Subpopulations of hyphae secrete proteins or resist heat stress in *Aspergillus oryzae* colonies

Martin Tegelaar,† Robert-Jan Bleichrodt,† Benjamin Nitsche,² Arthur F. J. Ram² and Han A. B. Wösten ①*©

¹Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.
²Molecular Microbiology and Biotechnology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands.

Summary

Hyphae at the outer part of colonies of *Aspergillus niger* and *Aspergillus oryzae* are heterogeneous with respect to transcriptional and translational activity. This heterogeneity is maintained by Woronin body mediated closure of septal pores that block inter-hyphal mixing of cytoplasm. Indeed, heterogeneity between hyphae is abolished in ΔhexA strains that lack Woronin bodies. The subpopulation of hyphae with high transcriptional and translational activity secretes enzymes that degrade the substrate resulting in breakdown products that serve as nutrients. The role of hyphae with low transcriptional and translational activity was not yet known. Here, we show that this subpopulation is more resistant to environmental stress in *A. oryzae*, in particular to temperature stress, when compared to hyphae with high transcriptional and translational activity. Notably, all hyphae of the ΔhexA strain of *A. oryzae* were sensitive to heat stress explained by the reduced heterogeneity in this strain. Together, we show that different subpopulations of hypha secrete proteins and resist heat stress showing the complexity of a fungal mycelium.

Introduction

Hyphae of filamentous fungi grow at their tips and branch subapically, which gives rise to a network of hyphae called mycelium. Mycelia are heterogeneous with respect to gene expression, growth and secretion (Wösten *et al.*, 2013). For instance, only part of the hyphae at the periphery of the mycelium of *Aspergillus niger* secrete glucoamylase (Wösten *et al.*, 1991) and express its encoding gene (Vinck *et al.*, 2005; de Bekker *et al.*, 2011). Hyphae with high glaA expression also highly express other genes encoding secreted proteins. Moreover, they have a high rRNA content and highly express the glyceraldehyde-3-phosphate dehydrogenase gene gpdA (Vinck *et al.*, 2011). From these studies, it was concluded that a subpopulation of hyphae with ‘high’ and a subpopulation of hyphae with ‘low’ transcriptional and translational activity can be distinguished at the outer part of the vegetative mycelium. Low activity would be sufficient to support growth but high activity would be needed to support secretion of high amounts of protein.

Hyphae of fungi belonging to the Ascomycota and the Basidiomycota are compartmentalized by porous septa. The diameter of the septal pore varies between 50 and 500 nm allowing passage of cytosol and even organelles (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). The septal pores of ascomycetes can be closed by peroxisome-like organelles called Woronin bodies. Deletion of hexA in *Neurospora crassa*, *Magnaporthe grisea*, *Aspergillus oryzae* and *A. niger* results in the absence of Woronin bodies. This abolishes closure of septa after hyphal damage (Jedd and Chua, 2000; Tenney *et al.*, 2000; Soundararajan *et al.*, 2004; Maruyama *et al.*, 2005) and results in excessive cytoplasmic bleeding. Woronin bodies can also plug septa of intact growing hyphae (Markham, 1994; Bleichrodt *et al.*, 2012, 2015a; Steinberg *et al.*, 2017a,b). Plugging of these pores has been shown to block intra- and intercompartmental cytoplasmic mixing (Bleichrodt *et al.*, 2012, 2015a). Consequently, heterogeneity in composition of hyphae and their compartments and hyphae is maintained (Bleichrodt *et al.*, 2012, 2015a).

So far, it has been shown that the subpopulation of hyphae that has high transcriptional and translational activity highly secrete proteins. A role of the lowly active hyphae was not yet clear. In this study, it is shown that these hyphae of *A. oryzae* are more resistant to environmental stress, in particular to heat.
Together, it is concluded that subpopulations of hyphae at the outer part of *A. oryzae* colonies have different functions.

**Results**

*Environmental conditions affect septal plugging in A. niger and A. oryzae*

Hyphae at the periphery of fungal colonies explore the non-colonized substrate. This substrate can be heterogeneous with respect to physical (e.g., temperature, osmotic potential) and chemical (e.g., presence or absence of C- or N-source or antibiotics secreted by other microbes in the substrate) conditions. The effect of environmental conditions on plugging of the apical septum of leading hyphae of *A. niger* and *A. oryzae* was assessed. To this end, wild-type strains N402 and RIB40 were grown for 2 days at 30 °C, after which they were either or not exposed to temperature stress, pH stress, hypo- or hypertonic conditions, 25 μg ml⁻¹ phleomycin or 4 mg ml⁻¹ hygromycin. Alternatively, strains were starved for 2 days for carbon or nitrogen. Septal plugging was assessed by laser dissection of the apical compartment (Fig. 1 and Experimental procedures). Significant changes in plugging incidence of the most apical septa were found when *A. oryzae* and *A. niger* were exposed to 45 °C for 1.5 h (Fig. 2). This temperature stress increased plugging incidence from 60% to 75% and 31% to 83% in the case of *A. niger* and *A. oryzae* respectively. Additionally, plugging incidence of apical septa in *A. niger* increased to 75% under hypertonic conditions and decreased to 45% when exposed to high pH. In the case of *A. oryzae*, apical plugging incidence increased to 48%, 86% and 48% under low pH conditions, C-starvation and N-starvation respectively. Together, these data show that environmental conditions affect septal closure of *A. niger* and *A. oryzae*. Yet, their response to such conditions is different.

![Fig. 1. Schematic representation of the way stress was induced in fungal colonies and how septal closure was scored. Septa were scored as being open when movement of organelles (grey ellipses) towards the septum was observed after laser dissection of the subapical compartment. Black dots represent Woronin bodies and their presumed location. Laser dissection in this example is performed in the apical compartment. The location of the disruption is represented by a dashed line.](image1)

![Fig. 2. Percentage of closed apical septa of growing hyphae at the periphery of *A. niger* N402 (A) and *A. oryzae* RIB40 (B) colonies that had been subjected to different stress conditions. Error bars indicate 95% CIs. Asterisks (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001) indicate significant differences between control conditions and treatment conditions. N = 100.](image2)
Growth of A. niger and A. oryzae ΔhexA strains can be affected by environmental conditions

The diameter of 24 h old colonies of control and ΔhexA strains of A. niger (N402 and N402ΔhexA, respectively) and A. oryzae (RB#153.1 and RB#149.1, respectively) was measured after growing at standard conditions or after carbon starvation. Colony diameter was 2 mm for all strains at standard growth conditions. This was also the case for the A. niger and A. oryzae control strains that had been grown under carbon limitation. In contrast, colony diameter of A. niger and A. oryzae ΔhexA strains was 2.4 mm and 0.6 mm, respectively, after 24 h of carbon starvation. This shows that growth of the A. oryzae ΔhexA strain but not the wild-type strain is affected by carbon starvation.

In the next step, 24 h old A. niger and A. oryzae colonies were either or not exposed for 1.5 h to stress conditions, which was followed by incubation for 24 h under normal growth conditions. Hyphae of the wild-type A. niger colonies had extended by 2.3 mm during the 25.5 h period, irrespective whether they had been exposed to stress (Fig. 3). The A. niger ΔhexA strain showed similar growth except for hyphae that had been exposed to 45 °C for 1.5 h. These hyphae had only extended by 1 mm. No significant differences were observed in the case of A. oryzae, irrespective of strain or heat treatment (Fig. 3). Together, this shows that A. oryzae can restore its growth rate after exposure to stress, if affected at all, irrespective of the presence of hexA. In contrast, sensitivity of A. niger ΔhexA cannot be restored by growing at standard growth conditions.

Hyphal heterogeneity promotes adaptability of the colony to stress conditions

Aspergillus oryzae was shown to recover from heat stress by growing for 24 h at standard growth conditions (see above). As a next step, growth of hyphae and glaA-driven green fluorescent protein (GFP) fluorescence was measured immediately after heat shock. This was done with the control (RB#140.1) and the ΔhexA (RB#141.3) strains that express gfp from the glucoamylase glaA promoter. Expression of glaA was induced by transfer to maltose containing medium. This was followed by either or not exposing the colonies at 45 °C for 1.5 h. Expression of glaA within hyphae at the periphery of colonies of the A. oryzae control and ΔhexA strain were best described by a bimodal distribution when grown under control conditions. The fraction of high fluorescent hyphae was 50% and 7.5% for the control and ΔhexA strain respectively. After heat shock, heterogeneity was reduced in the A. oryzae control strain and abolished in the ΔhexA strain (Fig. 4).

Mean fluorescence intensity of hyphae at the colony periphery had reduced 90 min after heat-shock from 958 to 844 and 262 to 234 arbitrary units for the A. oryzae control and ΔhexA strain respectively. No reduction in fluorescence intensity was observed for colonies exposed to control conditions. Low fluorescent hyphae of the control and the ΔhexA strain that had not been exposed to 45 °C had grown 39 and 67 μm h⁻¹, respectively, while the high GFP expressing hyphae had extended 63 and 82 μm h⁻¹ (Fig. 5). Growth of heat stress exposed hyphae that showed low GFP fluorescence was reduced to 18 and 7 μm h⁻¹ in the case of the control strain and the ΔhexA strain respectively. In contrast, extension of hyphae that were highly fluorescent was reduced to 2 μm h⁻¹ for both strains. Together, hyphae of the control A. oryzae strain that highly express glaA are more sensitive to heat stress than hyphae lowly expressing glaA. Furthermore, both lowly and highly expressing hyphae of the ΔhexA strain showed faster growth under control conditions, while they were more sensitive to temperature stress. This implies a trade-off between fast growth and adaptability to environmental stress.

Discussion

A subpopulation of hyphae with low transcriptional and translational activity and a subpopulation of hyphae with high such activities are found at the outer part of colonies.
of A. oryzae and A. niger (Vinc et al., 2005, 2011; Bleichrodt et al., 2015a,b). The highly active hyphae secrete proteins, while we here show that the lowly active hyphae are more resistant to environmental stress, in particular to high temperature stress.

Woronin bodies maintain the heterogeneity of hyphae at the outer part of the Aspergillus colony (Bleichrodt et al., 2012). As a consequence, GFP fluorescence intensity distribution resulting from glaA driven expression is heterogeneous (i.e., bimodal) between hyphae at the periphery of wild-type colonies but homogenous (i.e., unimodal) between ΔhexA hyphae that lack Woronin bodies. Here, we did find a bimodal GFP fluorescence intensity distribution for the ΔhexA strain. Yet, the subpopulation of the highly active hyphae was very small (only 7.5% of the total hyphae), while this subpopulation represents half the hyphae in the wild type. Thus, our data also support the role of Woronin bodies in maintenance of hyphal heterogeneity.

Part of the septa of vegetative hyphae of the basidiomycetes Schizophyllum commune and Rhizoctonia solani and the ascomycetes A. oryzae, A. niger and Zymoseptoria tritici a re closed (van Driel et al., 2008; van Peer et al., 2009, 2010; Bleichrodt et al., 2012, 2015a; Steinberg et al., 2017b). Incidence of septal closure in vegetative growing hyphae of S. commune depends on environmental conditions. Heat shock, hypertonic shock and presence of antibiotics promote septal plugging, while low glucose reduces plugging incidence (van Peer et al., 2009). Such an effect was not observed in A. oryzae (Bleichrodt et al., 2012). Here, we did show an effect of environmental conditions on septal plugging both for A. niger and A. oryzae. This is due to the use of a larger sample size and adapted statistical analysis. Plugging incidence of the apical septa of leading hyphae of A. oryzae was affected by heat treatment, low pH conditions, C-starvation and N-starvation. On the other hand, plugging incidence in A. niger was affected by exposure to heat, high pH and hypertonic conditions. Together, these data...
Aspergillus niger and A. oryzae strains with Woronin bodies were not affected in growth during carbon starvation. In contrast, growth of the A. oryzae ΔhexA strain was severely affected. Hyphal extension of the A. niger and A. oryzae ΔhexA strains was also affected by heat treatment. This effect was only found directly after the treatment in the case of A. oryzae ΔhexA, while in the case of the A. niger ΔhexA strain the effect of temperature was even observed after 24 h of recovery. It may well be that septal closure mediated hyphal heterogeneity is also instrumental in other environmental stresses. Deletion of a gene encoding the Woronin body associated transmembrane protein TmpL in Alternaria brassicicola and A. fumigatus results in hypersensitivity to oxidative stress (Kim et al., 2009). Moreover, a ΔHEXA strain of M. grisea is not able to survive N-starvation (Soundararajan et al., 2004). In addition, hexA is upregulated in Trichoderma atroviride stressed by the organophosphate pesticide dichlorvos (Tang et al., 2010).

Lack of Woronin bodies not only abolishes hyphal heterogeneity, but also increases growth rate. Tegelaar and Wösten (2017) found that wild-type A. niger hyphae grow at 84% of the growth rate of the A. niger ΔhexA strain. Here, a similar effect was shown for A. oryzae. Hyphae with high transcriptional and translational activity grew 23% slower than the ΔhexA strain, while this was even 42% in the case of lowly active hyphae. Our data thus suggest that fast growth comes with a reduced ability to respond to environmental stress. Together, it is concluded that the outer part of a wild-type A. oryzae colony consists of hyphae that are specialized in release of nutrients from the substrate by secreting proteins and hyphae that have higher stress resistance. Why would Aspergillus use this strategy? Organisms that live in a heterogeneous environment must adapt to survive. A clonal population of cells such as that in a mycelium can employ two main strategies to adapt. First, (phenotypically uniform) individuals can sense their environment followed by immediate switching their phenotype to the one most suited to the new condition. Alternatively, phenotypically diverse individuals colonize the substrate, of which some are not optimally suited for their current environment but may be well adapted to a potential change in environment, a phenomenon known as bet-hedging (Kussell and Leibler, 2005; Zacchetti et al., 2018). When the environment changes frequently, the former strategy is preferred, while the latter strategy is preferred when cells are exposed to infrequent changes. In nature, Aspergillus hyphae are exposed infrequently to environmental changes in relation to the length of their life cycle, being a few days. Indeed, we here showed a bet-hedging strategy for Aspergillus hyphae with respect to heat. Together, it is concluded that the existence of a subpopulation of persister-type hyphae at the periphery of the Aspergillus colony ensures efficient colonization of substrates.

Experimental procedures

Strains, media and spore isolation

Strains used in this study (Table 1) were grown on minimal medium (MM) (de Vries et al., 2004) with 200 mM xylose or 25 mM maltose as a carbon source or on CD + Met medium (Maruyama et al., 2010) supplemented with 1% glucose. Spores were harvested from 3 day old cultures that had been grown on complete medium (MM with 0.2% trypton, 0.1% casamino acids, 0.1% yeast extract, 0.05% yeast ribonucleic acids and 1% glucose) using saline tween [ST, 0.9% NaCl (wt/vol), 0.05% (vol/vol) Tween-20].

Construction of A. oryzae strains

NSRKu70-1-1AS and NSRΔHx5 were transformed with pNR10 harbouring the niaD gene (Yoon et al., 2009).

Table 1. Strains used in this study.

| Strain          | Parental strain | Genotype                          | Reference          |
|-----------------|-----------------|-----------------------------------|--------------------|
| A. oryzae RI40  |                 | Wild-type                         | Machida et al. (2005) |
| A. niger N402   | NRRL 3          | ΔcspA1                            | Bos et al. (1988)   |
| A. niger N402ΔhexA | N402            | ΔcspA1 ΔhexA hygR+               | This study         |
| A. niger RB652  | N402ΔhexA       | ΔcspA1 ΔhexA hygR+ AnhexA phleoR+ | This study         |
| A. oryzae RB140.1 | NSRKu70-1-1AS | Δku70 glaA::sGFP niaD+            | Bleichrodt et al., 2012 |
| A. oryzae RB141.3 | NSRKΔHx5      | Δku70 ΔAohex1 glaA::sGFP niaD+    | Bleichrodt et al., 2012 |
| A. oryzae RB153.1 | NSRKu70-1-1AS | Δku70 niaD+                       | This study         |
| A. oryzae RB149.1 | NSRKΔHx5      | Δku70 ΔAohex1 niaD+               | This study         |
The resulting strains RB#153.1 and RB#149.1 are prototrophic for nitrate.

Construction of A. niger strains

To inactivate hexA (An07g04570) of A. niger, its 0.9 kb upstream and 1.0 kb downstream flanking regions were amplified with primer pairs BN090 and BN091 and BN088 and BN089 (Supplementary Table 1), respectively, using genomic DNA of strain N402 as a template. The up- and down-stream sequences were inserted by BP recombination into pDONR™ P4-P1R and pDONR™ P2R-P3 (Invitrogen, www.invitrogen.com), respectively, generating the 5’ and 3’ entry clone plasmids pDONR_P-HexA and pDONR_T-HexA. The hygromycin resistance cassette (hygR) was amplified with primers hygroFW and hygroREV (Table S1) using pAN7.1 (Punt et al., 1987) as a template and inserted into pDONR™ P1-P2R (Invitrogen) by BP recombination generating the centre entry clone plasmid pDONR_hygR. The 5’, 3’ and centre entry constructs were subjected to LR clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pΔAnHexA. The DNA fragment including the hexA flanking regions interspersed by the hygromycin resistance cassette was amplified by poly chain reaction (PCR) using primers BN090 and BN089 and plasmid pΔAnHexA as a template and was introduced into A. niger strain N402, generating strain N402ΔhexA. Disruption of the hexA gene was confirmed by Southern blotting using genomic DNA that had been digested with BamHI and KpnI.

To complement the N402ΔhexA strain, the hexA gene was amplified by PCR with primers AnhexA-FW-NotI (TAGCTTATACCGGCAGTTGATCTAGCGCGGTGAGCC) and AnhexA-REV-PstI (TCGCTATACTGCAGTTACGA CGGCACGAAACGGC). The PCR product was digested with restriction enzymes NotI and PstI and introduced in plasmid pRB206 (Wang et al., 2015) that had been cut with the same enzymes. This resulted in plasmid pB065 that contains the hexA gene and a phleomycin resistance cassette. pB065 was transformed to the ΔhexA strain. Complementation of hexA was assessed by chromosomal DNA extraction and PCR with primers ORF-hexA-FW (ATGGGTATCTAC GACGACGACG) and ORF-hexA-REV (CATCCTCGAGG CCTCAGCG). Chromosomal DNA from N402 and ΔhexA strains was used as a control. PCR was positive for transformants RB65.2, RB65.3, RB65.4 and negative for the ΔhexA strain. Sequencing of the PCR bands confirmed hexA identity. To confirm phenotypic complementation, septal plugging of apical septa was assessed by laser microdissection. When the complemented strain RB#65.2 was grown under control conditions, 12% of apical septa were closed when cut in the apical compartment, thus confirming the strain was complemented.

Transformation of Aspergillus

Protoplasts of A. niger and A. oryzae were generated as described (de Bekker et al., 2009) and transformed using polyethylene glycol (Punt and van den Hondel, 1992). Nitrate prototrophic (niaD+) strains of A. oryzae were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5% agar). Transformants of A. niger were selected on MMS medium containing 100 μg ml⁻¹ hygromycin or 25 μg ml⁻¹ phleomycin (InvivoGen; www.invivogen.com).

Quantification of septal plugging

Aspergillus strains were grown under water saturating conditions in 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA, P35G-1.5-20-C) (Bleichrodt et al., 2012). To this end, 30 μl 60 °C CD + Met medium with 1% glucose and 1% agarose (CDMGA) was pipetted into the glass bottom dish that had been prewarmed at 50 °C. A total of 250 spores in 0.5 μl saline Tween were placed in the middle of a 18 mm cover slip and were left to dry. Cover slips were placed at the non-solidified CDMGA. When CDMGA had solidified, 2 ml of liquid CD + Met medium (CDMG) was added on top of the culture. Temperature stress was imposed by incubating 2 day old colonies at 4 °C or 45 °C. For pH stress, CDMG of 2 day old cultures was replaced by CDMG with a pH of 2 or 11 adjusted with HCl or NaOH respectively. For hypo- and hypertonic conditions, liquid CDMG of 2 day cultures was replaced with demi water or 1 M MgSO₄ respectively. Cultures that were C or N-starved were exposed to C or N-limiting conditions for 2 days starting at the time of inoculation. To this end, CDMG was used with 0.2% glucose instead of 1% glucose or CDMGA without methionine respectively. The liquid medium on top of the culture did not contain carbon or nitrogen source. For antibiotics treatment, CDMG and CDMGA were supplemented with either 25 μg ml⁻¹ phleomycin or 4 mg ml⁻¹ hygromycin. The second compartment (counting from the tip) was ruptured by laser dissection using the laser pressure catapulting function (LPC) of the P.A.L.M. laser dissecting microscope (Carl Zeiss AG; Oberkochen, Germany). To this end, 60%–70% of the power of the pulsed UV-laser was used. Dissecting the second compartment enabled us to assess the plugging state of the apical septum by monitoring the flow-out of cytoplasm from the apical to the compartment out of the damaged compartment (Bleichrodt et al., 2012). Each experiment was repeated five times for 100 apical septa in total.
Recovery of hyphal extension after exposure to stress conditions

Control and ΔhexA strains of *A. niger* and *A. oryzae* were grown in glass bottom dishes as in the previous section. After 24 h of growth, colonies were either or not exposed to 45 °C or hypertonic conditions for 1.5 h. For C-starvation, colonies were grown from the moment of inoculation in CDMGA with 0.2% glucose instead of 1% glucose. The liquid medium on top of the culture did not contain carbon source. To recover, liquid medium was replaced by fresh CDMG and left to grow for another 24 h. Hyphal extension within biological triplicates was monitored using P.A.L.M. software and the microscope of the P.A.L.M. laser dissection setup (Carl Zeiss AG; Oberkochen, Germany).

Hyphal extension during heat treatment

Lumox® film (25 μm thickness; Greiner BioOne, Frickenhausen, Germany) was cut into 18 mm diameter circles and 0.5 μl spore solution containing 50,000 spores of *A. oryzae* strain RB#140.1 or RB#141.3 was pipetted in the middle of the hydrophobic side of the lumox foil and allowed to dry. Lumox® film was then placed, hydrophobic side down, on a polycarbonate (PC) membrane on MM with 1.5% agarose and supplemented with 200 mM xylose (MMXA). After 2 days of growth at 30 °C, the Lumox® film with the adhering fungal colony was transferred, colony side down, to 500 μl MM supplemented with 25 mM maltose (MMM) in a Cellview™ cell culture dish (Greiner Bio-One PS, 35/10 MM, 627861) for 4 h at 30 °C. Colonies were then exposed to either 30 °C or 45 °C for 90 min. The cell culture dishes were then placed under the P.A.L.M. laser dissecting microscope (Carl Zeiss AG; Oberkochen, Germany) using a Plan-Neofluar 40x/0.6 objective and a CCD camera (AxioCam Icc 1, Carl Zeiss AG, Oberkochen, Germany). Fluorescent, growing, leading hyphae were selected, imaged and their growth was recorded for 90 min. Experiments were done in triplicate for each strain and treatment conditions with 30 selected hyphae per replicate.

Statistics

Differences in radial growth between control and treatment conditions were assessed using analysis of variance (ANOVA) with two-sided Dunnet's post hoc tests or Kruskal–Wallis test followed by pairwise comparisons. Differences in hyphal extension between strains were assessed using ANOVA with Bonferroni post hoc corrections. To assess differences between septal plugging proportions during stress conditions, a Chi-square test was used. To assess the relation between fluorescence intensity and growth speed, either or not under stress conditions, a test for bimodality was performed a described by and as described by Vinck et al. (2005). When populations could be described as bimodal, low fluorescence expressing hyphae were defined as those with a total corrected cellular fluorescence (Burgess et al., 2010) ≤ x1+ its upper 95% confidence interval (CI), intermediate fluorescence expressing hyphae as >x1+ its upper 95% CI and <x2 – its lower 95% CI and high fluorescence expressing hyphae as ≥x2 – its lower 95% CI. Impact of fluorescent hypha location and clustering on hyphal fluorescence intensity was assessed using multinomial logistic regression and Gaussian Mixture Clustering using the R package mclust (Scrucca et al., 2016), followed by Spearman’s rank correlation respectively. Growth speeds were bootstrapped 1000 times and a three-way ANOVA was carried out. Differences in fluorescence intensity were analyzed by repeated measures ANOVA or Kruskal–Wallis test. All analyses were carried out in IBM SPSS 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0; IBM Corp., Armonk, NY).

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Conflict of interest

The authors declare no conflict of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Supplementary Table 1** Primers used in this study. Underlined sequences indicate attB recombination sequences.