

# Fungal Gene Expression on Demand: an Inducible, Tunable, and Metabolism-Independent Expression System for *Aspergillus niger*<sup>∇†</sup>

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**Filamentous fungi are the cause of serious human and plant diseases but are also exploited in biotechnology as production platforms. Comparative genomics has documented their genetic diversity, and functional genomics and systems biology approaches are under way to understand the functions and interaction of fungal genes and proteins. In these approaches, gene functions are usually inferred from deletion or overexpression mutants. However, studies at these extreme points give only limited information. Moreover, many overexpression studies use metabolism-dependent promoters, often causing pleiotropic effects and thus limitations in their significance. We therefore established and systematically evaluated a tunable expression system for *Aspergillus niger* that is independent of carbon and nitrogen metabolism and silent under noninduced conditions. The system consists of two expression modules jointly targeted to a defined genomic locus. One module ensures constitutive expression of the tetracycline-dependent transactivator rtTA2<sup>S</sup>-M2, and one module harbors the rtTA2<sup>S</sup>-M2-dependent promoter that controls expression of the gene of interest (the Tet-on system). We show here that the system is tight, responds within minutes after inducer addition, and allows fine-tuning based on the inducer concentration or gene copy number up to expression levels higher than the expression levels of the *gpdA* promoter. We also validate the Tet-on system for the generation of conditional overexpression mutants and demonstrate its power when combined with a gene deletion approach. Finally, we show that the system is especially suitable when the functions of essential genes must be examined.**

The metabolic versatility of filamentous fungi makes them outstanding cell factories in biotechnology. Important fungal metabolites that are commercially produced include pharmaceuticals, such as antibiotics and immunosuppressants; bulk commodities, such as organic acids; and enzymes exploited in various industrial sectors (25). Also, some filamentous fungi are phytopathogenic, causing huge agricultural losses, while others are causative agents of devastating human diseases (10). Within the last years, genome sequences for most of the important industrial, agricultural, and medical filamentous fungi have been published (for reviews, see references 5 and 12), thus facilitating multiple research activities, including genome-mining approaches to spot new metabolites and enzymes (9, 47), bioinformatics developments to reconstruct metabolic pathways (2), functional genomics attempts to study the functions of genes (11), and systems biology experiments to dissect the interactions of genes and proteins (1, 3).

A key resource for these approaches and the understanding of gene functions and networks is knockdown or knockout mutant collections, which provide a wealth of information.

However, studies in *Saccharomyces cerevisiae* and higher eukaryotes, such as *Drosophila* and *Caenorhabditis elegans*, have shown that the majority of knockdown/knockout mutants have little, if any, discernible loss-of-function phenotype (13, 21). This observation is thought to result from the general robustness of biological systems and their buffering capacities against cellular variations, e.g., due to gene redundancies and compensatory pathways (19, 38). Moreover, mutants with essential genes deleted evade analysis, imposing further limitations on this approach. An alternative and complementary approach that overcomes these limitations is the generation of overexpression mutant collections. Attempts in yeast, plant, and insect systems have shown their power to assign gene functions where loss-of-function approaches have failed (38, 41, 48). However, there are limitations, as well. If metabolism-dependent promoters are used for overexpression studies, one is restricted in the choice of growth medium. In addition, the phenotypes observed might be ambiguous due to pleiotropic effects caused by metabolic changes. Moreover, if these systems mediate only an “on/off” situation, expression at a defined, user-specified level is not permitted. Therefore, tunable and metabolism-independent promoter systems have been developed for yeast, plant, insect, and mammalian systems, allowing more flexibility in experimental approaches (23, 24, 33, 46).

For filamentous fungi, an impressive set of promoters are available, allowing overexpression of a gene of interest (GOI). However, these promoters are either constitutively active and growth related (e.g., the *Aspergillus niger* glyceraldehyde-3-

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phosphate dehydrogenase promoter, *PgpdA*) or dependent on the carbon or nitrogen source (e.g., *A. niger* *PglaA* and *PinuE*, *Aspergillus nidulans* *PalcA*, *Neurospora crassa* *Pqa-2*, *Ustilago maydis* *Pcrg1* and *Pnar1*, and *Trichoderma reesei* *Pcbh1*), and some are also leaky (e.g., *PinuE* and *Pqa-2*) (7, 22, 36, 37). In search of promoters that are tight, tunable, and metabolism independent, three systems have recently been tested for application in filamentous fungi: the thiamine promoter system (*PthiA*) in *Aspergillus oryzae* (40), the human estrogen receptor (hER $\alpha$ ) system in *A. nidulans* and *A. niger* (32), and a system based on the *Escherichia coli* tetracycline resistance operon (Tet) in *Aspergillus fumigatus* (45). All three systems mediate gene expression in an inducer-dependent manner; however, the *PthiA* system is inactive under alkaline growth conditions, and hER $\alpha$  systems are either strongly inducible but leaky or tight but weakly inducible (32, 40). In comparison, the Tet system appeared to be the most promising for use in filamentous fungi (45).

Basically, the Tet system is a negative regulatory circuit of *E. coli* involving the repressor protein TetR, which binds to the operator sequence (*tetO*) of the Tn10 tetracycline resistance operon in the absence of tetracyclines. Transcription of the operon is thus shut down. However, the interaction of repressor and operator can be efficiently prevented by tetracycline, which forces TetR to dissociate from *tetO*. In consequence, transcription of the operon is initiated, thus conferring tetracycline resistance (4). Both TetR and *tetO* have been adapted for efficient use in eukaryotic systems (for detailed information, see references 15–18 and 42). In brief, TetR has been combined with the minimal transcriptional activation domain derived from the herpes simplex virus protein 16 (VP16). The generated hybrid transactivator, tTA, stimulates gene expression in the absence of tetracycline (the Tet-off system). This system has been modified so that tetracycline or its derivative doxycycline (Dox) induces but does not abolish binding to the operator sequence. For this purpose, the binding activities of tTA were modified and the reverse hybrid transactivator rtTA was generated (the Tet-on system). In order to increase the sensitivity of rtTA to Dox and to optimize rtTA expression in eukaryotic systems, additional mutations were introduced, eventually resulting in the reverse hybrid transactivator rtTA2<sup>S</sup>-M2. Finally, the tetracycline-responsive promoter was optimized for maximum expression levels by placing seven copies of the *tetO* sequence (*tetO7*) upstream of a minimal promoter (Pmin).

To judge, whether the Tet-on/Tet-off system is generally applicable to *A. fumigatus*, Vogt et al. constructed three plasmids, one containing the expression cassette ensuring constitutive expression of rtTA2<sup>S</sup>-M2 (*PgpdA*::rtTA2<sup>S</sup>-M2), one containing *PgpdA*::tTA, and one harboring the *tetO7* sequence linked to a 175-bp minimal promoter sequence of *PgpdA* (Pmin) upstream of the *E. coli* hygromycin resistance gene *hph* (*tetO7*::Pmin::hph). When plasmid pairs were cotransfected to *A. fumigatus*, hygromycin-resistant clones were detected in either the presence or absence of Dox, demonstrating that the Tet system is in principle functional in *A. fumigatus* (45). However, the dynamics of the system and the effects of gene dosage on the efficacy and tightness of the system were not studied.

As detailed understanding of the performance of the Tet system is indispensable for future applications in filamentous

fungi, we decided to redesign the Tet-on system and to systematically evaluate it using the industrial model fungus *A. niger* as the host and the codon-optimized luciferase gene *mluc* (14) as a reporter. Different *A. niger* transformants containing one or multiple copies of the newly constructed Tet-on system were generated. The strains were cultivated at microtiter plate (MTP) and bioreactor scales in the presence or absence of Dox, and luciferase activities were determined at minute intervals. The data obtained demonstrate that the Tet-on system is tight under noninduced conditions and can respond within minutes after Dox addition. It allows tunable gene control, which can be user specified by varying the Dox concentration or gene copy numbers. Most importantly, the system reaches high expression levels comparable to those of the strong *gpdA* promoter. To demonstrate the utility of this tool, we finally used our Tet-on version to establish overexpression mutants and report on a Tet-on-based strategy to establish mutants that have essential genes deleted but are viable under selected conditions. The data from this work suggest that the Tet-on system can serve as a general and excellent tool for functional genomics and systems biology approaches in filamentous fungi.

#### MATERIALS AND METHODS

**Strains, culture conditions, and molecular techniques.** The *A. niger* strains used in this study are given in Table 1 and were cultivated in minimal medium (MM) (6) containing 1% glucose as a carbon source or in complete medium (CM), consisting of MM supplemented with 1% yeast extract and 0.5% Casamino Acids. Uridine (10 mM) was added when required. Fermentation medium (FM) is composed of 0.75% glucose, 0.45% NH<sub>4</sub>Cl, 0.15% KH<sub>2</sub>PO<sub>4</sub>, 0.05% KCl, 0.05% MgSO<sub>4</sub>, 0.1% salt solution (6), and 0.003% yeast extract. The pH of the FM was adjusted to pH 3. General cloning procedures in *E. coli* were done according to the methods described by Sambrook and Russell (39). Transformation of *A. niger*, selection procedures, genomic-DNA extraction, and Southern analyses were performed using recently described protocols (28). Quantitative real-time PCR was carried out as described previously (31). The primer pairs for *mluc*, *gpdA*, and *H2B* (used as a reference gene) are summarized in Table S1 in the supplemental material.

**Cultivation in 96-well MTPs.** In each well of 96-well MTPs, 10<sup>4</sup> freshly harvested spores of *A. niger* transformants were inoculated in 100  $\mu$ l CM or MM and cultivated at 30°C for a maximum of 24 h. At 0, 6, or 16 h after inoculation, 30  $\mu$ l of Dox was added to induce the expression of *mluc* (diluted in MM or CM; final concentrations, 0 to 500  $\mu$ g/ml) and 70  $\mu$ l of the substrate luciferin (diluted in MM or CM; final concentration, 1.4 mM) to detect mLuc activity. Luminescence (luminescent counts per second [LCPS]) and biomass formation were measured *in situ* every 5 min using Victor<sup>3</sup> (Perkin Elmer) at 537 and 620 nm, respectively. Three independently growing cultures were analyzed for every experimental condition.

**Bioreactor cultivation.** Freshly harvested conidia (2.5  $\times$  10<sup>8</sup>) from the different *A. niger* strains were used to inoculate 1 liter FM. Cultivations were performed in a BioFlo/CelliGen 115 bioreactor (New Brunswick Scientific), where the temperature (set to 30°C), pH (set to 3), and agitation speed were controlled on line. The dissolved oxygen tension was at all times above 40% to ensure sufficient oxygen supply. The cultivation program followed two phases. (i) During the first 5 h of cultivation, the agitation speed was set to 250 rpm, and aeration was performed via the headspace (1 liter min<sup>-1</sup>). These precautions help to reduce loss of hydrophobic conidia via the exhaust gas. (ii) After this period, more than 90% of the spores had started to germinate. Therefore, the agitation speed was set to 750 rpm and air was supplied through a ring sparger system to allow optimal mixing and oxygen transfer (1 liter min<sup>-1</sup>). To avoid foam formation, 0.01% (vol/vol) polypropylene glycol was added as an antifoam agent to the culture broth. Dox was added to the culture (final concentration, 5 or 20  $\mu$ g/ml) during the early exponential growth phase, i.e., when the culture reached a density of 1 g (dry weight) biomass/kg culture broth (run time, about 17.5 h). Mycelial samples were taken after certain points in time (before and after Dox addition) to determine the biomass concentration and luciferase activity using a microtiter plate-based reading assay. In doing so, 130  $\mu$ l freshly harvested biomass samples were mixed with 70  $\mu$ l luciferin (diluted in FM; final concentration,

TABLE 1. *A. niger* strains used in this study

Strain	Code	Relevant genotype (comment)	Source
N402		Wild type	Laboratory collection
AB4.1		<i>pyrG</i> <sup>-</sup>	44
MA70.15		<i>pyrG</i> <sup>-</sup> $\Delta$ <i>kusA</i>	26
MA77.1		<i>pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA78.6		$\Delta$ <i>kusA</i> (N402 derivative)	8
MA169.4		<i>pyrG</i> <sup>-</sup> <i>kusA</i> <sup>-</sup>	8
VG5.1	L0	<i>pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (transformed with pVG2.2; single copy)	This work
VG6.1	L1	<i>pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (transformed with pVG4.1; single copy)	This work
VG6.3	L1	<i>pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (transformed with pVG4.1; single copy)	This work
VG7.2	L0	<i>pyrG</i> <sup>+</sup> (transformed with pVG2.2; single copy)	This work
VG8.27	L1	<i>pyrG</i> <sup>+</sup> (transformed with pVG4.1; single copy)	This work
VG8.1	L2	<i>pyrG</i> <sup>+</sup> (transformed with pVG4.1; double copy)	This work
VG8.2	L3	<i>pyrG</i> <sup>+</sup> (transformed with pVG4.1; triple copy)	This work
MA147.8	$\Delta$ <i>cfrA</i>	$\Delta$ <i>cfrA pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA148.7	$\Delta$ <i>cfrB</i>	$\Delta$ <i>cfrB pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA149.2	$\Delta$ <i>cfrC</i>	$\Delta$ <i>cfrC pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA151.1	$\Delta$ <i>cfrE</i>	$\Delta$ <i>cfrE pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA152.5	$\Delta$ <i>cfrF</i>	$\Delta$ <i>cfrF pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA153.1	$\Delta$ <i>cfrG</i>	$\Delta$ <i>cfrG pyrG</i> <sup>+</sup> $\Delta$ <i>kusA</i> (MA70.15 derivative)	This work
MA173.18	OE <i>cfrA</i>	<i>TetO7::Pmin::cfrA pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA174.5	OE <i>cfrB</i>	<i>TetO7::Pmin::cfrB pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA175.3	OE <i>cfrC</i>	<i>TetO7::Pmin::cfrC pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA177.6	OE <i>cfrE</i>	<i>TetO7::Pmin::cfrE pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA178.2	OE <i>cfrF</i>	<i>TetO7::Pmin::cfrF pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA179.6	OE <i>cfrG</i>	<i>TetO7::Pmin::cfrG pyrG</i> <sup>+</sup> (AB4.1 derivative)	This work
MA216.9	OE <i>actA</i>	<i>TetO7::Pmin::actA pyrG</i> <sup>+</sup> (MA169.4 derivative)	This work
MA224.1	$\Delta$ -OE <i>actA</i>	<i>TetO7::Pmin::actA pyrG</i> <sup>+</sup> $\Delta$ <i>actA hyg</i> <sup>r</sup> (MA216.9 derivative)	This work

1.4 mM), and the luminescence and biomass were measured at 537 and 620 nm, respectively, using Victor<sup>3</sup> (Perkin Elmer). All measurements were performed at least in triplicate.

**Cloning of a Tet-on reporter-control construct.** The expression vector pVG4.1 (*PgpdA::rtTA::TcgrA-tetO7::Pmin::mluc::TrpC-pyrG\**) and the negative-control vector pVG2.2, which lacks the *mluc* open reading frame (ORF) (*PgpdA::rtTA::TcgrA-tetO7::Pmin::TrpC-pyrG\**), were constructed as described in detail in Fig. S1 in the supplemental material. Both plasmids and information on the respective DNA sequences have been deposited at the Fungal Genetics Stock Center (FGSC) (<http://www.fgsc.net>). The *PgpdA::rtTA::TcgrA* fragment was taken from plasmid p474 (obtained from FGSC [45]) and *tetO7::Pmin*, as well as *TrpC* fragments from plasmid p500 (obtained from FGSC [45]). The *pyrG\** allele was taken from plasmid pAB94 (43) in order to facilitate integration of the constructs at the endogenous *pyrG* locus of *A. niger*. The *pyrG*<sup>-</sup> strains AB4.1 and MA70.15 were used as recipient strains for transformation. Strain MA70.15 is deleted in the *ku70*-homologous *kusA* gene, thus allowing targeted integration at high frequencies (26). Uracil prototroph transformants were selected, purified, and subjected to Southern analyses according to the method of Meyer et al. (28) to confirm homologous integrations of pVG2.2 and pVG4.1 at the *pyrG* locus. Isolated strains carrying one or more copies of both plasmids are given in Table 1.

**Construction of *cfr* deletion and overexpression strains.** Constructs to delete the *cfrA* (An08g08280), *cfrB* (An07g07170), *cfrC* (An16g05020), *cfrE* (An11g05330), *cfrF* (An18g03740), or *cfrG* (An17g02350) gene were made as follows: *cfrX* 5' flanking sequences (length, ~700 bp) were obtained as KpnI-XhoI fragments by PCR using genomic DNA from strain N402 as a template and the primer pairs *cfrXP1KpnI* and *cfrXP2XhoI*. *cfrX* 3' flanking sequences (length, ~700 bp) were obtained as HindIII-NotI fragments from N402 genomic DNA by PCR using the primer pairs *cfrXP3HindIII* and *cfrXP4NotI* (the only exception was *cfrG*; there, the 5' flanking sequence was NotI-HindIII and the 3' flanking sequence was XhoI-KpnI). The respective 5' PCR products were digested with KpnI and XhoI and ligated into KpnI-XhoI-opened pBluescript II SK(+) (Fermentas). A 1.7-kb XhoI-HindIII fragment of the *A. oryzae pyrG* gene was isolated from pMA172 (8), and the *cfrX* 3' flanking sequences were digested with HindIII and NotI. Both fragments were ligated into XhoI-NotI-opened pBluescript II SK(+) vector containing the respective *cfrX* 5' flanking sequence. The *pyrG*<sup>-</sup> strain MA70.15 was used as a recipient strain for transformation. Successful deletions were identified via Southern analyses.

*cfr* overexpression (OE*cfr*) constructs were made as follows. The ORF was

obtained by PCR using N402 genomic DNA as a template and the primer pairs *cfrXP5PmeI* and *cfrXP6PmeI*, which introduced PmeI overhangs. The PCR products were ligated into pJET1.2 (Fermentas), sequenced, and cloned in the right orientation via PmeI restriction into plasmid pVG2.2. The plasmids obtained (pVG2.2-*cfrX*) were transformed into the *pyrG*<sup>-</sup> strain AB4.1 using conferred uracil prototrophy for selection. Uracil prototroph transformants were purified and subjected to Southern analyses to confirm homologous integrations of pVG2.2-*cfrX* at the *pyrG* locus. The strains are summarized in Table 1.

**Construction of conditional actin overexpression and loss-of-function strains.** In order to establish a conditional *actA* overexpression strain, the *actA* ORF was cloned into pVG2.2, and the plasmid obtained, pVG2.2-*actA*, was used to transform the *pyrG*<sup>-</sup> strain MA169.4, which contains a transiently disrupted *kusA* allele for improved gene targeting (8). A *pyrG*<sup>+</sup> transformant carrying a single copy of *PgpdA::rtTA::TcgrA-tetO7::Pmin::actA::TrpC* at the *pyrG* locus was isolated by Southern analysis and used for further analysis. This strain, named MA216.9, was used as a recipient strain to delete the actin gene. In doing so, an actin disruption cassette was made, consisting of the hygromycin resistance cassette (a 3.1-kb fragment obtained from pAN7-1 [35]) via HindIII/XhoI restriction) flanked by ~700-bp-long sequence stretches of *actA*. Note that the 5' flanking sequence consisted of about 400 bp of the 5' untranslated region and the first 300 bp of the *actA* ORF. This construct was transformed into MA216.9, and putative *actA* loss-of-function strains were isolated and purified on plates containing 100  $\mu$ g/ml hygromycin and 4  $\mu$ g/ml Dox. Successful deletion of *actA* was verified by Southern analysis.

## RESULTS

**Establishing a Tet-on system for *A. niger*.** Aiming at the highest control of the genomic integration of the system, thus allowing direct comparison of different transformants, we decided to redesign the Tet-on system and to integrate all of its components into a single plasmid. In doing so, plasmid pVG4.1 was constructed, comprising both the *PgpdA::rtTA2<sup>S</sup>-M2* and the *tetO7::Pmin::mluc* expression cassette, as well as a third cassette, *pyrG\**, necessary for selection and gene targeting (Fig. 1; for cloning details, see Fig. S1 in the supplemental

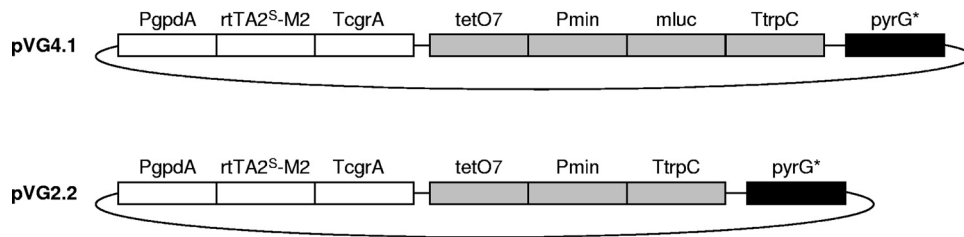


FIG. 1. The Tet-on system for *A. niger*. Plasmid pVG4.1 comprises one module ensuring constitutive expression of the tetracycline-dependent transactivator (*PgpdA*::*rtTA2<sup>S</sup>-M2*::*TcgrA*, with the *A. nidulans PgpdA* promoter and the *A. fumigatus crgA* terminator), a second module enabling *mluc* reporter gene expression in an *rtTA2-M2*-dependent manner (*tetO7*::*Pmin*::*mluc*::*TtrpC*, with *tetO7*::*Pmin* as an *rtTA2<sup>S</sup>-M2*-dependent promoter and the *A. nidulans trpC* terminator), and a third module, *pyrG\**, necessary for homologous integration of the plasmid at the *pyrG* locus of *A. niger*. The control plasmid pVG2.2 is identical to pVG4.1 but lacks the *mluc* gene. For cloning details and plasmid and fragment sizes, as well as restriction endonuclease sites, see Fig. S1 in the supplemental material.

material). As a control plasmid, pVG2.2, which is identical to pVG4.1 but lacks the *mluc* gene, was generated. Strain MA70.15 (*pyrG*<sup>-</sup>  $\Delta$ *kusA*) and strain AB4.1 (*pyrG*<sup>-</sup>) were used as recipient strains, in order to obtain single-copy or multicopy transformants, respectively (26). Transformants selected for growth in the absence of uridine were isolated and purified. Twenty transformants were screened via Southern hybridizations for the presence of single or multiple plasmid copies at the *pyrG* locus. Strains harboring one homologously integrated copy of the control construct pVG2.2 were named L0 (for no *mluc* copy), and strains harboring one or two copies of pVG4.1 at the *pyrG* locus were named L1 and L2 (for one or two *mluc* copies). We also identified one strain that contained three copies of pVG4.1 (L3), two of which integrated in tandem at the *pyrG* locus while one copy integrated heterologously in the genome of *A. niger* (Table 1; see Fig. S2 in the supplemental material).

To assess any possible influence of Dox on the growth of *A. niger*, 10<sup>4</sup> spores of wild-type strains (N402 and MA78.6) and selected transformants (L0 to L3) were spotted on MM and CM agar plates containing different Dox concentrations. The plates were incubated at 30°C for up to 7 days, and the colony diameter was measured daily. When Dox concentrations up to 125  $\mu$ g/ml were used, no effect on the growth rate and morphology was detectable. However, concentrations above this level inhibited growth of all *A. niger* strains (~20% growth inhibition in the presence of 200  $\mu$ g/ml Dox) (data not shown).

**System evaluation on an MTP scale.** In order to study the effect of *mluc* copy numbers on luciferase activity, L1 to L3 strains were cultivated in MM for 16 h, after which 0 or 50  $\mu$ g/ml Dox was added and luminescence values were recorded at minute intervals. In the absence of Dox, no luminescence values were detectable for strains L1, L2, and L3, suggesting that the Tet-on system is tight (Fig. 2A). When 50  $\mu$ g/ml Dox was present, *mluc* expression was induced in a gene dosage-dependent manner: luciferase activity was much more highly stimulated in L2 than in L1 but was highest in L3. Most importantly, two independent L1 clones (VG6.1 and VG6.3) showed exactly the same kinetics of luciferase expression, reflecting very good reproducibility of the Tet-on system (Fig. 2A).

To follow inducer-dependent responses, spores of a selected L1 strain were cultivated in MM in the presence of various Dox concentrations, and luminescence and cell density were re-

corded up to 20 h of cultivation. As depicted in Fig. 2B and C, 0.5  $\mu$ g/ml Dox was sufficient to induce *mluc* expression. When higher Dox concentrations were applied, the responses were faster and reached higher levels. To calculate the response time of the system, i.e., how long it takes for Dox to provoke measurable luminescence activities, we cultivated spores from an L0 strain (negative control) and an L1 and L2 strain in MM or CM and added different Dox concentrations after 6 h of growth. The response time was defined as the time when luminescence values of L1 and L2 exceeded the threshold level defined by L0. (Note that in L0, i.e., in the *mluc*-negative control strains VG5.1 and VG7.2, background luminescence values scatter around 50. This value could be a result of some nonspecific autoluminescence of *A. niger* and was set as the threshold value.) As shown in Table 2, the system responded within either a few minutes or a few hours, demonstrating that rapid or delayed induction and a strong or a weak response can be adjusted by varying gene copy numbers and/or inducer concentrations.

**System evaluation on a bioreactor scale.** The Tet-on system showed excellent dose-response characteristics in MTP cultivations, and we therefore wished to assess its performance under controlled growth conditions in bioreactors, i.e., under cultivation conditions required for systems biology research. For this purpose, we conducted single or duplicate batch runs of strains L0 to L3 (see Materials and Methods) and added different Dox concentrations when the cultures entered the early exponential growth phase (run time, about 17.5 h). Mycelial samples were taken till the end of the exponential growth phase (run time, about 26 h), and luciferase activities were determined. As shown in Fig. 3A, strains L0, L1, L2, and L3 reached similar maximum growth rates ( $\mu_{\max}$ ). This suggests that, as already observed during the MTP-based experiments, expression of the system components *rtTA2<sup>S</sup>-M2* and *mLuc*, as well as addition of Dox, did not impair the growth of *A. niger*. Figure 3B and Table 3 reflect the compilation of *mLuc* data obtained from runs in which expression of luciferase was induced in L0, L1, L2, and L3 cultures using 0 or 5  $\mu$ g/ml Dox. In agreement with the MTP data, increased copy numbers of *mluc* resulted in enhanced reporter activities. Most importantly, in the absence of Dox, virtually no reporter activities were measurable in L1, L2, and L3 strains, and the tightness of the system was determined to be higher than 99.5%. Once Dox

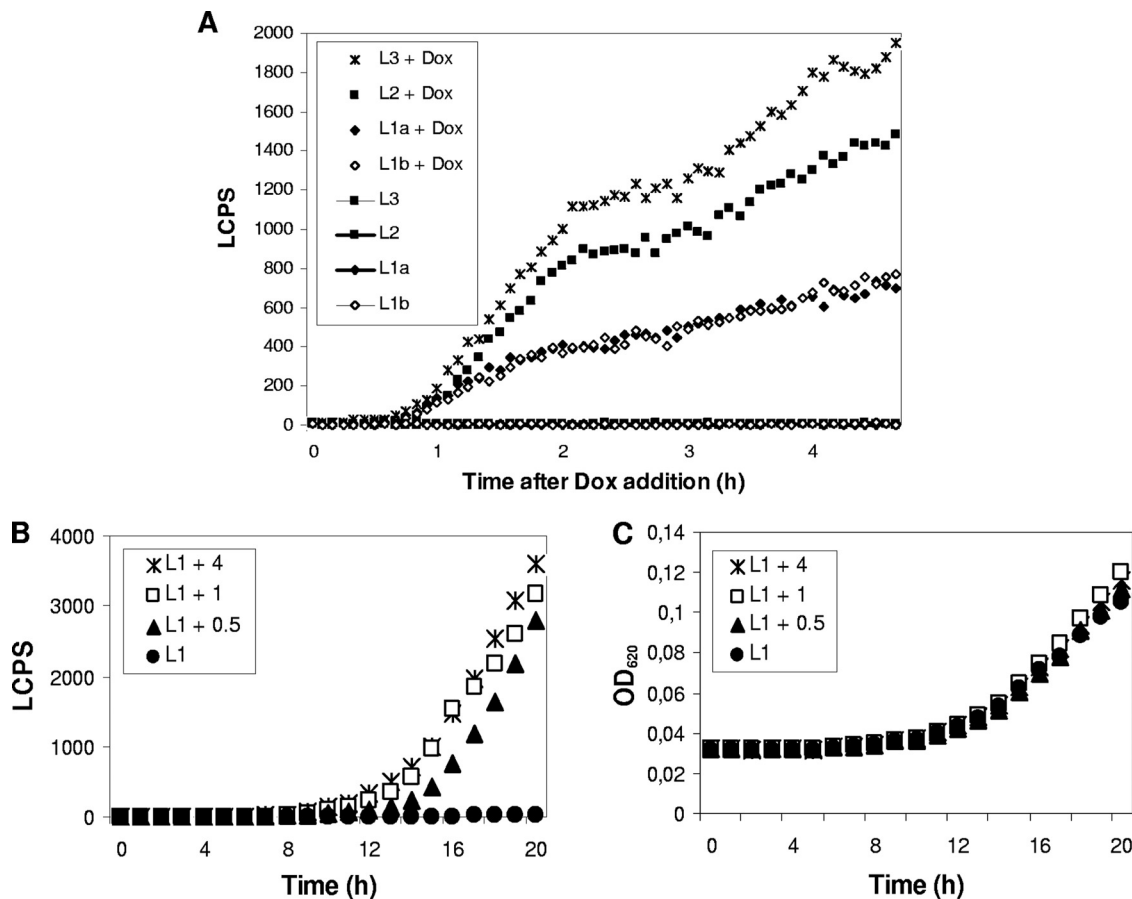


FIG. 2. Gene dosage- and concentration-dependent luciferase activities on an MTP scale. (A) One thousand spores of different strains were inoculated in MM for 16 h (30°C), after which 0 or 50 µg/ml Dox was added and luciferase activities were measured (in LCPS). The strains used were L1a (i.e., strain VG6.1), L1b (i.e., strain VG6.3), L2 (i.e., strain VG8.1), and L3 (i.e., strain VG8.2). (B and C) Different amounts of Dox were added directly to 10<sup>4</sup> spores of an L1 strain (VG6.1) in MM, and the LCPS and optical density at 620 nm (OD<sub>620</sub>) were recorded over time during cultivation. Note that luciferase activities became detectable only after germination had been completed (after ~7 to 8 h).

was added, however, *mluc* expression rapidly switched on and was induced several thousandfold.

To determine the response time under bioreactor conditions, we induced *mluc* expression in the L1 strain using 0, 5, or 20 µg/ml Dox and ascertained the time point when the L0 threshold level was crossed. Dox at a concentration of 5 µg/ml elicited mLuc activity after 9 min and 20 µg/ml Dox elicited mLuc activity after 3 min. In addition, the higher Dox amount

not only caused faster response of the system, it also facilitated higher mLuc levels (Fig. 3C), demonstrating that the system allows not only a qualitative and rapid on-off shift, but also quantitative fine-tuning.

Finally, we wished to know whether expression levels achieved with the Tet-on system can bear comparison with expression levels of the strong *gpdA* promoter, one of the most commonly used promoters for protein overproduction in filamentous fungi. We therefore used real-time PCR to measure *mluc* and *gpdA* mRNA amounts from selected samples, which were harvested from L1 and L2 cultures induced with 5 µg/ml Dox. As summarized in Table 4, the Tet-on system was able to induce *mluc* transcription up to levels comparable with *gpdA* transcript levels. As the transcript stabilities of both *mluc* and *gpdA* mRNAs might differ, a direct quantitative comparison is of course not legitimate. However, the fact that the values obtained for *mluc* and *gpdA* transcript amounts are on the same order of magnitude strongly suggests that the Tet-on system can also be used as a production system for controlled overexpression for proteins of interest.

**Application of the Tet-on system for overexpression studies.** To examine the utility of the Tet-on system for functional gene

TABLE 2. Reaction times of the Tet-on system in MM and CM

Concn of Dox (µg/ml)	Reaction time (h) <sup>a</sup>			
	L1		L2	
	MM	CM	MM	CM
0.5	6	6	2	1
2	3	3	1	1
4	2	2	1	0.1
20	1	ND	1	ND
40	1	ND	0.2	ND

<sup>a</sup> Reaction times were determined as the time points when the luminescence counts of the cultures exceeded the threshold value defined by L0. L1, strain VG6.1; L2, strain VG8.1; ND, not determined.

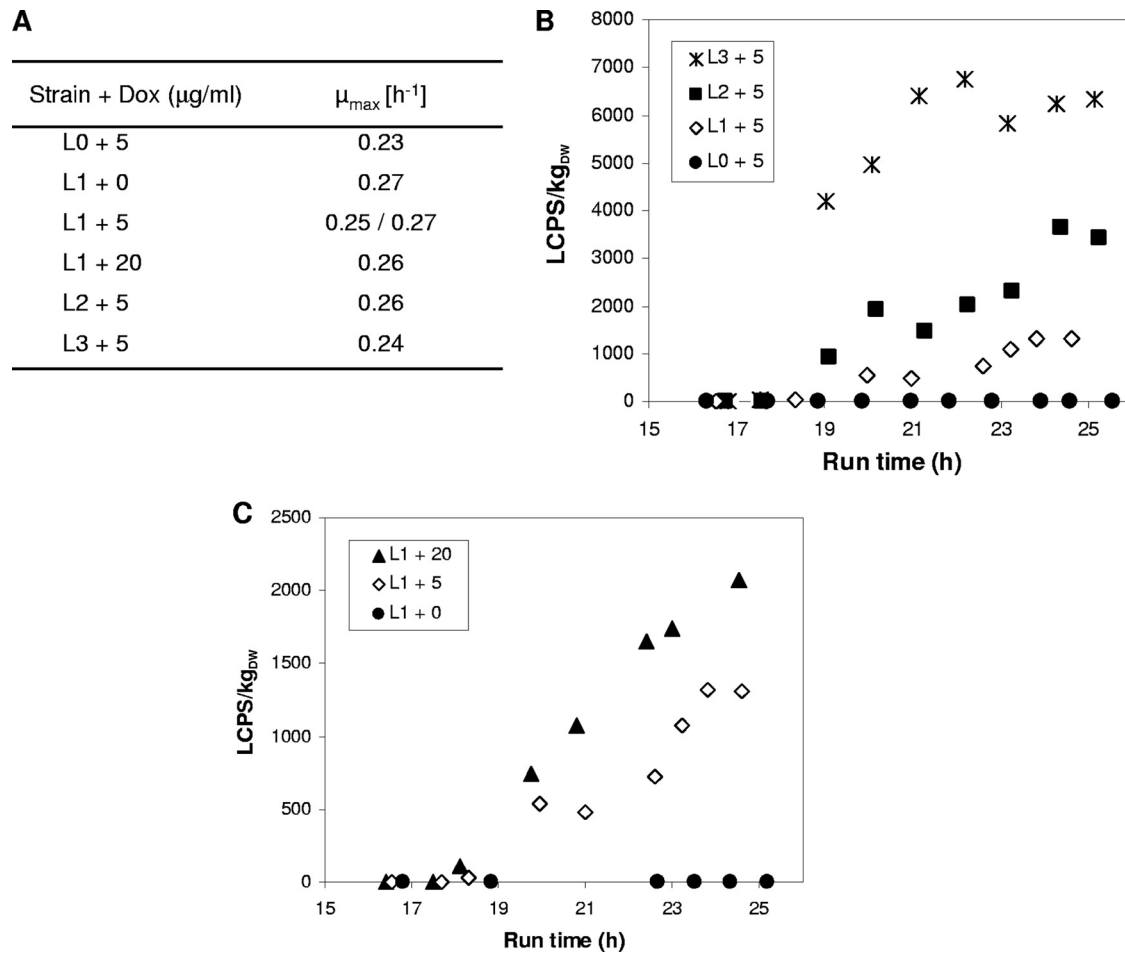


FIG. 3. Gene dosage- and concentration-dependent luciferase activities on a bioreactor scale. (A) Growth rates determined from different bioreactor runs in the presence or absence of Dox. (B) Luciferase profiles of different bioreactor runs. Reporter gene expression was induced after a run time of  $\sim 17.5$  h with  $5 \mu\text{g/ml}$  Dox. (C) Luciferase profiles of the Tet-on system dependent on different Dox concentrations (0, 5, or  $20 \mu\text{g/ml}$  Dox). L0, strain VG7.2; L1, strain VG8.27; L2, strain VG8.1; L3, strain VG8.2. DW, dry weight.

studies, we constructed overexpression mutants for six predicted ORFs selected from the genome sequence of *A. niger* (34). The functions of these ORFs, temporarily named *cfrA*, *cfrB*, *cfrC*, *cfrE*, *cfrF*, and *cfrG*, are completely unknown. However, our previous transcriptomic analyses suggested that these genes might play important roles in the survival of *A. niger*

under stress conditions (27). We have thus rationally selected these six candidate genes for our analyses. The sequences of the GOIs were integrated into pVG2.2, downstream of the *tetO7::Pmin* sequence, and the six plasmids obtained were used to transform strain AB4.1 (*pyrG*<sup>-</sup>). Transformants carrying integrations of *tetO7::Pmin::GOI* at the *pyrG* locus were iden-

TABLE 3. Gene dosage-dependent tightness and fold induction of the Tet-on system

Run code	Strain used	Concn of Dox ( $\mu\text{g/ml}$ ) <sup>a</sup>	LCPS/kg <sub>DW</sub> <sup>b</sup> before Dox addition (at 17 h)	Tightness <sup>c</sup> (%)	LCPS/kg <sub>DW</sub> (at 26 h)	Fold induction <sup>d</sup>
L0 + 5	VG7.2	5	0.82		1.41	
L0 + 5	VG7.2	5	0.80		1.35	
L1 + 0	VG8.27	0	1.39	99.96	1.38	0
L1 + 5	VG8.27	5	1.52	99.96	1,316	950
L1 + 5	VG8.27	5	0.85	99.96	1,312	950
L2 + 5	VG8.1	5	9.21	99.82	4,466	3,200
L3 + 5	VG8.2	5	32.1	99.51	6,403	4,600

<sup>a</sup> Dox was added after about 17.5 h run time.

<sup>b</sup> DW, dry weight.

<sup>c</sup> The tightness (%) of the system was calculated from the LCPS/kg<sub>DW</sub> values at 26 h ( $t_2$ ) and 17 h ( $t_1$ ) using the following formula:  $100 - 100 \times (\text{LN}_{t_1} - \text{L0}_{t_1}) / (\text{LN}_{t_2} - \text{L0}_{t_2})$ , where LN is L0, L1, L2, or L3.

<sup>d</sup> Fold induction was calculated as follows:  $\text{LN}_{t_2} / (\text{L1} + 0)_{t_2}$ .

TABLE 4. Strength of the Tet-on system compared to the *gpdA* promoter

Run code	Strain used	Run time (h)	Time after Dox addition (h)	<i>mluc</i> expression compared to <i>gpdA</i> expression (%) <sup>a</sup>
L1 + 5	VG8.27	20.9	3.5	18 ± 2
		24.4	6.9	72 ± 1
L2 + 5	VG8.1	21.2	3.7	84 ± 16
		25.1	7.6	810 ± 110

<sup>a</sup> Determined by quantitative real-time reverse transcription (RT)-PCR using *H2B* as a reference gene. Mean values from technical triplicates are given.

tified using Southern blot analyses (data not shown). For comparison, deletion strains of the GOIs using MA70.15 (*pyrG*<sup>-</sup>  $\Delta$ *kusA*) as a recipient strain were constructed, as well (Table 1 and data not shown).

The OE*cfr* and *cfr* deletion ( $\Delta$ *cfr*) strains were cultivated on MM agar plates in the absence or presence of 1 M NaCl (Fig. 4). Phenotypic analysis of the  $\Delta$ *cfr* strains revealed that only two out of six  $\Delta$ *cfr* strains ( $\Delta$ *cfrF* and  $\Delta$ *cfrG*) showed a growth- and sporulation-inhibited phenotype, implying that only *cfrF* and *cfrG*, but not the other four *cfr* genes, are important for the survival of *A. niger* under salt stress. In contrast, all six OE*cfr* strains showed a clear stress phenotype under induced conditions (MM agar plates supplemented with Dox and NaCl), strongly indicating that each of the six *cfr* genes is important for the *A. niger* salt stress response and not only *cfrF* and *cfrG*, as inferred from the deletion analysis. Hence, the conditional

Tet-on system can uncover functional information for *A. niger* genes that lack a loss-of-function phenotype.

**Application of the Tet-On system for loss-of-function studies.** We finally tested the applicability of the system for the establishment of conditional knockout mutants. Our interest was especially focused on the question of whether it is possible to construct mutants that are viable under certain conditions although with essential genes deleted. The approach pursued the idea that deletion of an essential gene of interest in a *tetO7::Pmin::GOI* background strain could result in a viable strain when cultivated in the presence of Dox but in a lethal phenotype when cultivated in medium lacking Dox. To test this approach, we selected the  $\gamma$ -actin gene (*actA*), which is essential for filamentous fungi, as a candidate gene. Following the approach described above for the OE*cfr* strains, we constructed an OE*actA* strain, which was indistinguishable from the control strain, L0, in the absence and presence of Dox (Fig. 5). This OE*actA* strain was transformed with an *actA* disruption cassette gene containing hygromycin as a selection marker (see Materials and Methods). Three primary transformants ( $\Delta$ -OE*actA*) were isolated and purified on plates containing 100  $\mu$ g/ml hygromycin and 4  $\mu$ g/ml Dox and were verified by Southern analysis to lack *actA* (data not shown). As none of the three  $\Delta$ -OE*actA* strains was able to grow on medium lacking Dox (Fig. 5 and data not shown), we concluded that this condition indeed led to a complete knockout situation. However, growth of the  $\Delta$ -OE*actA* strains was rescued in medium containing increasing amounts of Dox (Fig. 5), most likely because expression of *actA* was induced by the Tet-on system. Hence, the approach followed here can be used to generate

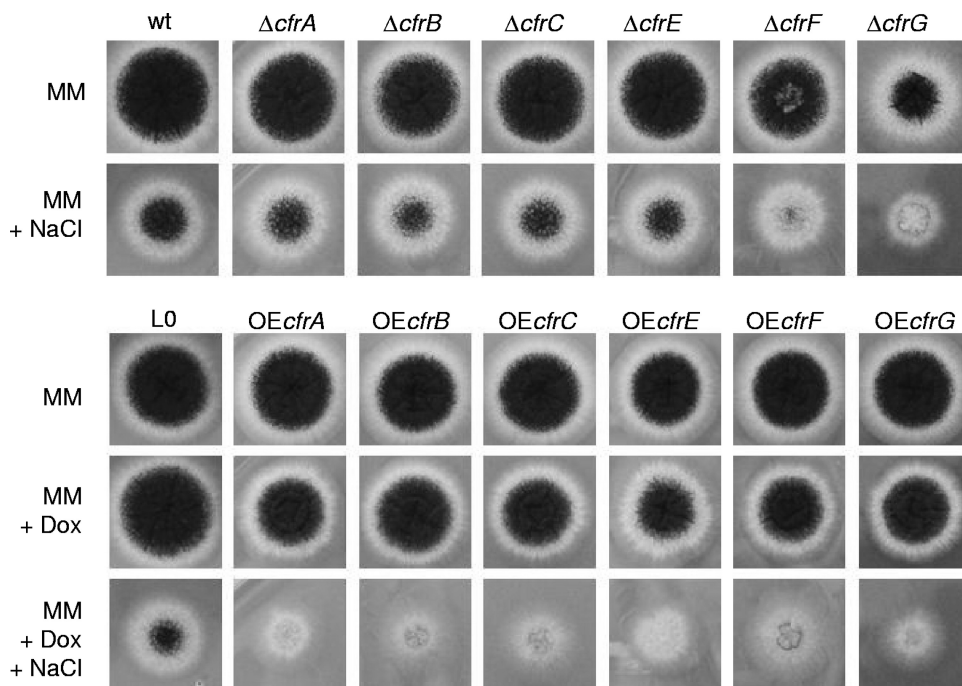


FIG. 4. Phenotypes of  $\Delta$ *cfr* and OE*cfr* strains under different growth conditions. One thousand spores of the different strains were inoculated on MM supplemented with 1 M NaCl and/or 20  $\mu$ g/ml Dox. The plates were cultivated for 72 h at 30°C. MA77.1 (wild type [wt]) and VG5.1 (L0) were used as corresponding control strains. Note that none of the six OE*cfr* strains displayed a mutant phenotype when cultivated under noninduced conditions (MM agar plates lacking Dox), demonstrating that the Tet-on system is indeed tight.

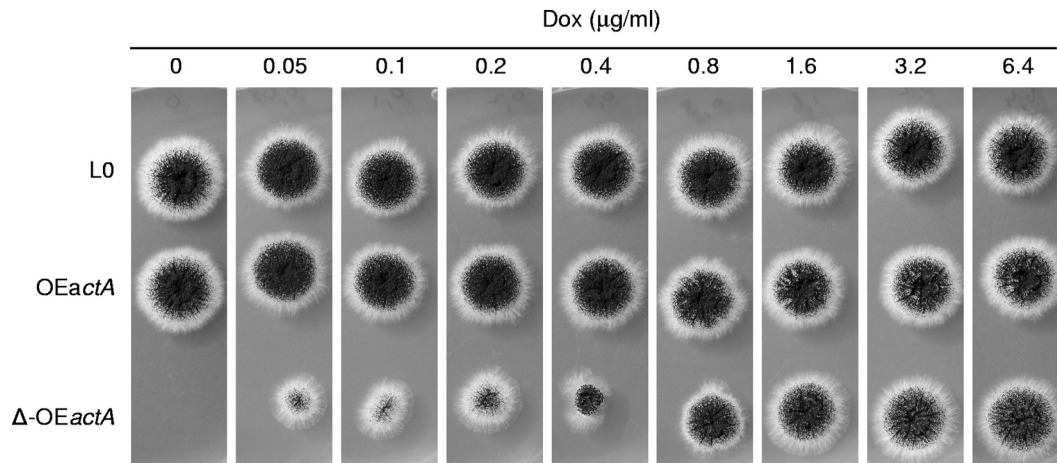


FIG. 5. Phenotypes of *OEactA* and  $\Delta$ -*OEactA* strains in the presence or absence of Dox. One thousand spores of both strains were inoculated on MM supplemented with the indicated Dox concentrations. The plates were cultivated for 65 h at 30°C. VG5.1 (L0) was used as a control strain.

and study three different phenotypes in a single strain: the knockout phenotype, the wild-type phenotype, and the overexpression phenotype.

## DISCUSSION

The postgenomic era calls for new approaches to substantially understand the growth, metabolism, and behavior of filamentous fungi. To better exploit these organisms in biotechnology and to understand the emergence and progression of fungal diseases, functional genomics and systems biology attempts are under way to uncover the relationship between fungal genomes and phenotypes. Both approaches require that expression of the genes of interest can be controlled quantitatively and temporally. However, the ability to control gene expression in filamentous fungi is so far restricted to metabolism-dependent promoters and/or to promoters that do not allow fine-tuning.

In this study, we developed an artificial gene expression system based on the Tet-on system for the industrial model fungus *A. niger* and systematically evaluated its performance using different settings. As a reporter gene, we used the luciferase gene *mluc*, encoding a reporter that is sensitive and nondestructive and has a short half-life (14, 29), thus being especially suited to monitor and measure temporal changes in transcription on line. We demonstrated that on both MTP and bioreactor scales, the Tet-on system can be kept silent in the absence of Dox but becomes rapidly and strongly induced after Dox addition in a gene dosage- and inducer concentration-dependent manner. Hence, the system enables precise gene expression control, which is an essential prerequisite for systems biology approaches. In addition, the Tet-on system provides a wide range of promoter strengths, from very low to very high levels, which are competitive even with the strong *gpdA* promoter. Most importantly, the newly designed Tet-on system for *A. niger* has significant advantages over the previously described Tet-on system for *A. fumigatus* (45). On one hand, it allows site-specific integration of the system at the *pyrG* locus, which ensures stringent control over gene expression. On the other hand, the presence of both the regulator and response

modules on a single plasmid ensures that the transactivator *rtTA2<sup>S</sup>-M2* cannot be titrated away if multicopy integrations are aimed for (e.g., when very high expression levels of the GOI are intended and some background expression is acceptable). Irrespective of which of the two *Aspergillus* Tet-on systems is applied, the overall performance and efficiency of the system depends on the activity of the *gpdA* promoter, which has to ensure high-level expression of *rtTA2<sup>S</sup>-M2*. To the best of our knowledge, no condition under which the *gpdA* promoter is silent is known.

We foresee many implementations of the Tet-on system in filamentous fungi, and we demonstrated in this work the proof-of-principle for two possible applications in *A. niger*. First, we showed that the Tet-on system can be used for the establishment of conditional overexpression mutants. As discussed above, loss-of-function screens in yeast, flies, and worms in the majority of genes did not uncover gene functions, in contrast to gain-of-function screens (38, 41). We thus rationally selected six *cfr* genes from the genome of *A. niger* and constructed the respective deletion and Tet-on-based overexpression strains. Based on our previous transcriptomic analyses (27), we suspected that these *cfr* genes might be important for the survival of *A. niger* under stress conditions. Whereas the analysis of  $\Delta$ *cfr* strains illuminated a potential role for two out of six *cfr* genes in stress resistance, all six *OEcfr* strains displayed a salt stress phenotype, suggesting that Tet-on-based overexpression can indeed be applied as an effective functional genomics tool for *A. niger*. Second, we demonstrated the power of combining the Tet-on system with a deletion approach. Using the gene *actA* as an example, we showed that it is possible to turn *actA* expression on or off in a  $\Delta$ -*OEactA* strain and that it is thus possible to generate a conditional viable strain, although with an essential gene deleted. This approach permits unprecedented flexibility in dissecting gene functions; within a single strain, a loss-of-function, an overexpression, and a wild-type situation can be adjusted in a Dox-dependent manner.

Many more applications of the Tet-on system can be envisioned for *A. niger* and other filamentous fungi. For example, conditional knockdown mutants can be generated by expressing the gene of interest in an antisense direction from the



*tetO7::Pmin* promoter. For systems biology approaches, the Tet-on system offers excellent options as well, e.g., for studying the interaction and cross talk of signaling cascades and metabolic pathways. For example, the expression of a gene of interest (e.g., encoding a signaling protein) can be rapidly modulated at a specific time, and the temporal response of the respective signaling cascade(s) and the performance of gene/protein networks can be studied. Finally, the Tet-on system appears to be perfectly suited for the production of recombinant proteins in *A. niger*. Controlled gene expression in microbial production strains is often needed for maximum protein yield and quality (20, 30) and has to be monitored, especially when expressing potentially toxic products. The tightness and strong inducibility of the Tet-on system suggests that it can become an efficient tool for protein overproduction in filamentous fungi.

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