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Upgrading Fungal Gene Expression on Demand: Improved Systems for Doxycycline-Dependent Silencing in *Aspergillus fumigatus*

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Conditional gene expression is key for functional studies in any given microorganism. To allow tight regulation in the pathogenic mold *Aspergillus fumigatus*, improved versions of the doxycycline-dependent Tet-On system were generated by replacing functional elements of the precursor module, thereby circumventing the former problem of leakiness due to intramolecular recombination.

Deletion or hyperexpression of a gene product is a central strategy for characterization of its function in any given organism. Conditional gene expression has evolved as a valuable tool of molecular biology when addressing the intracellular role of uncategorized proteins, as it allows the discovery of relevant phenotypes that might correlate with the expression level. In general, two major approaches have been followed to adjust or condition protein expression: expression of conditional alleles that result in functionally altered gene products dependent on external cues or manipulation of the expression level of the encoding gene. Especially the latter approach is advantageous, as it may allow various levels of expression, resulting in specific phenotypes based on gene dosage effects. For instance, the complete shutdown of expression can result in a pronounced null phenotype or demonstrate essentiality of the gene of interest (1), whereas forced overexpression could give additional hints on the function of the encoded protein and the involved cellular processes.

Conditional gene expression is generally based on the replacement of the endogenous gene promoter by an adjustable one that drives transcription of the gene in question. The controllable promoter responds to changing environmental conditions, such as alternative nutritional sources or the presence/absence of specific molecules. For the latter, tetracycline-dependent systems have been designed and validated in a widespread and highly successful manner, with relevant cellular systems ranging from viral ones over unicellular microbes to mammalian cell lines and transgenic animals (2–5).

For filamentous fungi of the genus *Aspergillus*, such a conditional expression system was established by expressing a tetracycline-binding transcriptional activator protein constitutively and fusing the coding sequence of interest behind a minimal promoter sequence preceded by several operator (*tetO*) sites that the transactivator would bind to (6). Two variants of this tetracycline-responsive transcriptional activator protein exist: the tetracycline transactivator (tTA) is inactivated through binding of tetracycline, whereas the reverse tetracycline transactivator (rtTA) will be activated through binding this molecule. These systems are generally referred to as Tet-Off and Tet-On systems, respectively. Due to its superior stability, doxycycline is commonly used instead of tetracycline.

While the initial system for *A. fumigatus* was assembled by two separate constructs that need to be integrated in the recipient's genome, a recently streamlined Tet-On version comprises all regulatory and coding elements in one cassette (7). Functionality of this module was proven in *A. niger* to allow tightly regulated, linear "gene expression on demand" and later again in the human-pathogenic mold *A. fumigatus* when demonstrating the essentiality of the Rho GTPase Rho1 (8).

In the course of testing essential genes, we became aware of an inherent drawback of this recent construct that resulted in an apparent leakiness of the expression module: when plating conidia from a strain carrying an essential gene under the control of the Tet-On system on selective media, a certain fraction (app. 0.01%) was able to germinate and form colonies that would grow in an unrestricted fashion (our unpublished results). Inspection of such clones by diagnostic PCR on genomic DNA revealed that a recombination event had taken place, based on a direct repeat sequence from the *gpdA* promoter region (^{p}*gpdA*). This *A. nidulans* promoter drives constitutive transcription of the transactivator-encoding sequence, and a short segment (174 bp) of it also serves as the minimal promoter element downstream of the *tetO* sites and upstream of the gene of interest. As a consequence, homologous recombination of these direct repeats leads to excision and placement of the corresponding gene behind the reconstituted *gpdA* promoter to result in constitutive transcription at high levels. Depending on the frequency, such recombinants may interfere with reliable phenotype analysis of the transgenic strain as they dilute its clonality.

In order to circumvent this issue of leakiness due to recombination in the Tet-On module, we followed two distinct approaches. One aimed at the replacement of the constitutive *gpdA* promoter, the other, alternatively, of the minimal promoter segment derived from the same regulatory element. Accordingly, two derivative constructs were generated by conventional cloning

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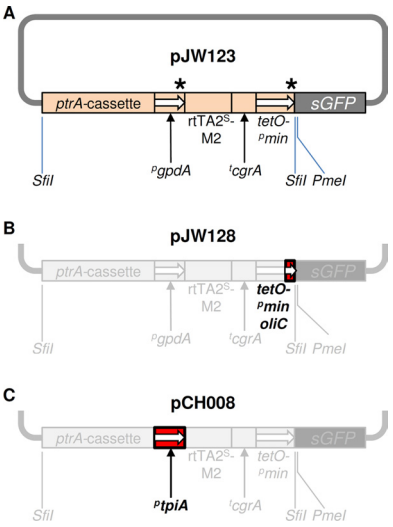

FIG 1 Tet-On expression systems for conditional gene expression in *Aspergillus*. (A) The original Tet-On cassette in pJW123 is enclosed in two incompatible SfiI digestion sites and harbors the pyrithiamine resistance gene (*ptrA* cassette), the *gpdA* promoter of *A. nidulans* (^{P}*gpdA*), the reverse tetracycline transactivator rtTA2^{S}-M2, the terminating region of *cgrA* from *A. fumigatus* (^{t}*cgrA*), and the chimeric ^{P}*gpdA*-based *tetO-*^{P}*min* promoter. Asterisks mark the sites of homologous recombination. (B) In pJW128, the chimeric ^{P}*gpdA*-based *tetO-*^{P}*min* promoter was replaced by a minimal promoter based in the *oliC* promoter of *A. nidulans* (*tetO-*^{P}*min-oliC*). (C) In pCH008, the ^{P}*gpdA* was exchanged for the *tpiA* promoter of *A. nidulans* (^{P}*tpiA*).

techniques using plasmid pJW123 (8) as the starting template (Fig. 1A): in pJW128, the 174-bp ^{P}*gpdA* fragment downstream of the *tetO* sites was replaced by a 160-bp fragment from the *A. nidulans oliC* promoter that had been mapped as the minimal region to drive transcription of downstream sequences (9, 10). In parallel, the *gpdA* promoter region of pJW123 was replaced by the 506-bp upstream sequence of the *A. nidulans tpiA* gene, which encodes a triosephosphate isomerase and was shown to be constitutively expressed (11), yielding pCH008. After validation of both constructs, pJW128 (Fig. 1B) and pCH008 (Fig. 1C), by restriction mapping and sequence analysis, each one was incorporated into a replacement cassette for the promoter of the essential *A. fumigatus rho1* gene. The cassettes were transformed and integrated at the homologous locus in the genome of recipient strain AfS35 (12). Several transgenic clonal isolates were screened for correct promoter replacement by diagnostic PCR (not shown), and two validated and independent strains for each construct were subjected to further analyses.

First, growth capacities in the presence or absence of the inducing agent doxycycline at various concentrations were monitored (Fig. 2). Whereas the wild-type progenitor displayed accurate radial extension of its mycelium accompanied by abundant conidiation, conidia from the *rho1*$_{tetOn}$ strains were unable to grow when doxycycline was omitted from the culture plates. Only in the presence of doxycycline were these strains able to form a mycelium from which mature conidia developed. A fundamental difference became apparent with respect to the threshold concentration that allowed germination and growth: the ^{P}*tpiA* derivatives required higher doses (5 $\mu g \cdot ml^{-1}$) than the ^{P}*gpdA* progenitor strain (0.25 to 0.5 $\mu g \cdot ml^{-1}$), while the ^{P}*oliC*$_{min}$ strains displayed growth at lower concentrations (0.05 $\mu g \cdot ml^{-1}$).

Furthermore, we were interested in validating the presumed absence of recombination in the new Tet-On derivatives due to the lack of direct repeats in the module. For this purpose, defined amounts of freshly harvested conidia from each *rho1*$_{tetOn}$ strain were plated on minimal culture medium containing 5 $\mu g \cdot ml^{-1}$ doxycycline or being devoid of it and incubated at 37°C. All plates were tracked for 96 h, and numbers of single colonies were determined to assess the frequency of recombination and degree of leakiness (Fig. 3A and data not shown). The initial isolate displayed a pronounced and reproducible proportion of spores that were able to grow like the wild type under restrictive conditions (approximately 1 out of 10,000 conidia), while conidia harvested from the derivative strain carrying the ^{P}*tpiA* fragment were unable to do so. Interestingly, we observed occasional growth of the strain carrying the *oliC* minimal promoter fragment. These colonies always had a prominent growth phenotype, i.e., slow growth and drastically reduced conidiation. Since conidia isolated from these colonies phenotypically did not differ from the parental strain (i.e., doxycycline dependency), we assume this growth is attributed to a higher basal expression driven by the minimal ^{P}*oliC* region. Notably, this phenotype specifically depended on the inoculum density (Fig. 3B). As expected, microscopic inspection of freshly germinated spores of all *rho1*$_{tetOn}$ strains revealed frequent

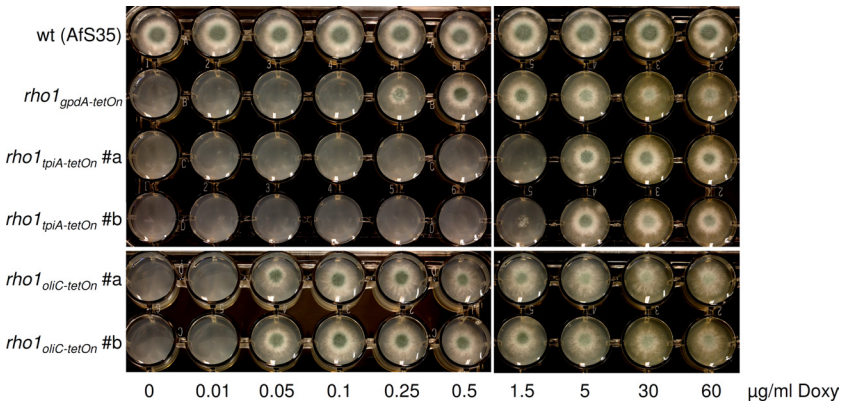

FIG 2 Doxycycline-dependent growth of conditional *rho1*$_{tetOn}$ strains carrying different versions of the gene silencing module. From suspensions of 5×10^{5} conidia $\cdot$ ml^{-1} of the indicated strains, aliquots of 3 μl ($\triangleq$1,500 conidia) were inoculated on AMM agar supplemented with the indicated amount of doxycycline (Doxy) and incubated at 37°C for 36 h.

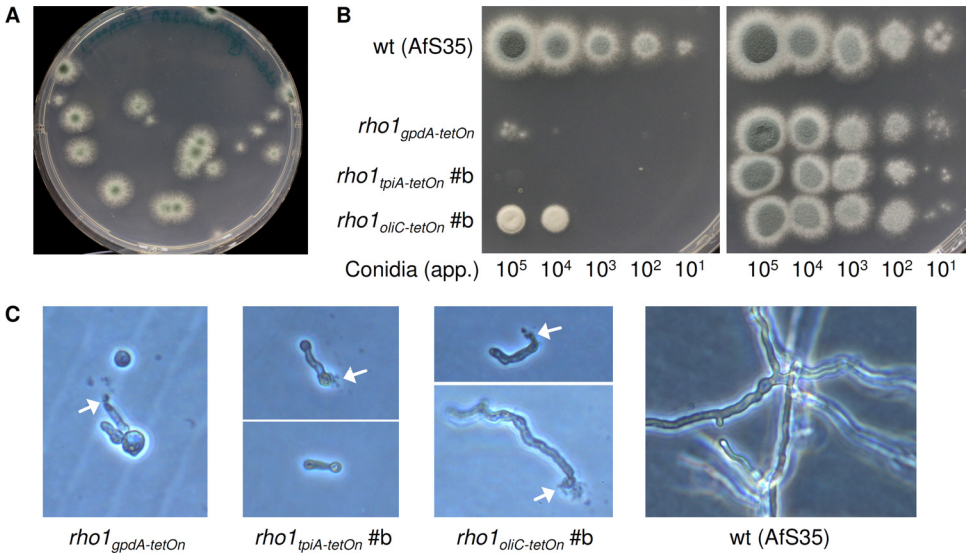

FIG 3 Evaluation of recombination rates and growth phenotypes. (A) Exemplary image. A total of 250,000 conidia of the $rho1_{gpdA\text{-}tetOn}$ strain were spread on AMM agar and incubated at 37°C for at least 48 h. (B) In a series of 10-fold dilutions derived from a starting suspension of 3×10^7 conidia · ml^{-1} of the indicated strains, 3-μl aliquots were inoculated on AMM agar supplemented with no (left) or 5 μg · ml^{-1} doxycycline (right) and incubated at 37°C for 36 h. (C) Exemplary microscopy images of the indicated strains. Conidia were inoculated in AMM (5×10^3 conidia · ml^{-1}) and incubated at 37°C in the absence of doxycycline for 16 h. White arrows indicate cytoplasmic leakage.

cytoplasmic leakage and death in the absence of doxycycline (8) (Fig. 3C).

In conclusion, we were able to generate improved versions of the recently introduced tetracycline-dependent system for adjustable gene expression in *Aspergillus*. Both constructs represent a further development of the validated system by V. Meyer and coworkers (7), as they do not contain repetitive sequences that entail the risk of intramolecular recombination. Accordingly, both Tet-On modules allow gene silencing, which is essential for studying the cellular function of any gene of interest by conditional promoter replacement. This screening approach has proven extremely useful for the identification of virulence determinants and drug targets in fungal pathogens (1, 13) and represents a superior alternative to the commonly used gene deletion strategy or the recent RNA interference (RNAi)-mediated gene silencing approach (14, 15). Of special interest is the fact that both Tet-On constructs differ in their response toward the endogenous inducer doxycycline, with the $^{P}oliC$ version resulting in apparently sufficient expression of the *rho1* gene product at lower concentrations at the costs of an apparently higher basal expression. In contrast, the precursor construct and the newly designed $^{P}tpiA$ version are more tightly regulated but also require higher concentrations of doxycycline. The exact reason for these differences is unclear and may be due to differing expression levels of the transactivator protein or binding affinities to the chimeric *tetO-*^{P}min promoters. However, both Tet-On constructs trigger transcription of a downstream sequence above a certain threshold level of doxycycline, although each at a different concentration range. As the $^{P}tpiA$-derived construct requires higher doses of doxycycline, the resulting transcription rates of certain genes might not be sufficient for full complementation, as could be demonstrated for the glucan synthase-encoding *fks1* gene (our unpublished observation). Accordingly, each module might be more or less appropriate for conditional gene silencing or expression purposes, but both expand the toolbox of *Aspergillus* molecular biology (16, 17).

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