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Telomere position effect is regulated by heterochromatin-associated proteins and NkuA in *Aspergillus nidulans*

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Gene-silencing mechanisms are being shown to be associated with an increasing number of fungal developmental processes. Telomere position effect (TPE) is a eukaryotic phenomenon resulting in gene repression in areas immediately adjacent to telomere caps. Here, TPE is shown to regulate expression of transgenes on the left arm of chromosome III and the right arm of chromosome VI in *Aspergillus nidulans*. Phenotypes found to be associated with transgene repression included reduction in radial growth and the absence of sexual spores; however, these pleiotropic phenotypes were remedied when cultures were grown on media with appropriate supplementation. Simple radial growth and ascosporogenesis assays provided insights into the mechanism of TPE, including a means to determine its extent. These experiments revealed that the KU70 homologue (NkuA) and the heterochromatin-associated proteins HepA, ClrD and HdaA were partially required for transgene silencing. This study indicates that TPE extends at least 30 kb on chromosome III, suggesting that this phenomenon may be important for gene regulation in subtelomeric regions of *A. nidulans*.

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INTRODUCTION

In recent years, research in fungal biology has provided a view that several gene-silencing strategies are important in organismal development. There are the classic studies of silent mating-type loci (HML and HMR) in budding yeast (Laurenson & Rine, 1992), RNAi-mediated heterochromatin formation in the fission yeast *Schizosaccharomyces pombe* (Bühler & Moazed, 2007), and telomere position effect (TPE) in several fungi including *Saccharomyces cerevisiae*, *Candida* spp. and *Neurospora crassa* (Castaño *et al.*, 2005; Gottschling *et al.*, 1990; Rosas-Hernández *et al.*, 2008; Smith *et al.*, 2008). One of the common mechanisms underlying this form of transcriptional silencing has been shown to be chromatin-level control, a process that involves repositioning of nucleosomes and/or post-translational modifications of histone tail residues, which function to alter the availability of DNA to transcriptional machinery (reviewed by Grewal & Jia, 2007; Jenuwein & Allis, 2001).

Possibly the best-understood silencing phenomenon is TPE, as it has been demonstrated in several organisms from yeast to humans despite variable chromatin structure among organisms. For example, yeast heterochromatin consists of Sir-protein complexes while in higher eukaryotes

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Abbreviations: AspGD, *Aspergillus* Genome Database; TPE, telomere position effect; UU, uracil and uridine.

A supplementary table of primers and three supplementary figures are available with the online version of this paper.

heterochromatin is composed of histone H3 methylated at lysine 9 and heterochromatin protein 1 (Schoeftner & Blasco, 2009). These differing systems of heterochromatic silencing utilize different proteins, yet the underlying similarity is that chromatin is compacted in areas that are transcriptionally silent. Moreover, several of the TPE-regulated genes have important functions for their respective organisms. For example, a subset of the *FLO* genes of *S. cerevisiae* involved with adhering to surfaces are located near telomeres and thus regulated under this mechanism (Barrales *et al.*, 2008). Additionally, a polygalacturonase gene (*PGU1*) is located ~25 kb from a yeast telomere and thus subject to epigenetic regulation (Louw *et al.*, 2010). In *Candida glabrata* binding to human cells is a key factor for pathogenicity, and adhesion depends on the telomerically located adhesins, which in turn are regulated by telomeric silencing (Castaño *et al.*, 2005). TPE has also been shown to be variable at yeast telomeres, suggesting that not all telomeres have the same silencing capacity (Mondoux & Zakian, 2007).

Filamentous fungi have also been shown to have chromosomal location effects (Greenstein *et al.*, 2006; Palmer & Keller, 2010; Robellet *et al.*, 2010); however, telomeric silencing mechanisms have thus far only been studied in *N. crassa* (Smith *et al.*, 2008). Positional effects have been studied genetically through utilization of null mutants in chromatin-associated enzymes, and several of these mutants have been shown to have various pleiotropic effects in *N. crassa* and *Aspergillus fumigatus*, indicating that primary metabolism may be regulated by this mechanism (Adhvaryu *et al.*, 2005; Freitag *et al.*, 2004; Palmer *et al.*, 2008; Tamaru & Selker, 2001; Tamaru *et al.*, 2003). Secondary-metabolite gene clusters are also partially regulated by an altered chromatin landscape; specifically a histone deacetylase (HdaA), the H3K9 methyltransferase (ClrD) and heterochromatin protein 1 (HepA) are involved in regulation of the sterigmatocystin and penicillin gene clusters (Reyes-Dominguez *et al.*, 2010; Shwab *et al.*, 2007), while CclA, a Bre2 orthologue involved in histone 3 lysine 4 methylation, is implicated in silencing of the newly identified emodin and F9775A/B gene clusters (Bok *et al.*, 2009).

Here we present evidence for TPE regulation in *A. nidulans*. We show that transgenes located within 20 kb of the telomere of the left arm of chromosome III in *A. nidulans* are repressed and that silencing extends at least to the SpoC1 cluster boundary (~30 kb distal from the telomere). Additionally, we describe TPE at the right arm of chromosome VI near the penicillin gene cluster. NkuA (a KU70 homologue) has a role in TPE, as a null NkuA mutant partially relieves silencing. In addition, proteins involved in heterochromatin maintenance are involved in TPE, as partial derepression was observed in null mutant backgrounds of HepA, ClrD and HdaA.

METHODS

General. Fungal strains used in this study are listed in Table 1; all primers used are listed in Supplementary Table S1, available with the online version of this paper. All strains were maintained on glucose minimal medium (GMM) (Shimizu & Keller, 2001) at 37 °C and when appropriate were supplemented with 1.2 g uracil l^{-1}, 1.2 g uridine l^{-1}, 0.5 μM pyridoxine.HCl, 2.5 μM riboflavin.HCl, 100 μM arginine and 1 μM *p*-aminobenzoate. *In silico* analysis was done using the *Aspergillus* Genome Database, AspGD (http://www.aspergillusgenome.org), and all locus identification numbers correspond to the current annotation of AspGD (Arnaud *et al.*, 2010).

Creation of fungal strains. Gene replacement mutants were generated by transformation of suitable recipient strains with gene replacement cassettes constructed using fusion PCR (Szewczyk *et al.*, 2006; Yang *et al.*, 2004). Transformation was done essentially as in Miller *et al.* (1985), with the exception of embedding protoplasts in top agar (0.75 %). For example, AN5091 was disrupted with a PCR product consisting of a 0.88 kb upstream flanking region, a 0.92 kb downstream flanking region, and a 1.97 kb PCR fragment of the *A. fumigatus pyrG* gene as a marker gene to create TJMP6.9. All original mutants were created essentially in the same fashion according to the following: TDP1-1, 1-2, 2-7 and 2-12 (ΔAN5092::Af*pyrG*), TSM18-3 (ΔAN5092::*Gryp*fA), TMM11 (ΔAN5495::Af*pyrG*), TSM11-3, 11-4 and 11-10 (ΔAN5092::Af*pyroA*) and TSM3-1 (ΔAN4432::Af*pyroA*) were created in a TN02A7 background, and TJMP6.9 (ΔAN5091::Af*pyrG*) was created in the RJMP1.49 background. Replacement of the target sequence with the selectable marker was confirmed by Southern blot analysis and by PCR using primers lying outside the gene replacement cassettes. Prototrophic strains were constructed by independently crossing TJMP6.9 and TDP1-1 to RJMP101.5 to construct RJMP115.3 and RJMP116.3. TJMP16.1 was constructed by transformation of TJMP6.9 with pJW53 harbouring the *A. nidulans pyroA* gene (Tsitsigiannis *et al.*, 2004). Crossing RJMP1.35 with SMC73.1-105 created RJMP121.7 and RJMP121.4, while RJMP122.6, RJMP123.3, RJMP125.20 and RJMP127.4 were created by independently crossing RJMP1.59 with SMC72-1, SMC73.1-5, SMC58-7 and SMC79-13 respectively. Finally, crosses between TJMP50.3 and SMC58-7 yielded RJMP135.11, and TJMP50.3 and SMC79-13 yielded RJMP131.7.

Semi-quantitative reverse transcriptase PCR. Equal numbers of conidia from all strains were inoculated in YSC medium (Lies *et al.*, 1998) containing uracil and uridine (UU) and incubated at 30 °C overnight. Cells were harvested, frozen in liquid nitrogen, and total RNA was isolated using the procedure of Timberlake (1980). Ten micrograms of total RNA was used to make single-stranded cDNA using the Superscript III kit and the protocol recommended by the manufacturer (Invitrogen). Serial 1 : 10 dilutions of single-stranded cDNA were used as template in PCRs, and equal volumes of the PCRs were separated on a 1 % (w/v) agarose gel. The gel was stained with ethidium bromide and imaged using a Typhoon 8600 scanner. The intensity of the bands was quantified using ImageQuant software. The band intensity of each Af*pyrG* PCR product was divided by the actin band intensity from the same dilution to give an expression ratio Af*pyrG*/actin for each sample. The Af*pyrG*/actin ratios from the 0.1 × cDNA reactions for each TDP sample were divided by the ratio from the TMM11 control to give a relative expression level for each sample.

Physiology experiments. Prototrophic strains in a wild-type *veA* (*veA*$^{+}$) genetic background were used for radial growth measurements and quantification of ascospores. Radial growth assays were conducted at 37 °C by measuring colony diameter after 3 days on plates of solid medium [GMM and GMM with appropriate supplementation (uridine and uracil, UU; pyridoxine, P)] that were centrally point-inoculated with ~2000 spores. Quantification of ascospores was done on overlay-inoculated cultures that were set up by pipetting 1×10^6 conidia into 0.75 % molten agar that was subsequently poured over 1.5 % solid agar in Petri dishes. Cultures were incubated at 37 °C in the dark for 5–7 days and agar cores were taken from the plates with a 1.2 cm cork

Table 1. Strains used in this study

Strain	Genotype	Source
TN02A7	*pyrG89 pyroA4 riboB2* Δ*nkuA*::*argB veA1*	Nayak *et al.* (2006)
TDP1-1	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyrG* Δ*nkuA*::*argB veA1*	This study
TDP1-2	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyrG* Δ*nkuA*::*argB veA1*	This study
TDP2-7	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyrG* Δ*nkuA*::*argB veA1*	This study
TDP2-12	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyrG* Δ*nkuA*::*argB veA1*	This study
TMM11	*pyrG89 pyroA4 riboB2* ΔAN5495::Af*pyrG* Δ*nkuA*::*argB veA1*	This study
TSM18-3	*pyrG89 pyroA4 riboB2* ΔAN5092::*Grypf*A Δ*nkuA*::*argB veA1*	This study
TSM11-3	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyroA* Δ*nkuA*::*argB veA1*	This study
TSM11-4	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyroA* Δ*nkuA*::*argB veA1*	This study
TSM11-10	*pyrG89 pyroA4 riboB2* ΔAN5092::Af*pyroA* Δ*nkuA*::*argB veA1*	This study
TSM3-1	*pyrG89 pyroA4 riboB2* ΔAN4432::Af*pyroA* Δ*nkuA*::*argB veA1*	This study
RJMP1.49	*pyrG89 pyroA4* Δ*nkuA*::*argB*	Shaaban *et al.* (2010)
TJMP6.9	*pyrG89 pyroA4* ΔAN5091::Af*pyrG* Δ*nkuA*::*argB*	This study
RJMP101.5	*pyrG89 wA3*	This study
RJMP115.3	*pyrG89* ΔAN5091::Af*pyrG*	This study
RJMP116.3	*pyrG89* ΔAN5092::Af*pyrG*	This study
TJMP45.2	Δ*nkuA*::*argB*	Shaaban *et al.* (2010)
TJMP16.1	*pyrG89* ΔAN5091::Af*pyrG* Δ*nkuA*::*argB*	This study
RJMP1.19	*pyroA4* Δ*nkuA*::*argB*	Shaaban *et al.* (2010)
TMSII2.4	*pyroA4 pyrG89* ΔPbII::Ap*pyrG* Δ*nkuA*::*argB*	Shaaban *et al.* (2010)
TMS8.2	*pyroA4 pyrG89 Ap pyrG* Δ*nkuA*::*argB*	M. Shaaban & N. P. Keller, unpublished
SMC58-7	ΔAN5092::Af*pyroA pyroA4* Δ*clrD*::Af*pyrG pyrG89 argB2 yA2 veA1*	This study
SMC73.1-5	*riboB2* ΔAN5092::Af*pyroA pyroA4* Δ*hdaA*::Af*pyrG pyrG89 veA1*	This study
SMC72-1	*pyrG89* Δ*hepA*::Af*pyrG* ΔAN5092::Af*pyro pyroA4 riboB2 wA2 yA2 veA1*	This study
SMC73.1-105	ΔAN5092::Af*pyroA pyroA4 pyrG89 argB2 riboB2 wA2 veA1*	This study
SMC79-13	*pyrG89* Δ*hstA*::*argB* ΔAN5092::Af*pyro pyroA4 riboB2 pabaA1 yA2 veA1*	This study
RJMP1.59	*pyrG89 pyroA4*	This study
TJMP50.3	*pyrG89*	This study
RJMP1.35	*pyroA4 trpC801*	This study
RDIT9.32	Wild-type	Tsitsigiannis *et al.* (2004)
RJMP121.7	*pyroA4* ΔAN5092::Af*pyroA*	This study
RJMP121.4	*pyroA4* ΔAN5092::Af*pyroA veA1*	This study
RJMP122.6	*pyroA4* ΔAN5092::Af*pyroA* Δ*hepA*::Af*pyrG*	This study
RJW110.4	Δ*hepA*::Af*pyrG*	Reyes-Dominguez *et al.* (2010)
RJMP123.3	*pyroA4* ΔAN5092::Af*pyroA* Δ*hdaA*::Af*pyrG*	This study
RMS1.22	Δ*hdaA*::Af*pyrG*	Shaaban *et al.* (2010)
RJMP125.20	*pyroA4* ΔAN5092::Af*pyroA* Δ*clrD*::Af*pyrG*	This study
RJMP135.11	Δ*clrD*::Af*pyrG*	This study
RJMP127.4	*pyroA4* ΔAN5092::Af*pyroA* Δ*hstA*::*argB*	This study
RJMP131.7	Δ*hstA*::*argB*	This study

borer. This method of inoculation allows cleistothecia to develop evenly across the Petri dish. Ascospores were quantified using a haemocytometer and represented as ascospores per square millimetre. All experiments were completed at least in triplicate and statistical analysis was done using Prism 5 software.

RESULTS

Discovery of TPE at the left arm of chromosome III

We were originally interested in AN5092 because of its putative function assigned by the genome annotation. AN5092 is predicted to encode a full-length RecQ protein, which is an orthologue of the telomere-linked helicase (TLH) gene family described in *Magnaporthe oryzae* (Rehmeyer *et al.*, 2009). TLH genes are found in close proximity to telomeres of some species of filamentous fungi and are similar in sequence to telomerically located Y′ elements in *S. cerevisiae* (Rehmeyer *et al.*, 2006). Analysis of TLH-related DNA sequences in *A. nidulans* revealed that they were present near the ends of six chromosomes (Clutterbuck & Farman, 2008); however, only AN5092 appears to be full-length according to the criteria of Rehmeyer *et al.* (2009).

To investigate the role of AN5092, we replaced the ORF with the *A. fumigatus pyrG* gene (Af*pyrG*), which complements the uracil/uridine auxotrophy conferred by *pyrG89*. A Δ*nkuA* genetic background (TN02A7) was used to increase the likelihood of obtaining the desired mutant (Nayak *et al.*,

2006). Four of thirteen transformants screened by Southern blot and PCR analyses had simple gene replacements (AN5092::Af*pyrG*). All four strains (TDP1-1, 1-2, 2-7 and 2-12) produced smaller colonies on minimal medium (GMM) than control transformants, where Af*pyrG* was used to replace the nonessential, non-telomerically located gene AN5495 (Fig. 1a). We hypothesized that partial repression of the Af*pyrG* gene could be responsible for this phenotype. Consistent with this idea, the colony size defect of all four AN5092::Af*pyrG* strains was completely relieved by supplementing the media with uracil and uridine (UU) (Fig. 1a). Moreover, we have used Af*pyrG* for gene replacements at several other locations in the genome without observing a growth defect (data not shown). We therefore attributed the growth defect of the TDP strains to partial uracil auxotrophy, not the loss of the ORF corresponding to AN5092.

The partial uracil auxotrophy of the TDP strains suggested that expression of Af*pyrG* is reduced in these strains compared to controls. To test this, we analysed Af*pyrG* mRNA levels in our strains by semiquantitative RT-PCR. Fig. 1(b) shows that expression of the Af*pyrG* transgene is reduced by approximately one-half in TDP strains compared to a control strain. These results indicated that the reduced colony growth rate of TDP strains is likely due to reduced levels of Af*pyrG* expression.

Repression is independent of transgene or transgene orientation

To determine whether the orientation of the transgene at AN5092 is required for the reduced expression described above, we repeated the deletion of AN5092 using a construct in which the direction of Af*pyrG* transcription was opposite to that in the AN5092::Af*pyrG* strains (schematically drawn in Fig. 2a). A transformant (TSM18-3) was obtained and confirmed by PCR and Southern analysis (Fig. 2b). Like TDP1-1, the AN5092::*Grypf*A strain produced small colonies on minimal medium and normal-sized colonies on medium containing UU (data not shown). Thus, a specific orientation of the transgene inserted near the telomere of chromosome III was not required for silencing.

Additionally, we reasoned that the growth defect could be specific to uracil metabolism; thus, we repeated the deletion of AN5092 using an alternative selectable marker, *A. fumigatus pyroA (*Af*pyroA)*. Three strains (TSM11-3, 11-4 and 11-10) were isolated and confirmed by PCR and Southern analysis (Fig. 2b). The nonessential gene AN4432 (centrally located in chromosome III) was disrupted with Af*pyroA* and served as a control strain (data not shown). All three strains (TSM11-3, 11-4 and 11-10) produced small colonies on medium lacking pyridoxine and the growth defect was remedied by addition of pyridoxine to the medium, whereas the control strain produced normal-sized colonies on both media (Supplementary Fig. S1).

In silico analysis of the telomere-proximal left arm of chromosome III

To gain potential insight into the silencing of the transgene located at AN5092, we employed an *in silico* approach to determine the chromosomal context of AN5092. Using the AspGD (http://www.aspergillusgenome.org), we determined

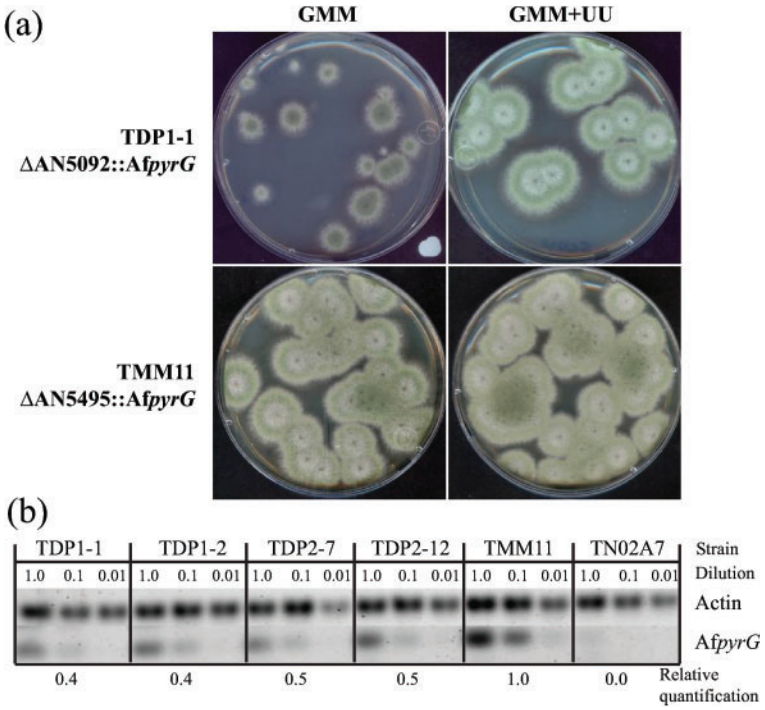

Fig. 1. (a) Gene replacement of AN5092 with the *A. fumigatus pyrG* gene resulted in a strain with visible growth defects on minimal medium compared to a control strain (TMM11). The observed phenotype was remedied when cultures were grown on medium containing the appropriate supplements, in this case uridine and uracil. (b) The growth reduction on minimal medium correlates with repression of the Af*pyrG* transgene located at AN5092. The control strain (TMM11) contains Af*pyrG* integrated at the nonessential gene AN5495. Semiquantitative RT-PCR revealed that relative Af*pyrG* expression levels were 0.4 for TDP1-1 and TDP1-2 and 0.5 for TDP2-7 and TDP2-12.

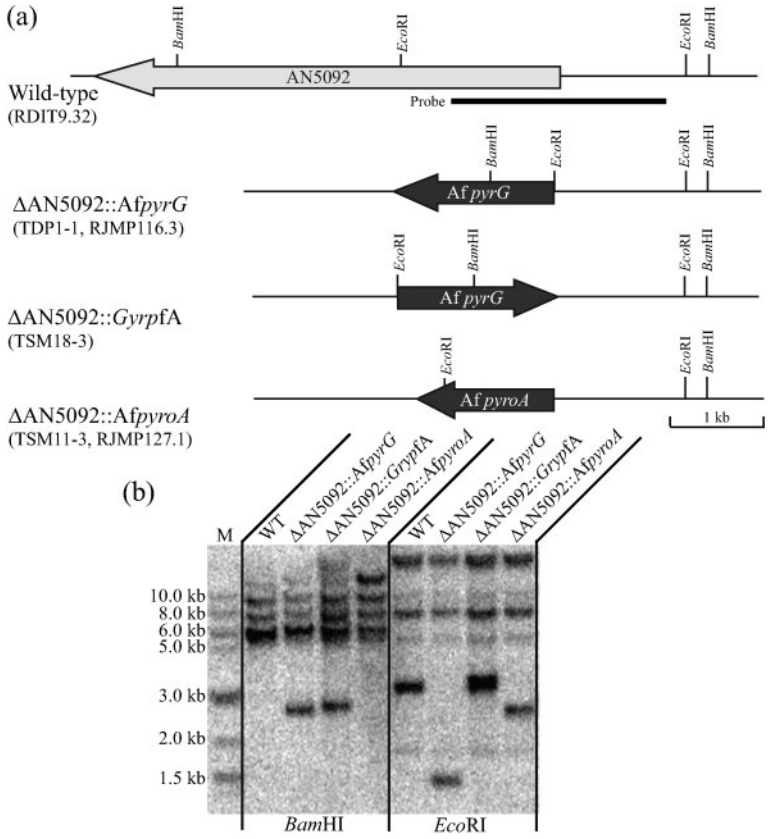

Fig. 2. Schematic of gene disruption strategies (a) and Southern analysis of mutants constructed at the AN5092 locus (b). The AN5092 locus was replaced with three constructs: *A. fumigatus pyrG*, *A. fumigatus pyrG* in the opposite orientation (*Grypf*A), and the *A. fumigatus pyroA* gene. Due to the repetitive nature of TLH-like sequences, Southern blots produced a considerable amount of background. Strains used for Southern blots were: WT, RDIT9.32; ΔAN5092::Af*pyrG*, RJMP116.3; ΔAN5092::*Grypf*A, TSM18-3; and ΔAN5092::Af*pyroA*, RJMP121.7. Using a radiolabelled probe corresponding to a portion of the AN5092 locus and the 5′ flanking region, in a *Bam*HI digestion the following bands were expected: WT, 5.6 kb; ΔAN5092::Af*pyrG*, 2.6 kb; ΔAN5092::*Grypf*A, 2.8 kb; and ΔAN5092::Af*pyroA*, >10 kb. An *Eco*RI digestion predicted WT, 3.0 kb; ΔAN5092::Af*pyrG*, 1.4 kb; ΔAN5092::*Grypf*A, 3.2 kb; and ΔAN5092::Af*pyroA*, 2.5 kb.

that AN5092 is located ~2 kb from the end of chromosome III. According to Clutterbuck & Farman (2008), there is a sequence gap of 18.4 kb between the telomere cap and the genome annotation. Taken together, these analyses place AN5092 approximately 20 kb from the telomere of chromosome III (Fig. 3). Further analysis revealed the presence of the SpoC1 cluster telomere distal to AN5092. The SpoC1 cluster, described by Gwynne *et al.* (1984), is a 38 kb cluster of developmentally regulated transcripts, which are flanked by two 1.1 kb repetitive sequences (RPT3). While two of the SpoC1 cluster genes are annotated in the AspGD genome sequence, the remaining genes are absent. Our *in silico* analysis indicates that the RPT3 flanking sequences of the SpoC1 cluster are located near the current annotation of AN5091 and AN5081. Expanding on these data, analysis of the orientation of genes presented by Gwynne *et al.* (1984) matches perfectly with the annotation found in the AspGD. Therefore we conclude that the SpoC1 cluster corresponds to a 38 kb stretch located on chromosome III and the boundary is located 30 kb from the telomere (Fig. 3). In addition to AN5092 there are two putative ORFS located between the SpoC1 cluster and the end of chromosome III. AN5093 is likely not a functional gene as it is composed of repetitive DNA sequence, and AN5091 encodes a putative methyltransferase with some similarity to LaeA, a protein involved in regulation of secondary metabolite gene clusters (Bok & Keller, 2004).

Repression extends at least to the boundary of the SpoC1 cluster

Via Northern analysis, we were unable to detect expression during a developmental time-course in a wild-type strain (RDIT9.32) of the putative telomere-distal gene AN5091, which is more than 30 kb from the telomere (Fig. 3, Supplementary Fig. S2). This indicated that silencing might extend beyond the AN5092 locus. In a similar fashion to disruption of AN5092, the AN5091 locus was replaced with the Af*pyrG* gene and the replacement confirmed by Southern analysis (Fig. 4). The resulting phenotype was nearly identical to that of the AN5092::Af*pyrG* mutant, with transformants requiring UU supplementation for normal growth and ascosporogenesis (Fig. 5a, b). Taken together, our data suggest that TPE functions regardless of the transgene or orientation, and extends at least to the SpoC1 cluster boundary.

TPE can be assayed via radial growth and ascospore production

In order to study TPE in prototrophic strains and a wild-type background, we attempted to outcross TDP1-1 (AN5092::Af*pyrG*). Heterokaryons were formed and selected on minimal medium, and cleistothecia were allowed to form. All cleistothecia examined from the attempted outcross were devoid of ascospores. Outcrosses of the other three AN5092::Af*pyrG* mutants gave the same

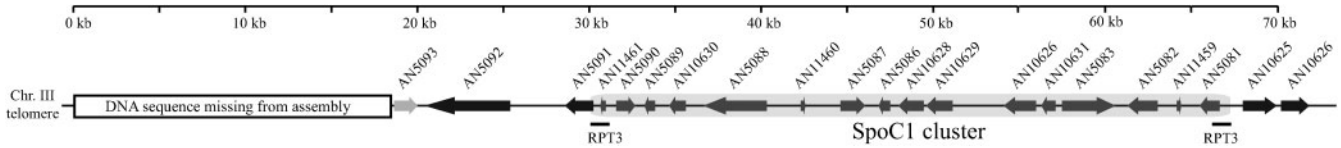

Fig. 3. Scale schematic representation of the left telomere of chromosome III of *A. nidulans*. AN5092 is located ~20 kb from the telomere of the left arm of chromosome III. Clutterbuck & Farman (2008) describe that 18.4 kb of DNA is missing between the telomere of chromosome III and the genome assembly. Bioinformatic analysis places the SpoC1 cluster approximately 30 kb from the telomere of chromosome III, spanning the current annotation from AN5091 to AN5081. Three putative open reading frames exist between the SpoC1 cluster and the end of the current annotation in the AspGD: AN5093 is unlikely to be a functional gene as it contains repetitive sequences, AN5092 is a putative TLH-like gene, and AN5091 encodes a methyltransferase with sequence homology to LaeA.

result. Since TDP1-1 required UU for normal vegetative growth and the transgene located at AN5092 was the only functional *pyrG* gene in the cross, we hypothesized that repression of Af*pyrG* was responsible for the ascosporogenesis defect. Indeed, supplementing the medium with UU remedied the ascosporogenesis defect. Analogous results were obtained with crosses involving TSM11 strains, in which Af*pyroA* at AN5092 was the only functional *pyroA* gene. These crosses produced ascospores only when pyridoxine was included in the medium. Thus, the limited expression of the transgene remedying a nutritional deficiency prevents ascosporogenesis.

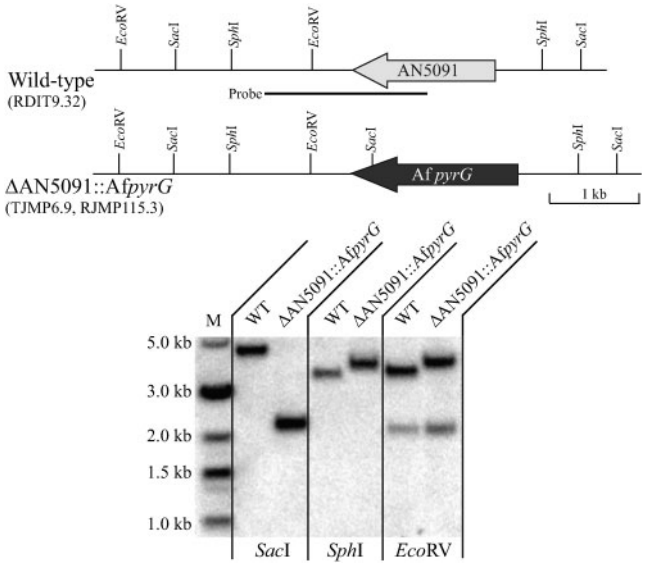

Fig. 4. The AN5091 locus was replaced with the *A. fumigatus pyrG* gene as drawn schematically. Strains were confirmed by Southern analysis. Strains used were WT (RDIT9.32) and ΔAN5091 : : Af*pyrG* (RJMP115.3). A *Sac*I digestion predicted bands of WT 4.5 kb and ΔAN5091 : : Af*pyrG* 2.2 kb; a *Sph*I digestion predicted WT 3.4 kb and ΔAN5091 : : Af*pyrG* 4.0 kb; while an *Eco*RV digestion predicted WT 3.4 kb + 2.1 kb and ΔAN5091 : : Af*pyrG* 4.0 kb + 2.1 kb.

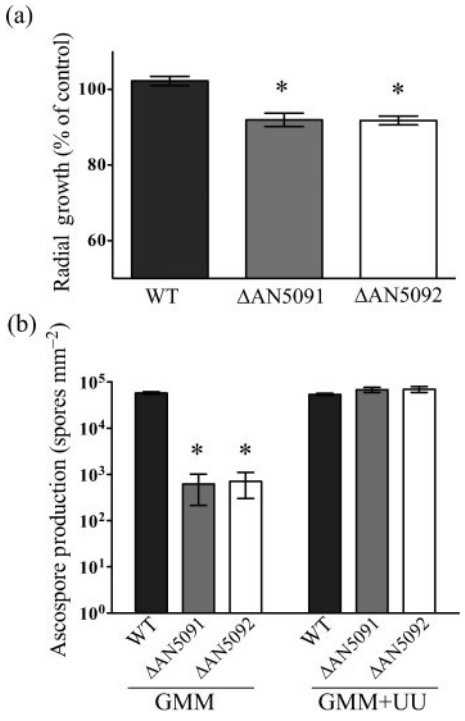

Fig. 5. (a) Prototrophic strains exhibiting TPE display a quantifiable growth defect on minimal medium versus minimal medium with supplements. Integration of Af*pyrG* at either AN5092 or AN5091 results in the same phenotype, suggesting that TPE extends at least 30 kb from the telomere. WT, RDIT9.32; ΔAN5091, RJMP115.3; ΔAN5092, RJMP116.3. (b) Sexual development is aberrant in TPE strains. Macroscopic cleistothecial development is unaffected by integration of Af*pyrG* at either the AN5092 or the AN5091 locus; however, ascospore production is severely debilitated. Quantification of ascosporogenesis indicates that TPE strains produce three orders of magnitude fewer ascospores when grown on minimal medium compared to medium with appropriate supplements. WT, RDIT9.32; ΔAN5091, RJMP115.3; and ΔAN5092, RJMP116.3. Note that the *y*-axis scale is logarithmic. Means ± SD are plotted; asterisks indicate statistically significant differences between wild-type and other strains ($P<0.001$ using Student's *t*-test).

Most laboratory strains of *A. nidulans* harbour the *veA1* allele, which makes them essentially blind to light so they produce asexual conidia regardless of whether grown in the light or dark, with limited sexual development. Therefore, to further examine TPE in the context of sexual development we used wild-type (veA^{+}) strains. TPE repression of Af*pyrG* at the AN5092 locus (RJMP116.3) reduced production of ascospores by approximately three orders of magnitude on minimal medium compared to medium supplemented with UU (GMM=618±405 ascospores mm^{-2}; GMM+UU=68 083±8527 ascospores mm^{-2}) (Fig. 5b). In order to confirm that silencing of transgenes was solely responsible for the observed pleiotropic phenotypes, we grew a *pyroA4* mutant (RJMP1.59) on medium with decreasing concentrations of pyridoxine and were able to replicate the reduction in radial growth and loss of ascospore production observed in the AN5092::Af*pyroA* strain (Supplementary Fig. S3).

TPE exists at the right arm of chromosome VI

A recent study aimed at assessing the role of repetitive sequences flanking the penicillin gene cluster resulted in creation of a mutant with a transgene integrated in close proximity to the telomere of chromosome VI (Shaaban *et al.*, 2010). In this mutant (ΔPbII, TMSII2.4), a 30 kb piece of DNA ~8.5 kb from the telomere was replaced with the *A. parasiticus pyrG* gene (Shaaban *et al.*, 2010). Thus this strain allowed us to assay putative TPE silencing effects at chromosome VI. Similar to results from analysis of transgene repression on chromosome III, a reduction in radial growth and ascospore production was quantifiable and was partially remedied by appropriate supplementation of the medium (Fig. 6a, b). A growth defect remains in this strain with UU supplementation, hypothesized to be due to loss of uncharacterized ORFs (Shaaban *et al.*, 2010).

Heterochromatin-associated proteins and NkuA are involved in TPE

Several proteins have been shown to be required for TPE in other organisms, including KU70/80 (NkuA/B) proteins (Boulton & Jackson, 1998; Mishra & Shore, 1999; Rosas-Hernández *et al.*, 2008), as well as heterochromatin protein 1, the lysine 9 histone 3 methyltransferase, and histone deacetylases (Ottaviani *et al.*, 2008). By utilizing radial growth and ascosporogenesis assays we were able to elucidate the involvement of several heterochromatin-associated proteins (HepA, ClrD, HdaA and HstA) in TPE at chromosome III by creating double mutants of AN5092::Af*pyroA* and null mutants of HepA, ClrD, HdaA or HstA. Fig. 7 shows that both radial growth and ascosporogensis were identical to wild-type in the single chromatin mutants but were partially derepressed in HepA, ClrD and HdaA AN5092::Af*pyroA* double mutants, thus implicating all three of these proteins as players in TPE regulation. HdaA, a histone deacetylase, had the strongest derepressive effect. However the Δ*hstA* AN5092::Af*pyroA* double mutant did not show increased radial growth or ascospore production compared to the control strain (Fig. 7a, b, c).

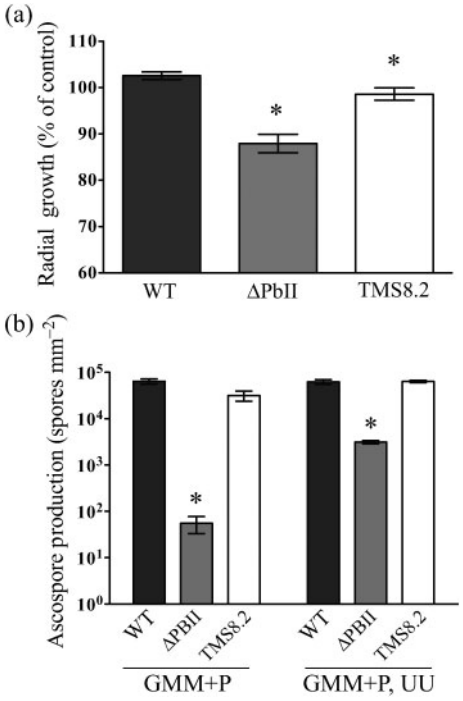

Fig. 6. Physiological experiments with mutants created by Shaaban *et al.* (2010) allowed for determination of TPE at chromosome VI. (a) A radial growth assay illustrates the quantifiable difference in growth of ΔPbII (*A. parasiticus pyrG* integrated ~8.5 kb from the telomere of chromosome VI-R) when grown on GMM versus GMM+UU. The isogenic control strain (TMS8.2) contains the *A. parasiticus pyrG* gene located ectopically and also shows a slight reduction in growth on GMM versus GMM+UU compared to WT (RJMP1.19); however, radial growth of ΔPbII is significantly reduced compared to TMS8.2. (b) Ascosporogenesis assays mirror the radial growth assay, where ΔPbII produces significantly fewer ascospores than either the WT or TMS8.2. These data illustrate TPE repression of the *A. parasiticus pyrG* transgene when located near the telomere of chromosome VI. Means±SD are plotted; asterisks indicate statistically significant differences between wild-type and other strains at $P<0.001$ using Student's *t*-test.

Using a similar approach to test the involvement of NkuA we created a double AN5091::Af*pyrG* Δ*nkuA* mutant. Consistently, this mutant produced more ascospores than the single AN5091::Af*pyrG* mutant by one order of magnitude (Fig. 7d). Together, these data suggest that NkuA, HepA, ClrD, HstA and HdaA are not involved in radial growth or ascospore production under the conditions tested; however, in Δ*nkuA*, Δ*hepA*, Δ*clrD* and Δ*hdaA* genetic backgrounds there is partial derepression of the telomerically located transgenes. None of the double mutants were able to restore growth or ascospore production

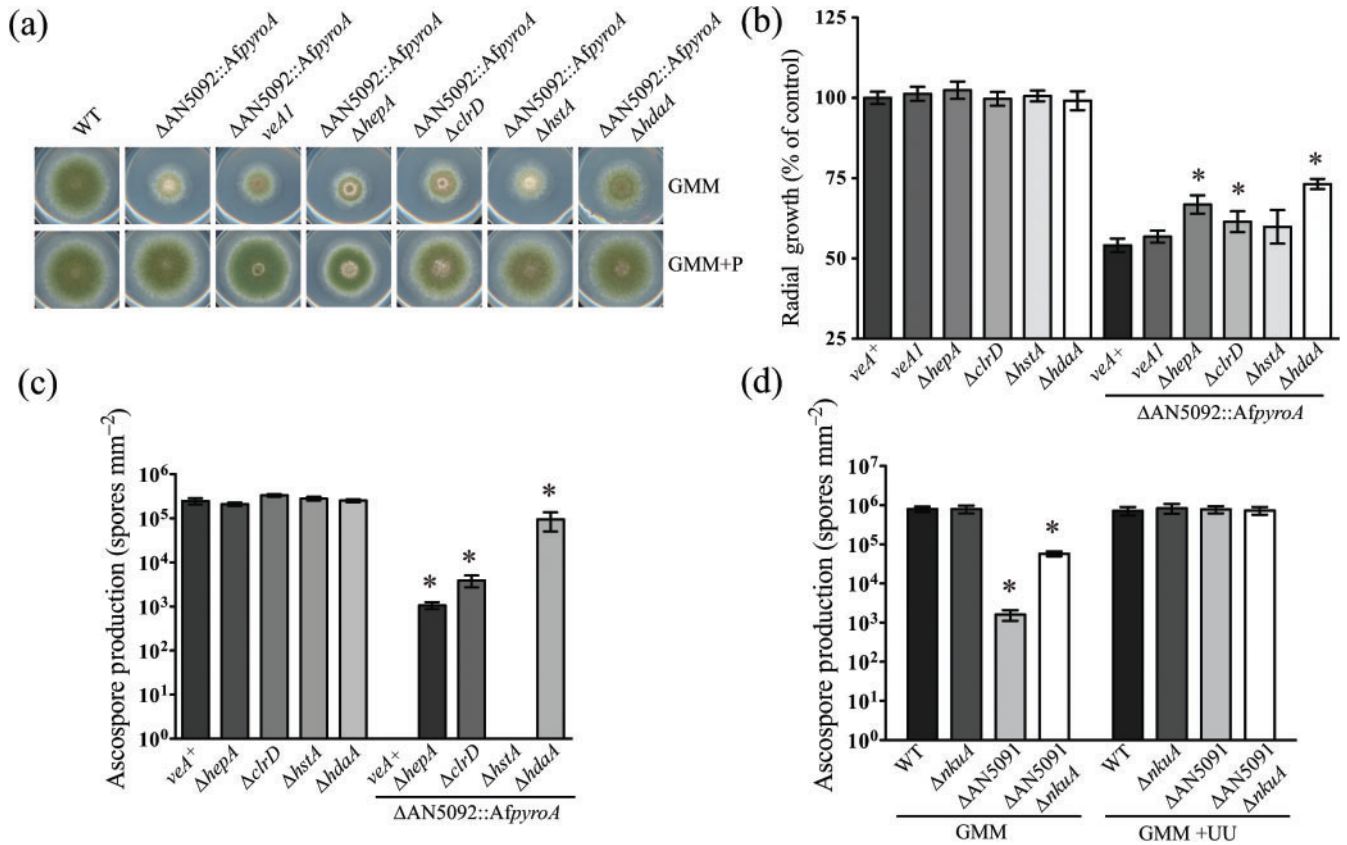

Fig. 7. In order to address the mechanism of TPE in *A. nidulans*, radial growth and ascosporogenesis assays were conducted on double mutants. (a) Radial growth assays illustrate the reduction in growth on GMM versus GMM + pyridoxine (P) of strains harbouring ΔAN5092 : : Af*pyroA*. (b) Quantification of the radial growth assay indicates that HepA, ClrD and HdaA derepress the Af*pyroA* transgene, while VeA1 and HstA have no effect on Af*pyroA* repression. The single mutants (*veA1*, Δ*hepA*, Δ*clrD*, Δ*hstA* and Δ*hdaA*) show no effects on radial growth in this assay. (c) Ascosporogenesis assays match the radial growth assays, providing further evidence for the involvement of HepA, ClrD and HdaA in TPE. The single mutants (*veA1*, Δ*hepA*, Δ*clrD*, Δ*hstA* and Δ*hdaA*) show no effect on ascospore production in this assay. (d) Ascospore production is partially rescued in a double mutant (ΔAN5091 Δ*nkuA*) compared to the single ΔAN5091 mutant; however, ascospore production does not reach wild-type levels. WT (*veA*$^{+}$), RDIT9.32; ΔAN5092 : : Af*pyroA*, RJMP121.7; ΔAN5092 : : Af*pyroA veA1*, RJMP121.4; ΔAN5092 : : Af*pyroA* Δ*hepA*, RJMP122.6; ΔAN5092 : : Af*pyroA* Δ*clrD*, RJMP125.20; ΔAN5092 : : Af*pyroA* Δ*hstA*, RJMP127.4; ΔAN5092 : : Af*pyroA* Δ*hdaA*, RJMP123.3; *veA1*, RDIT2.3; Δ*hepA*, RJW110.4; Δ*clrD*, RJMP135.11; Δ*hstA*, RJMP131.7; Δ*hdaA*, RMS1.22; Δ*nkuA*, TJMP45.2; ΔAN5091, RJMP115.3; and ΔAN5091Δ*nkuA*, TJMP16.1. Note that the *y*-axis scale is logarithmic in panels (c) and (d). Means ± SD are plotted in panels (b–d); asterisks indicate statistically significant differences between wild-type and other strains at $P<0.001$ using Student's *t*-test.

to wild-type levels, thus indicating that TPE is polygenic in *A. nidulans*.

DISCUSSION

TPE is a widespread phenomenon amongst diverse organisms and this work supports the conservation of this silencing mechanism in *A. nidulans*. Our experiments indicate that transgenes are silenced when placed at the telomere of chromosome III and that this silencing extends at least 30 kb from the telomere cap to the SpoC1 cluster. We have also identified TPE at the telomere of the right arm of chromosome VI. Additionally, we have shown that HepA, ClrD, HdaA and NkuA are involved in regulation of TPE at chromosome III.

Our unexpected observation of impaired ascospore development and a reduction in radial growth due to partial repression of Af*pyrG* and Af*pyroA* transgenes led us to exploit these phenotypes to assess TPE at two different telomeres as well as establish a role for NkuA, HepA, ClrD and HdaA in regulating TPE. These simple phenotypic assays are advantageous because they potentially provide a quantitative measure of transgene repression. Sexual development is a complex process in *A. nidulans*, requiring proper formation of several differentiated cell types that make up a cleistothecium (reviewed by Braus *et al.*, 2002).

Normal development of the sexual cycle requires adequate nutrient supply, as illustrated by amino acid starvation repression of sexual development (Eckert *et al.*, 1999; Hoffmann *et al.*, 2000), and Bruggeman *et al.* (2004) reported that many auxotrophic strains are unable to complete the sexual cycle. Extending this observation, the repressed sexual development observed in this study was likely a consequence of inadequate pyridoxine metabolism (*pyroA* transgene) or pyrimidine metabolism (*pyrG* transgene); this was substantiated by the finding that growing a *pyroA4* mutant on a limited amount of pyridoxine results in loss of ascospore production and eventually a reduction in radial growth (Supplementary Fig. S3 and data not shown).

The KU70/KU80 heterodimer has been shown to be required for non-homologous end joining recombination and normal maintenance of telomeres (Boulton & Jackson, 1998). The KU heterodimer binds telomeres and is thought to facilitate telomerase activity (reviewed by Dubrana *et al.*, 2001). In the absence of either of the KU proteins, normal telomere function is altered, resulting in lack of heterochromatin complexes and subsequently increased transcription of genes located near telomeres. Similarly to what has been demonstrated in other organisms (Boulton & Jackson, 1998; Mishra & Shore, 1999; Rosas-Hernández *et al.*, 2008), we report here that the *A. nidulans* KU70 homologue (NkuA) partially suppresses TPE as evidenced by increased ascosporogenesis, while at the same time showing that NkuA has no effect on normal ascosporogenesis.

Based on TPE models proposed in other eukaryotic systems, we characterized the involvement of several heterochromatin proteins in TPE of *A. nidulans*. In fission yeast and *N. crassa*, methylation of lysine 9 of histone 3, heterochromatin protein 1 and histone deacetylase enzymes are involved in regulation of TPE (Ottaviani *et al.*, 2008; Smith *et al.*, 2008). Our results in *A. nidulans* indicate that there is considerable mechanistic conservation of TPE between fungal species, including TPE regulation by core heterochromatin-activating/maintenance proteins (HepA, ClrD and HdaA). However, there are some differences as well. For example, here we see that NkuA plays a role in *A. nidulans* TPE, unlike fission yeast, where the KU70 homologue is not involved in TPE. Additionally, Smith *et al.* (2008) reported that multiple sirtuins (class III histone deacetylases) were involved in regulation of TPE in *N. crassa*, yet we were unable to establish that the *A. nidulans* SIR2 orthologue (HstA) is involved in TPE at chromosome III. Our data suggest involvement of several *trans*-acting factors in *A. nidulans* TPE including a Nku complex and histone-remodelling associates.

Chromatin structure has been shown to play a role in transcriptional regulation of genes based on their chromosomal location in several organisms, including *A. nidulans* (Palmer & Keller, 2010). Here we demonstrate that TPE exists in *A. nidulans* and show that radial growth and ascosporogenesis can be used as sensitive, quantitative assays to determine the extent of silencing or to identify suppressors of silencing. Future efforts will focus on elucidating any impact of TPE on secondary metabolite gene clusters, which have a tendency to be located in subtelomeric regions (Hoffmeister & Keller, 2007) and are regulated by some of the same proteins involved in *A. nidulans* TPE (e.g. HdaA, ClrD, HepA) (Reyes-Dominguez *et al.*, 2010; Shwab *et al.*, 2007). Similarly to what has been described in other organisms, we have shown that TPE exists at two telomeres in *A. nidulans* and predict that TPE likely exists at most telomeres; however, the extent of silencing at an individual telomere may be variable (Mondoux & Zakian, 2007).

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