Fenhexamid - an efficient and inexpensive fungicide for selection of *Magnaporthe oryzae* transformants

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Accepted: 23 November 2021 / Published online: 1 December 2021 © The Author(s) 2021

Abstract *Magnaporthe oryzae* is one of the most economically important phytopathogenic fungi, and is used as a model organism to study plant-pathogen interactions. To unravel the infection process, forward and reverse genetic approaches are essential, but are often hindered by the lack of a straightforward selection procedure for transformants. Here we report on the use of fenhexamid, an inhibitor of ergosterol biosynthesis, for selection of *M. oryzae* transformants. An allele of the sterol 3-ketoreductase gene of *Fusarium fujikuroi* (*FfERG27*), known to confer resistance to fenhexamid, has already been used successfully with transformants of *Botrytis cinerea*. Our results demonstrate that expression of the *FfERG27* allele in *M. oryzae* also enables highly efficient selection of transformants on fenhexamid-containing media. The use of fenhexamid is an inexpensive alternative for selection as compared to commonly used antibiotics like hygromycin. No impact on growth and infection phenotypes of fenhexamid resistant *M. oryzae* mutants was detected, which underpins its usefulness for selecting *M. oryzae* transformants.

Keywords *Magnaporthe oryzae* · fenhexamid · *ERG27* · sterol 3-ketoreductase · selection · resistance marker

Introduction

The ascomycete *M. oryzae*, best known as the causal agent of rice blast disease, has become a major threat not only for rice but also for wheat cultivation (Martinez et al., 2021; Portz et al., 2021). For functional genetic analyses, selection markers are necessary to identify respective mutants. However, for *M. oryzae*, only a few markers are well-established which e.g. limits the option of multiple gene deletions. For example, hygromycin is commonly used but a drawback is its high price and the relative high amount (0.2 to 0.6 mg ml⁻¹) needed for selection of *M. oryzae* transformants (Jeong et al., 2007; Leung et al., 1990; Zhang et al., 2019). Alternatives for selection based e.g. on the use of glufosinate, benomyl or carboxin have rarely been reported (Guo et al., 2016; Jacob et al., 2015; Kachroo et al., 1997).

Here, we report on the use of fenhexamid resistance conferred by the gene *FfERG27* of *Fusarium fujikuroi* to select transgenic *M. oryzae* mutants as described previously for *Botrytis cinerea* (Cohrs et al., 2017). Fenhexamid
(N-(2,3-Dichlor-4-hydroxyphenyl)-1-methylcyclohexancarboxamid), marketed by Bayer CropScience as Teldor®, is a fungicide effective against B. cinerea, Sclerotinia sclerotiorum and Monilinia spp. (Cohrs et al., 2017; Billard et al., 2011; Debieu et al., 2013). Fenhexamid inhibits the biosynthesis of sterols and belongs to the chemical family of hydroxyanilides (Billard et al., 2011; Debieu et al., 2001). Sterols, especially ergosterols, are known to be important for membrane integrity in fungi. Fenhexamid inhibits the C4-demethylation of ergosterol by blocking the sterol 3-ketoreductase, a conserved enzyme present in all euascomycetes (Billard et al., 2011; Debieu et al., 2001). Due to intensive use of the fungicide, fenhexamid resistant B. cinerea isolates have been observed in the field (Billard et al., 2011; Grabke et al., 2013). The fenhexamid resistance was associated with changes in the amino acid sequence of the sterol 3-ketoreductase at position 412 and two other amino acid positions that have a slightly less impact on fungicide resistance (Billard et al., 2011; Fillinger et al., 2008). Also for F. fujikuroi a field isolates (IMI 58289) was identified with an allele of the ERG27 gene (FfERG27) that mediates resistance against fenhexamid and which was used for selection of B. cinerea transformants (Cohrs et al., 2017). Using this knowledge, we broaden the tool box for M. oryzae by establishing fenhexamid as an effective and cost-efficient selection marker in transformation.

**Experimental procedures**

**Fungal cultivation**

The fungus was cultivated on complete medium (Talbot et al., 1993) or potato dextrose agar (PDA; Sigma Aldrich St. Louis, USA) at 23-25 °C in the dark.

**Sporulation and inoculation**

For sporulation a piece of mycelium was transferred to oatmeal agar (20 g l⁻¹ agar, 2 g l⁻¹ yeast extract, 10 g l⁻¹ starch, 30 g l⁻¹ oat flakes) and incubated at 26 °C under fluorescent tubes (36W/840, Osram, Germany) mixed with blacklight tubes (TLD 36W/08 BLB UV, Phillips, Netherlands) for seven to fourteen days. Conidia were washed from agar plates in ddH₂O and filtered through three layers of gauze. For inoculation of barley plants, concentration of conidia was adjusted to 40000 conidia ml⁻¹ and diluted 1:1 with a solution containing 1 g l⁻¹ gelatin and 0.5 ml l⁻¹ Tween 20. The suspension was sprayed onto seven-day-old primary leaves of barley cv. Ingred and plants were cultivated in a growth chamber as described in Delventhal et al. (2014). Seven days post inoculation, leaves were harvested, placed on water agar plates and photographed using a Panasonic DMC-TZ61 camera. To determine disease severity, the infected part of each leaf was calculated in relation to the total leaf area using the image software Assess 2.0 (Image analysis software for plant disease quantification 2.0, https://my.apsnet.org/ItemDetail?ProductCode=43696). To analyze the infection structures in vitro, 10 µl of a spore suspension were placed on a glass slides and examined by microscopy after 14 or 24 hours.

**Blast-analysis, alignments and database search**

NCBI protein blast was used to obtain protein sequences from public depositories (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Sequences were aligned using Jalview and Muscle with default settings (http://www.jalview.org/). Genomic sequences of strain 70-15 were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/) and sequences of other Magnaporthe isolates were obtained from GEMO (http://genome.jouy.inra.fr/gemo/). Sequences of the ERG27 gene of the M. oryzae isolates CM28, PH14 and INA72 were analyzed after amplification by PCR using Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with primers MoErg27_F and MoErg27_R (Table S1). PCR products were sequenced by Microsynth Seqlab, Göttingen, Germany.

**Generation of MoALB1 deletion mutants and FenR mutants**

Generation of M. oryzae Guy11 protoplasts was done by glucanex treatment as described by Leisen et al. (2020). The donor template, containing the FenR-cassette (PtrpC, FfERG27, TniaD), was amplified with Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with primers MoAlb1MH_F and R (Table S1) and pTEL-Fen
as template (Leisen et al., 2020). A 50 bp overhang, homologous to sequences at the MoALB1 locus, was added to primer sequences to enable homologous recombination similar to the procedure described by Foster et al. (2018). The PCR product was purified with a PCR-clean-up kit (NucleoSpin™ Gel and PCR Clean-up Kit, Macherey-Nagel, Dueren, Germany) and solved in Tris-CaCl2 buffer (10 mM Tris-HCl, 1 mM EDTA, 40 mM CaCl2, pH 6.3) at a final concentration of 1 μg μl−1. The sgRNA was processed and purified as described in Leisen et al. (2020) using the primer sgRNA-MoAlb1. For the transformation, 120 μl protoplast suspension with a concentration of 1.5*10⁸ protoplast ml⁻¹ were mixed with 6 μg donor DNA and the ribonucleoprotein (RNP) in a total volume of 60 μl Tris-CaCl2 buffer. For RNP formation, 2 μg of sgRNA were pre-incubated with 6 μg Cas9-enzyme for 1 h at 37 °C. For a non-directed integration, the resistance cassette was amplified with primers Fen-F and Fen-R (Table S1) and transformed without Cas9-enzyme. Thereafter, protoplasts were poured into 15 ml CM agar, containing 1.2 M sucrose for osmotic stabilisation. The next day, 20 ml top agar were added containing fenhexamid (Bayer CropScience, Monheim, Germany) in different concentrations in DMSO and added at different concentrations to CM agar. A piece of mycelium from donor plates or conidia was transferred to these agar plates. Growth assays were performed at 25 °C in the dark for four to seven days. Colony area was quantified using the software ImageJ (Schneider et al., 2012). The concentration at which growth was inhibited by approximately 50 % (EC₅₀) in comparison to growth on plates without fenhexamid was determined graphically.

Growth inhibition assays

Commercially available fenhexamid was solved in DMSO and added at different concentrations to CM agar. A piece of mycelium from donor plates or conidia were transferred to these agar plates. Growth assays were performed at 25 °C in the dark for four to seven days. Colony area was quantified using the software ImageJ (Schneider et al., 2012). The concentration at which growth was inhibited by approximately 50 % (EC₅₀) in comparison to growth on plates without fenhexamid was determined graphically.

Verification of FenR integration and dermination of gene copy number

To analyze the integration site of the resistance cassette in the genome, genomic DNA of mutants was extracted as described in Leisen et al. (2020). Verification of FenR integration was done by PCR using primers Fen_F and Fen_R. For analysis of MoALB1 gene deletion mutants, primers binding to the border regions up- or downstream the integration site were used (MoALB1_F and MoALB1_R) and respective PCR products were sequenced to validate integration of the resistance gene into the MoALB1-locus.

Genomic DNA was used to determine the copy number of the FenR-gene in an assay based on an study described in Solomon et al. (2008). For normalization of gene copy number, the single copy gene MoACTIN was selected as reference gene. qPCR was performed in technical triplicates with primers MoACTIN_F/MoACTIN_R and FfERG_F/ FfERG_R, at a concentration of 0.5 μM, for genes MoACTIN and FfERG27, respectively, in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, California, USA). For analysis, 5 μl iTaq™ Universal SYBR® Green Supermix (BioRad, Hercules, California, USA) were used according to the manufacturer’s instruction. Serial dilutions of genomic DNA from a FenR mutant (50 ng, 5 ng, 0.5 ng and 0.05 ng DNA in the reaction mixture) allowed to calculate the primer efficiencies for E_MoAKTIN = 2.002 and E_FfERG27 = 2.012 using the equation E = 10^[−1/slope]. By this, an amount of 0.5 to 5 ng of genomic DNA was determined as optimal for each reaction in the qPCR. Further, we checked the primer efficiency of MoAKTIN and FfERG27 for each probe using 0.5 ng and 5 ng of genomic DNA in the reaction mixture. The following qPCR conditions were chosen: 95 °C for 3 minutes, 40 cycles of 3 seconds at 95 °C and 30 seconds at 60 °C.

To validate that this method can be used for determination of gene copy numbers, we first determined in a randomly selected FenR mutant the relative ratio of MoACTIN to FfERG27 using the Δct-method and the equation \( \text{Ratio} = 2^{\Delta C_T(\text{calibrator} - \text{sample})} \) (Livak & Schmittgen, 2001). In this case the ratio was close to 1:1, indicating the presence of a single insertion of FfERG27 in the genome of this mutant. For calculation of our data we wanted to use the ΔACT-method (Pfaffl, 2001) which is more precise because the primer efficiencies of each probe is considered. The ratio was calculated using the equation \( \text{Ratio} = \frac{E_{\text{target}}^{\Delta C_T(\text{calibrator} - \text{sample})}}{E_{\text{ref}}^{\Delta C_T(\text{calibrator} - \text{sample})}} \). For “calibrator”, the value for the previously mentioned FenR mutant with a 1:1 ratio of the transcripts from MoACTIN and FfERG27 was selected.

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Results

Fenhexamid inhibits the growth of *Magnaporthe oryzae*

We tested whether *M. oryzae* isolate Guy11 was sensitive to fenhexamid. Therefore, mycelial growth, germination of conidia and regeneration of protoplasts were analyzed on CM agar supplemented with concentrations of fenhexamid in the range between 0.001 and 100 mg l\(^{-1}\) (Fig. 1). Inhibition of protoplast regeneration started at 1 mg l\(^{-1}\) fenhexamid and at 10-100 mg l\(^{-1}\) no mycelial growth was observed (Fig. 1a). Similar inhibitory concentrations were determined for germination of conidia (Fig. 1b). Microscopy revealed that while at 1 mg l\(^{-1}\) fenhexamid conidia still germinated no germination was found at higher concentrations (> 10 mg l\(^{-1}\)). The same tendencies were observed for the inhibition of vegetative growth of mycelia (Fig. 1c). Therefore, 10-50 mg l\(^{-1}\) fenhexamid were determined as an optimal concentration for distinction of fenhexamid resistant (Fen\(^{R}\)) or sensitive (Fen\(^{S}\)) *M. oryzae* genotypes.

Expression of *FfERG27* leads to fenhexamid resistance of *M. oryzae*

To determine whether *FfERG27* confers fenhexamid resistance in *M. oryzae*, a construct containing the *FfERG27* expression cassette was amplified from

![Table of concentrations of fenhexamid](image)

**Fig. 1** Growth inhibition of *M. oryzae* Guy11 by fenhexamid. Concentrations of fenhexamid ranging from 0.001 to 100 mg l\(^{-1}\) were tested for an inhibitor effect on different *M. oryzae* structures. **a** Protoplasts of *M. oryzae* were poured in osmotic stabilized CM agar overlayed by a thin layer of CM agar supplemented with fenhexamid as indicated. Photographs were taken after six days. **b** A suspension with conidia (10000 conidia ml\(^{-1}\)) was placed on agar plates containing fenhexamid. After four days, conidia were analyzed by microscopy for germination and formation of vegetative hyphae. Scale bars: 60 μm. **c** A piece of *M. oryzae* mycelium was placed on CM agar supplemented with different concentrations of fenhexamid and colony size was recorded after six days. Results shown are from a single experiment which was repeated with similar results.
the telomere vector pTEL-Fen (Fig. 2a). The purified PCR-product was introduced into *M. oryzae* Guy11 by protoplast transformation. In this way, we were able to generate *Fen*<sup>R</sup> *M. oryzae* transformants (*MoFen*<sup>R</sup>) using a concentration of 30 mg l<sup>-1</sup> fenhexamid in the top agar for selection (Fig. 2b). Transformation efficiency varied between 8 and 20 colonies per μg donor-DNA. EC<sub>50</sub> values for sensitivity against fenhexamid were determined to increase from 1.5 to 8 mg l<sup>-1</sup> fenhexamid for the wild type and transformants with a single insertion of the resistance gene, respectively (Fig. 2c). Transformants with two insertions of the resistance gene showed an increased resistance level against the fungicide (Figure S1). Based on these results, and in agreement with data shown in Fig. 1, a concentration of 30 mg l<sup>-1</sup> fenhexamid in the top-agar, at which wild type colonies did not grow, was used for selection in subsequent experiments.

Expression of *FfERG27* does not alter growth and infection phenotype of *M. oryzae*

Next, we tested the versatility of the *Fen*<sup>R</sup> marker gene (*FfERG27*) by investigating potential effects on vegetative growth under normal or stress conditions using the three aforementioned mutants with a single insertion of the resistance gene. No differences were observed in growth on agar plates or formation of *in vitro* infection structures for the transformants compared to the wild type strain (Figure 3a and b). Similarly, different pH and osmotic or oxidative stresses did not cause any growth alteration between the wild type strain and *MoFen*<sup>R</sup> mutants (Fig. 3c). Additionally, we tested the infection phenotype of *Fen*<sup>R</sup> transformants on barley plants which, again, revealed no substantial differences in virulence compared to the wild type isolate (Fig. 4a-c).

Generation of gene deletion mutants by gene replacement

To ensure that the selection system can be used for generation of a targeted gene-deletion, fenhexamid resistant mutants of *M. oryzae* strain Guy11ku80 were generated using protoplast transformation as described in Leisen et al. (2020).

For this, the Cas9 endonuclease was pre-incubated with a sgRNA targeting *MoALB1*, a gene coding for a polyketide synthase which is involved in melanin biosynthesis. A deletion of this gene leads to whitish albino mutants (Foster et al., 2018). A co-transformation assay was done with a repair template containing (MoFenR-1/-2/-4) with a single insertion of the resistance gene. (MoFenR-1/-2/-4) without fenhexamid (MoWT) and three independently generated transformants (Fig. 2a). Schematic presentation of the *FfERG27* (*Fen*<sup>R</sup>) expression construct. *b* Pictures of *M. oryzae* colonies regenerated form wild type protoplasts on plates without or with fenhexamid (30 mg l<sup>-1</sup>). Protoplasts transformed with the *FfERG27* grew on fenhexamid selection media. Photographs were taken five days after sub-cultivation. *c* Comparison of mycelial growth of the wild type isolate *M. oryzae* Guy11 (MoWT) and three independently generated transformants (MoFenR-1/-2/-4) with a single insertion of the resistance gene. Growth area on plates with different concentrations of fenhexamid was calculated relatively to those without the fungicide which were set to 100 %. Evaluation was done using the image processing software ImageJ. Data shown are based on three independently evaluated wild type and transformed genotypes, respectively, and error bars present the standard errors from three technical replicates for each isolate.

![Fig. 2 Usage of fenhexamid for selection of *M. oryzae* transformants.](image-url)
homologous flanks to the genomic region of MoALB1 and the expression cassette for FfERG27 (Fig. 5a). Using a concentration of 30 mg l⁻¹ fenhexamid for selection, as determined above, whitish transformants were observed (Fig. 5b). Randomly picked transformants were confirmed by PCR and sequencing to contain the FenR gene at the expected integration site in the MoALB1 locus (Fig. 5b, c). A qPCR assay for determination of gene copy numbers revealed that out of ten randomly selected transformants nine had a single insertion of the resistance gene, most likely by homologous recombination, while one had two insertions, the second of which probably is due to ectopic integration in the genome (Figure S1).

Distribution of Fen⁸/Fen₈ in different Magnaporthe-isolates

Next, we tested whether the Fen⁸ phenotype is similarly efficient for selection with additional isolates of the Magnaporthe-species complex. Different isolates were cultivated on agar containing 30 mg l⁻¹ fenhexamid (Figure 6). Growth of Magnaporthe-isolates 70-15, BF17, BR116.5, BR29, BR32, CD180, Dsa1J, FR13, Guy11, H373, MoAR06, Mo33AR, TH16 und NaOH or HCL, 30 mg l⁻¹ fenhexamid (Fen) was added to test for Fen⁸, 0.5 M and 1 M NaCl and 1.4 M Sorbitol were applied to change the osmotic potential and 10 mM H₂O₂ and 2 mM DTT was added to generate oxidative stress. After five days photographs were taken to calculate the colony area using the software ImageJ. The experiment was performed three times. Columns represent the mean values and standard errors from three technical replicates of a representative experiment. No statistically significant differences could be found by Student’s t-test (p<0.01, MoWT vs. MoFen⁸), except for the growth in presents of fenhexamid.
Fig. 4  Infection phenotype of MoWT and MoFenR mutants on barley. Leaves of barley cultivar Ingrid were inoculated with the wild type (MoWT) or transformants (MoFenR) and pictures were taken at seven days post inoculation (a). A magnification of disease symptoms is shown on the right side. The image based software Assess 2.0. was used to distinguish infected leaf area from green tissue as shown exemplarily in (b). Data generated this way were used for quantification of infected area per leaf (c). Data shown in the boxplot diagram are from 15 leaves for each genotype collected in three biological replicates. No statistically significant differences were found by Student’s t-test between MoWT and each of the MoFenR mutants.
TH6772 were strongly inhibited with a reduction in growth area higher than 85% compared to their growth on agar without fenhexamid. Interestingly, isolates CD73.1, CM28, Dsa7J, INA 72 or PH55 seemed to be slightly less sensitive to fenhexamid with an inhibition of the mycelial growth less than 80% (Figure 6).

To test whether this phenotype correlates with differences in the nucleotide sequences among ERG27 alleles, amplification from genomic DNA and sequencing was done for isolates CM28, PH14 and INA72. For comparison, sequences of Magnaporthe isolates 70-15, Guy11, BR29, FR13, BR32, TH16 and CD156 were obtained from the Magnaporthe genome database GEMO (http://genome.jouy.inra.fr).

Fig. 5 Generation of FenR<sup>+</sup> MoALB1 gene deletion mutants by gene replacement. a Schematic illustration of the CRISPR/Cas<sup>9</sup> mediated gene replacement of MoALB1 by a fenhexamid resistance cassette (FenR<sup>+</sup>) by homologous recombination. Positions of primers used for validation of the insertion of the repair template are shown. b 16 transformants were isolated from selection plates in each of two independent experiments which had a whitish mycelium in comparison to the wild type strain Guy11ku80. c Two independent experiments were performed to yield MoALB1 gene deletion mutants. Concentration of RNP and DNA-repair template are indicated. A total of 58 and 47 mutants were counted in the first and second experiment to be resistant to fenhexamid, respectively. From each experiment 16 of these transformants were further cultivated and all of them had a whitish mycelium. Randomly selected individuals (five for each experiment) were confirmed by PCR to contain the FenR<sup>+</sup> gene. From these PCR-products, three were randomly selected and sequenced. This verified the expected insertion of the DNA-repair template at the MoALB1 locus by homologous recombination.

Fig. 6 Inhibition of mycelial growth by fenhexamid for different Magnaporthe-isolates. Growth assay with different Magnaporthe-isolates was performed in the presence of 30 mg L<sup>-1</sup> fenhexamid. Seven days after sub-culture photographs were taken and the colony area was calculated using the software ImageJ. The growths inhibition rate for each isolate was calculated by comparing the growth area on media with or without fenhexamid. Results shown are the mean and standard error from a representative experiment with three replicates for each isolate. The experiment was repeated with similar results.
fr/gemo)). This revealed that deduced amino acid sequences of Erg27 were identical for isolates, except for BR29 which, instead, was highly similar to Erg27 of Pyricularia grisea strain N907 (Figure S2). With respect to the different levels of sensitivity towards fenhexamid among the tested isolates, we conclude that other factors than the sequence of the ERG27 gene are also involved in modulation of sensitivity of M. oryzae against fenhexamid. This, in turn, underpins the need to test fenhexamid sensitivity for each wild type isolate for which fenhexamid will be used during selection of transformants.

Discussion

Fenhexamid in combination with the resistance-conferring gene FfERG27 of F. fujikuroi is well-established for selection of B. cinerea transformants (Cohrs et al., 2017). The fungicide can be used in its industrial formulation and is less expensive compared to commonly used antibiotics, such as e.g. hygromycin. M. oryzae isolate Guy11 is sensitive to fenhexamid with an EC50 value of 1.5 mg l−1 fenhexamid for growth of vegetative mycelium on artificial media (Figure 1). This is more than a magnitude higher compared to B. cinerea, for which about 0.1 mg l−1 is sufficient to inhibit mycelial growth by 50% (EC50) (Cohrs et al., 2017; Saito et al., 2014). We established that expression of a FfERG27 construct led to fenhexamid resistance in M. oryzae (Figure 2) as previously described for B. cinerea (Cohrs et al., 2017). While transformants were still able to grow at a concentration of 30 mg l−1 fenhexamid provided in the top agar, no “background” growth, e.g. caused by spontaneous mutations, were observed for the wild type (Figure 2). We further demonstrated that the FfERG27 resistance cassette can be efficiently used as repair template using homologous recombination in a CRISPR/Cas9 gene-deletion assay (Figure 5). The precision and efficiency was comparable to results obtained with the use of a hygromycin resistance cassette (Foster et al., 2018; Leisen et al., 2020).

As key requirement, a useful resistance marker should not affect the phenotype under investigation. For FenR strains of B. cinerea (HydR3+) a fitness penalty is reported and because ergosterols are involved in PAMP-triggered immunity an influence on pathogenicity cannot be excluded (Billard et al., 2012; Billard et al., 2011; Rodrigues, 2018; Ziogas et al., 2003). M. oryzae FenR transforms generated in this study, by contrast, were proved to be as virulent as the wild type strain on the host plant barley (Figure 4). Similarly, no differences were observed between FenR transformants and the wild type isolate in response to different pH in the medium and osmotic or oxidative stresses (Figure 3). Based on these data, fenhexamid can be recommended without reservation for selection of M. oryzae transformants. However, a precautionary note has to be added because different M. oryzae isolates or species from the Magnaporthe-species complex differed in their sensitivity towards the fungicide (Figure 6). Notably, strains, such as Guy11 or 70-15, which still build the backbone for experimental use in laboratories, were more sensitive to fenhexamid than the more rarely used isolates CM28, Dsa1J and Ina72. Sequencing of these isolates or data mining in public depositories revealed, except for isolate BR29, identical MoErg27 sequences (Figure S2). Therefore, we concluded that the different degree of fenhexamid sensitivity among isolates must result from a yet unknown mechanism, such as e.g. different capabilities for detoxification or efflux carriers (Deising et al., 2008).

Taken together, we demonstrated that the use of fenhexamid in combination with the resistance-conferring gene FfErg27 represents an efficient selection system for transformants of M. oryzae. The availability of such novel selection markers is an important step forward to facilitate the generation of multiple gene deletions in M. oryzae.

Acknowledgments The Magnaporthe oryzae strains Guy11 and Guy1ku80 were kindly provided by D. Thareau (CIRAD, Montpellier, France). Further Magnaporthe-isolates were kindly provided by Y. Tosa (Kobe University, Japan), A. Perello (Universidad Nacional de La Plata, Argentina) or obtained from the Faculty of Agriculture, Tamagawa University, Machida-shi, Japan. The plasmid pNDF-OCT, encoding the fenhexamid marker gene used in this study, was kindly made available by J. Schumacher (Westfälische Wilhelms-Universität Münster, Germany) to Matthias Hahn (Kaiserslautern University). Alan Slusarenko is kindly acknowledged for critical reading of the manuscript.

Availability of data and material The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author’s contribution All authors have been personally involved in this study. Alex Wegner and Louisa Wirtz
performed most of the experiments. Alex Wegner drafted the manuscript and interpreted the results. Thomas Leisen and Matthias Hahn were involved in the design of experiments and helped in interpretation of results. Ulrich Schaffrath designed experiments and finalized the manuscript. All co-authors red and approved the final version.

**Funding** Open Access funding enabled and organized by Projekt DEAL. Alex Wegner was funded by a RWTH Aachen University scholarships for Doctoral Students.

**Declarations**

**Conflicts of interest** The authors declare that they have no competing interests.

**Ethical declarations** The study does not involve any human participants and/or animals.

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