wild type timing were seen—and additionally dependent on F₁ genotype ($P = 5.2 \times 10^{-6}$ for *ahpA* Δ , 1.3×10^{-4} for *msnA* Δ , two-sided Wilcoxon rank sum test). Quantitative and highly variable heritability of competence timing is therefore not an artifact of background variation segregating in *prcA* strains, but is inherent to the precocious phenotype.

Discussion

We have outlined the environmental, genetic, and transcriptional basis of a threshold governing reproductive maturity in *A. nidulans*, likely conserved throughout filamentous Ascomycetes at least. Asexual development, as the earliest manifestation of competence and by far the better studied

Figure 4 Competence timing is inherited as a continuous trait for precocious mutants. Conidiation timing (proportion of colonies conidiating after 22 hr growth) for a sample of F₁ progeny from crosses between *prcA* and wild type (WIM27 × WIM126, left), and *prcA; prcB* and wild type (WIM28 × WIM126, right), mean ± SE from 2–3 biological replicates. Shaded bars indicate parental timing (green for wild type; WT).

reproductive mode, has been our focus, though an additional, much later threshold for competence to respond to sexual development has also been demonstrated using a temperature sensitive allele of the pivotal developmental integrator *velvetA* (AN1052) (Champe *et al.* 1981).

Transcriptional and hyphal differentiation during development

Molecular genetic studies have shown the expression of many genes changes, in quantity or localization, during early vegetative growth (Miller *et al.* 1991; Kawasaki and Aguirre 2001; Tsitsigiannis 2004; Soid-Raggi *et al.* 2006; Etxebeste *et al.* 2008, 2009; Harispe *et al.* 2008; Garzia *et al.* 2009; Kwon *et al.* 2010), 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 is often 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 *Aspergilli* examined. Although there are caveats to measuring gene expression at the whole-organism level (discussed below), correlations with multiple, independent single gene and genome-wide studies suggests the trends we report are robust and likely extend across the genus.

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), as well as a 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 messenger RNA and protein abundance is generally good, where it has been tested, in fungi ($r = 0.4$ – 0.7) (Lee *et al.* 2011; Vogel *et al.* 2011; Vogel and Marcotte

2012), 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.

While multiple physiological correlates of competence acquisition show nonlinear, switch-like dynamics, we observed a predominantly monotonic transition in gene expression over the 4-hr window. Altered symmetry at the level of the colony (*e.g.*, changes in growing:developing zones) might be a trivial explanation for some of the broad patterns of gene expression observed. Kinetics and morphology of early filamentous growth have been studied extensively (Trinci 1974; Fiddy *et al.* 1976; Dynesen and Nielsen 2003; Rittenour *et al.* 2009; Roper *et al.* 2011), but over a wide range of conditions and rarely with regard to competence state, complicating inference. Of particular relevance here, Dynesen and Nielsen (2003) 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 hr in rich medium). 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 during hyphal polarization include establishment of apical-high gradients of reactive oxygen species, Ca²⁺, and protons (Roncal *et al.* 1993; Robson *et al.* 1996; Semighini and Harris 2008; Rodríguez-Urra *et al.* 2012). While there is little or no functional data directly linking

Figure 5 Genetic analysis of defined precocious mutants. (A) RasB (AN5832) 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 hr ($P < 0.05$ by two-sided Wilcoxon rank sum test), maturation at 3.5 hr ($P < 0.05$), and for conidiation ($P < 0.005$ for a linear model including all time points, strain effect $P < 10^{-15}$ by ANOVA). Data are mean percent-ages ($\pm$ SE) 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 interpolation ($\pm$ additive SE). (C) Competence timing for the F₁ of backcrosses of precocious deletion mutants *ahpAΔ* and *msnAΔ* to their wild-type transformation strains. In both cases, F₁ timing is shifted toward that of the precocious parent, mean timing by genotype is significantly different ($P = 5.2 \times 10^{-6}$ and 1.3×10^{-4} , by Wilcoxon rank sum test), and progeny transgress the precocious parent in range. Data are mean $\pm$ SE of three biological replicates. WT, wild type.

competence-associated genes *msnA* (AN1652), *ahpA* (AN2020), *palH* (AN6886), and *rasB* (AN5832) to altered patterns of biochemistry at the hyphal scale; there are suggestions of involvement in all three of these processes. The C₂H₂ transcription factor *msnA* and its *A. fumigatus* ortholog, *seba* (Afu4g09080), are transcriptionally induced by osmotic, oxidative, and heat stress (Han and Prade 2002; Dinamarco *et al.* 2012). *Seba* was additionally shown to be concentrated in the nucleus under stress, and subject to Ca²⁺-CrzA-calcieneurin controls (Dinamarco *et al.* 2012). The *A. fumigatus* *ahpA* ortholog, *htfA* (Afu4g10110), is expressed at higher levels, particularly during early growth, in a *calAΔ* (Afu5g09360) calcieneurin mutant, and is required for induction of putative transporters *pmcA* (Afu1g10880) and *pmcB* (Afu3g10690) in response to a Ca²⁺ pulse (Malavazi *et al.* 2009). Ca²⁺ controls actin assembly and endocytosis rates in many organisms, and shows age-dependent oscillations at hyphal apices in filamentous fungi (Kim *et al.* 2012), warranting future attention. *PalH* is a seven-transmembrane pH sensor required for proteolytic activation of transcription factor PacC (AN2855) under alkaline conditions (Negrete-Urtasun *et al.* 1999), and pH homeostasis has recently emerged as a central signal regulating growth rate through Ras/PKA in *S. cerevisiae* (Dechant *et al.* 2014). Disruption of hyphal ion homeostasis at multiple levels may therefore be a common mechanism underlying shifts in competence timing, due either to accelerated nutrient uptake

(as seen in *prcA* and *prcA*; *prcB*) (Kurtz and Champe 1979; Kurtz 1980), or to alteration in sensitivity to a competence-associated signal. Effects on vacuole or mitochondrial ion homeostasis may also be of relevance to the inferred change in iron localization or redox state (Hall and Axelrod 1978).

Importantly, an apical FlbB/FlbE (AN7542/AN0721) regulatory complex appears to transition first from the hyphal tip to subapical nuclei in “mature” vegetative hyphae, and then, upon developmental induction, to nuclei throughout the colony (Etxebeste *et al.* 2008, 2009); potentially linking induction capacity with the morphogenetic, positive feedback, central development pathway. Association of the bZIP transcription factor FlbB with FlbE requires a conserved cysteine residue in the FlbB C terminus, potentially through competence-associated redox control (Herrero-García *et al.* 2015). It remains, however, to establish the precise timing of documented hyphal differentiation events, including transcriptional differentiation, with respect to density and competence, before consideration of the mechanistic basis of competence timing and its variation in precocious mutants.

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

that some precocious strains might not be true genetic mutants, but carriers of transiently heritable epigenetic states, or that the effect of mutational perturbation might be compensated over time if competence is a genetically robust trait.

Genetic analysis of *prcA* and *prcA*; *prcB* strains—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. Additionally, 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 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 *ahpAΔ* (AN2020) and *msnAΔ* (AN1652) 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 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 assayed. 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 fungi (Calo *et al.* 2014) and, as in analogous juvenile to adult 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 the timing of maturity 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-histories have historically been delineated as simple (direct) or complex (indirect) (Moran 1994; Wiegmann *et al.* 2009). One defining characteristic of this complexity put forward is an abrupt change in morphology, physiology, and behavior, usually associated with a change in habitat (Wilbur 1980), while others have elevated ecological criteria (Istock 1967). Clearly, a narrow definition is more broadly defensible, 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). While apparently restricted to differentiation in transcription and biochemistry, 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 the temperature range optimal for growth, on preferred carbon and nitrogen sources (according to hierarchical catabolite repression systems; see 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, might well 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 in filamentous fungi (Adams *et al.* 1988; Adams and Timberlake 1990). This may be achieved 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 binary bang-bang strategy—full allocation to vegetative growth, followed by a resource-dependent, complete switch to 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 patches of fixed resource levels, which involves trade-offs between spore production schedule and quantity, as well as propagule quantity and quality. The expected time required to exhaust a patch of resource is a particularly critical factor. Small patches, or intense competition, should increase selective pressure on shortening the time to competence. Conversely, large patches, or rare competition (niche specialization), may favor a greater allocation toward rapid vegetative growth, and delayed competence. A genetically assimilated competence schedule should then reflect the species' ecological history, and its level of predictability, particularly for critical factors such as effective patch size, duration, and distribution. Variance in these factors (Gilchrist *et al.* 2006), as well as advantages accruing from more costly, delayed 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 postcompetence 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 for a single spore at 24 hr, although it remains to see if this holds outside laboratory conditions, and for other species of similar niche.

The existence of a threshold-limited reproductive maturity trait in diverse fungi, coupled with increasingly dense phylogenetic sampling and genomic resources in a number of clades, offers some 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 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 could provide quantitative data for integration with life-history theory, which is rarely applied to fungi. Lastly, although nutrient-limited thresholds are ubiquitous in 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 genetic systems, and the elaboration of complex life-histories from simple beginnings.

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Supplementary Figure 1.

Microarray circuit, where each line represents a single competitive hybridization and each sample comparison includes dye-swapped technical replicates. Under the medium density culture conditions used, wild-type acquires competence between 14 and 15 hours, while *prcA* acquires competence between 12 and 14 hours.

Supplementary Figure 2.

Venn diagram of competence regulated gene expression concordant between *A. nidulans* (16 hours) and *A. fumigatus* (12 hours). Overlap represents number of orthologs – upregulated in green, downregulated in red. Lower diagrams show significantly differentially expressed genes by species.

Supplementary Figure 3.

Gene expression for selected genes involved in central carbon metabolism across three studies. Values are log2 fold changes relative to precompetent samples (12 hours for wild-type and *prcA*, 8 hours for *A. fumigatus*) or to high-density vegetative growth (*A. nidulans* 5h vs. 3h).

Supplementary Figure 4.

Correlation in gene expression between wild-type *A. nidulans* and *prcA* at 12 and 16 hours. A LOESS regression is plotted over coefficients for a 10 gene sliding window. Centromere positions are indicated by shaded bars.

Supplementary Figure 5.

Variant frequencies in wild-type and *precocious* sequenced bulks (12 genomes each). Point size is scaled by skew, and points for each cell of the 13 x 13 matrix are dispersed to minimise overplotting.

Supplementary Figure 6.

Variant differentiation between wild-type and *precocious* sequenced bulk genomes. Two high frequency variants on chromosomes II and III show evidence of linkage disequilibrium extending over several hundred kilobases. Centromere positions are indicated by shaded bars.

Supplementary Table 1

Name	Strain ID	Genotype	Source
MSF	58	<i>yA2, adE20, suA(adE20), AcrA1, galA1, pyroA4, facA303, sB3, nicB8, riboB2, veA1</i>	FGSC
A770	10076	<i>pyrG89, pabaB22, riboB2, veA1</i>	FGSC
A234	10380	<i>yA2, pabaA1, veA1</i>	FGSC
catABΔ	10383	<i>biA1, catAΔ(argB), metG1, catBΔ(argB) veA1</i>	Kawasaki et al. (1997) - CLK14
msnAΔ	10542	<i>biA1, pabaB22, msnAΔ(Af pyrG), veA1 niiA4</i>	Suzuki (unpublished)
TNO2A21	11036	<i>pyroA4, riboB2, veA1, nkuAΔ</i>	Hynes (unpublished)
TNO2A25	11037	<i>pyrG89, pabaB22 nkuAΔ(argB), veA1 riboB2</i>	Hynes (unpublished)
MUS51BARB12	11068	<i>pyroA4, pyrG89, veA1, nkuAΔ</i>	Hynes (unpublished)
WIM28	11597	<i>prcA, prcB</i>	L. N. Yager
WIM27	11598	<i>prcA</i>	L. N. Yager
WIM126	11599	<i>yA2 pabaA1</i>	L. N. Yager
W27UPR24	11774	<i>prcA, pyrG89, pabaB22, riboB2</i>	WIM27 x A770
PP3	11892	<i>yA2, prcA, pabaA1</i>	WIM27 x A234
WIM27PNG15	12016	<i>wA3, prcA, pabaA1, niiA4, gatA312</i>	PP3 x RSB12
PPU+	12042	<i>yA2 pabaA1 pyrG89, pyroA4 nkuAΔ(Bar)</i>	WIM126 x MUS51BARB12
PPU-	12043	<i>yA2 pabaA1 pyrG89, pyroA4 nkuAΔ(Bar), veA1</i>	WIM126 x MUS51BARB12
AN1060Δ9	12062	<i>pyrG89, pabaB22 nkuAΔ(argB), veA1 kdmAΔ(Nc pyr-4) riboB2</i>	Transformation (TNO2A25)
AN1060Δ12	12063	<i>pyrG89, pabaB22 nkuAΔ(argB), veA1 kdmAΔ(Nc pyr-4) riboB2</i>	Transformation (TNO2A25)
W271/2	12076	<i>prcA, pyrG89</i>	WIM27 x WIM27UPR24
W28UR2/5	12077	<i>prcA, prcB, pyrG89, riboB2</i>	WIM28 x WIM27UPR24
	12096	W27PNG15/MSF diploid	12016/58
PUTA29-	12097	<i>yA2, alcA(P)-brlA, pabaA1, pyrG89, nkuAΔ(bar), veA1</i>	Transformation (pTA29 -> PPU-)
PUTA29+	12098	<i>yA2, alcA(P)-brlA, pabaA1, pyrG89, nkuAΔ(bar)</i>	Transformation (pTA29 -> PPU+)
WIM126 5-AC	12199	<i>yA2, pabaA1</i>	5-azacytidine treatment (WIM126)
W28A103/4	12200	<i>yA1, niiA4, prcB, veA1</i>	WIM28 x MUS51BARA10
dmrAΔ	12209	<i>yA2 pabaA1 pyroA4 pyrG89 nkuAΔ(bar) dmrAΔ(Ncr pyr-4)</i>	Transformation (PPU+)
AN1060ΔPV+	12211	<i>pabaA1 pyrG89, kdmAΔ(Nc pyr-4)</i>	AN1060K012 x WIM126
csnEΔ13	12212	<i>yA2, pabaA1, csnEΔ(Nc pyr-4), pyroA4, nkuAΔ</i>	Transformation (PPU+)
RJW75.2	12270	<i>pyroA4 stcEΔ bre2Δ(Af pyroA) veA1</i>	N. P. Keller/J. Strauss
RJW77.3	12271	<i>pyroA4 stcEΔ lsdAΔ(Af pyroA) veA1</i>	N. P. Keller/J. Strauss
RJW80.5	12272	<i>pyroA4 stcEΔ rtf1Δ(Af pyroA) veA1</i>	N. P. Keller/J. Strauss
RJW89.4	12273	<i>pyroA4 stcEΔ cdc73Δ(Af pyroA) veA1</i>	N. P. Keller/J. Strauss
AN1060xP10	12289	<i>yA::[pLN7807] pyrG89, pyroA4, veA1</i>	Transformation (pLN7807 -> MUS51BARB12)
AN1060xP11	12290	<i>pyrG89, pyroA4, veA1 AN1060::[pLN7807]</i>	Transformation (pLN7807 -> MUS51BARB12)
LO1946	12324	<i>pyrG89, pyroA4, hhoA-GFP(Af pyroA), veA1 riboB2</i>	Oakley Lab. - LO1946
PRENB16	12422	<i>wA3, niiA4, gatA312, prcA, veA1</i>	WIM27 x RSB12
kdmAGFP16	12465	<i>pyrG89, hhoA-GFP(Af pyroA), kdmAΔ(Nc pyr-4)</i>	KOPV+ x LO1946
	12470	PRENB16/MSF diploid	12422/58
PBNB10	12489	<i>wA3, prcA, prcB, niiA4, biA1</i>	RSB12 x WIM28
WH1GFP6	12508	<i>pabaA1, hhoA-GFP(Af pyroA)</i>	WIM126 x LO1946
PAH1GFP5	12509	<i>prcA, histone H1-GFP(Af pyroA), pyroA?, nkuA?</i>	WIM27 x LO1946
PABH1GFP1	12510	<i>prcA, prcB, hhoA-GFP(Af pyroA), pyroA?, nkuA?</i>	WIM28 x LO1946
csnEH1GFP7	12515	<i>csnEΔ(Nc pyr-4), hhoA-GFP(Af pyroA), pyroA?, nkuA?</i>	csnEKO7 x LO1946
	12516	PBNB10/MSF diploid	12489/58
PABH13	12595	<i>prcA, prcB, hhoA-GFP(Af pyroA)</i>	PABH1GFP1 x WIM126
xPkdm+	12600	<i>pabaA1 pyrG89, AN1060::[pLN7807]</i>	AN1060xP11x WIM126
AN0888Δ	12632	<i>biA1, wA3, argB2, pyroA4, AN0888Δ(argB) veA1 riboB2</i>	J. Strauss
AN8211Δ	12633	<i>pabaA1 pyrG89, AN8211Δ(riboB), argB2, nkuAΔ(argB), veA1 riboB2</i>	J. Strauss
aodAΔ	12650	<i>nkuAΔ(argB), AN2099Δ(Af pyroA), veA1 niiA4</i>	Hynes (unpublished)
xPkdmAPU13	12809	<i>yA2 pabaA1 pyrG89, AN1060::[pLN7807]</i>	xPkdm+ x WIM126
YPU16	12810	<i>yA2 pabaA1 pyrG89</i>	xPkdm+ x WIM126
1Δ11-1	12915	<i>AN1997-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
2F7-1	12916	<i>AN9060-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
1E3-1	12917	<i>AN2012-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
1Δ9-3	12918	<i>AN1944-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
2Δ1-1	12919	<i>AN7170-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
2F1-1	12920	<i>AN8636-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
2E7-2	12921	<i>AN7873-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
1F9-2	12922	<i>AN3280-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)
1A11-1	12923	<i>AN0785-Af pyroA (TNO2A21 background)</i>	Transformation (disruption -> TNO2A21)

Name	Strain ID	Genotype	Source
2G3-1	12924	AN9397-Af pyroA (TNO2A21 background)	Transformation (disruption -> TNO2A21)
2F9-2	12925	AN9090-Af pyroA (TNO2A21 background)	Transformation (disruption -> TNO2A21)
2F9-1	12926	AN9090-Af pyroA (TNO2A21 background)	Transformation (disruption -> TNO2A21)
1F3-1	12927	AN2893-Af pyroA (TNO2A21 background)	Transformation (disruption -> TNO2A21)
1F7-1	12928	AN3075-Af pyroA (TNO2A21 background)	Transformation (disruption -> TNO2A21)
2-2	12929	AN1569-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
3-2	12930	AN2001-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
3-6	12931	AN2001-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
4-5	12932	AN2036-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
5-2	12933	AN3075-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
5-5	12934	AN3075-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
7-1	12935	AN3433-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
8-5	12936	AN5775-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
8-9	12937	AN5775-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
9-1	12938	AN5870-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
9-2	12939	AN5870-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
9-3	12940	AN5870-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
11-1	12941	AN7896-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
11-2	12942	AN7896-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
12-1	12943	AN9147-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
13-5	12944	AN10906-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
19-2	12945	AN9060-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
19-3	12946	AN9060-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
21-1	12947	AN4878-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
21-2	12948	AN4878-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
21-4	12949	AN4878-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
22-1	12950	AN5209-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
22-2	12951	AN5209-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
22-4	12952	AN5209-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
48-1	12953	AN2893-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
48-3	12954	AN2893-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
54-2	12955	AN3688-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
54-11	12956	AN3688-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
76-1	12957	AN7170-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
76-6	12958	AN7170-Af pyroA (PPU+ background)	Transformation (deletion -> PPU+)
25-5	12959	AN0307-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
33-2	12960	AN1170-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
32-1	12961	AN1160-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
15-1	12962	AN0709-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
54-1	12963	AN3688-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
16-4	12964	AN1875-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
77-2	12965	AN7173-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
33-1	12966	AN1170-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
34-2	12967	AN1267-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
34-1	12968	AN1267-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
27-6	12969	AN0557-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
75-1	12970	AN7169-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
58-1	12971	AN4051-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
35-1	12972	AN1338-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
77-1	12973	AN7173-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
75-4	12974	AN7169-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
54-2	12975	AN3688-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
64-1	12976	AN5130-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
55-1	12977	AN3799-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
16-3	12978	AN1875-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
55-4	12979	AN3799-Af pyroA (MUS51BARB12 background)	Transformation (deletion -> MUS51BARB12)
PCV9	12980	prcC, yA2, pabaA1, pyroA4, pyrG89, nkuAΔ	Mutagenesis (PUTA29+)

File S1: Effect of temperature on competence, Figure 2A. (.txt, 3 KB)

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File S2: Effect of inoculum density on competence, Figure 2B. (.txt, 5 KB)

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File S3: Effect of pH on competence, Figure 2C. (.txt, 4 KB)

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File S4: Processed gene expression data, Figure 3A. (.txt, 5 MB)

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File S5: Model selection data, Figure 3B. (.txt, 904 KB)

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File S6: prcA and prcA; prcB outcross competence data, Figure 4. (.txt, 12 KB)

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File S7: rasB assay data, Figure 5A-B. (.txt, 6 KB)

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File S8: msnA and ahpA assay data, Figure 5C. (.txt, 2 KB)

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