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Repetitive genomic sequences as a substrate for homologous integration in the *Rhizopus oryzae* genome

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ABSTRACT

The vast number of repetitive genomic elements was identified in the genome of *Rhizopus oryzae*. Such genomic repeats can be used as homologous regions for integration of plasmids. Here, we evaluated the use of two different repeats: the short (575 bp) rptZ, widely distributed (about 34 copies per genome) and the long (2053 bp) rptH, less prevalent (about 15 copies). The plasmid carrying rptZ integrated, but did so through a 2256-bp region of homology to the *pyrG* locus, a unique genomic sequence. Thus, the length of rptZ was below the minimal requirements for homologous strand exchange in this fungus. In contrast, rptH was used efficiently for homologous integration. The plasmid bearing this repeat integrated in multicopy fashion, with up to 25 copies arranged in tandem. The latter vector, pPyrG-H, could be a valuable tool for integration at homologous sequences, for such purposes as high-level expression of proteins.

Introduction

Zygomycetes are one of the most ancient groups of multicellular fungi (James *et al.* 2006), and it is believable that coenocytic mycelium reflects peculiarity of the evolutionary pathway of this kingdom to multicellularity (Ma *et al.* 2009). Publication of the first zygomycetous genome revealed that *Rhizopus oryzae* RA 99-880 shares more ancestral genes with metazoan than the latter does with either ascomycetes or basidiomycetes (Ma *et al.* 2009). Thus, *R. oryzae* may provide a model of molecular processes that occur in higher eukaryotes. *Rhizopus oryzae* can cause mucormycosis, a disease that generally occurs in immunocompromised patients (Ribes *et al.* 2000). Study of this organism is driven by strong interest in the field of industrial microbiology (Meussen *et al.* 2012a). In the fungal lineage, *Rhizopus* strains are the main natural producers of fumaric and lactic acids (Magnuson & Lasure 2004; Goldberg *et al.* 2006). In addition, *Rhizopus* produces several essential hydrolytic enzymes, including peptidase, glucoamylase, and the widely employed *R. oryzae* lipase (Schmidt-Dannert 1999; Yuzbashev *et al.* 2012).

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Abbreviations; LTR, long terminal repeat; rpt, repeat
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Despite strong interest, a limited number of studies have described genetically stable integrative modifications in *R. oryzae* (Skory 2002; Michielse *et al.* 2004; Skory 2004; Skory 2005; Meussen *et al.* 2012b; Zhang *et al.* 2012). Integration of transformed constructs in this organism is rare, presenting an obstacle. Typically, introduced DNA amplifies extrachromosomally in the form of a high-molecular-weight concatemer that spontaneously disappears after sporulation or incubation under non-selective conditions (Takaya *et al.* 1996;Skory 2002). On Southern blots of undigested DNA, the concatenated plasmid co-migrates with genomic DNA; this led to the incorrect conclusion that the construct integrated in multiple tandem arrays (Horiuchi *et al.* 1995). Nevertheless, transformation with a plasmid containing an extended region of homology yields a small proportion of additively integrated DNA at a homologous locus (Skory 2002). Integration can be improved to some extent through linearization of the plasmid by cutting within the homologous region. However, in many cases, the plasmid re-ligates through a non-homologous end joining mechanism, preventing integration. A detailed study revealed that homologous recombination in *R. oryzae* occurs mainly through non-crossover mechanisms (Skory 2004). Therefore, the broken copy of DNA is repaired, while the homologous partner is merely used as template. This redirection protects the *R. oryzae* genome from gross chromosomal aberrations, because it contains an unusually large number of repetitive elements (Ma *et al.* 2009). Recombination between homologous genomic repeats is a major source of genomic instability (Inbar & Kupiec 1999; Prado *et al.* 2003). As a result, large numbers of mitotically unstable transformants mask the presence of rare integrative transformants.

In this study, we evaluated the use of naturally abundant genomic repeats as a substrate for homologous integration in the genome of *R. oryzae*. The position within the genome strongly affects gene expression in eukaryotes. This approach allowed us to expand the spectrum of *R. oryzae* genomic loci available for integration; previously, only the *pyrG* and *pyrF* loci were adapted for this purpose. The frequency of integrative transformation in *Saccharomyces cerevisiae* positively correlates with the number of homologous targets on the chromosome (Wilson *et al.* 1994). Repetitive genomic sequences such as rDNA or the long terminal repeats (LTRs) of transposable elements have been used successfully as targets for multicopy integration in yeast (Le Dall *et al.* 1994; Juretzek *et al.* 2001). Furthermore, the inclusion of LTRs on plasmids facilitated isolation of the first integrative transformant of the mucoralean fungus *Absidia glauca* (Burmester *et al.* 1990).

Materials and methods

Strains and media

Escherichia coli XL1-Blue (Bullock *et al.* 1987) were used for plasmid construction and propagation. Cells were grown at 37 °C in Luria–Bertani broth and on agar plates (Sambrook *et al.* 1989). Ampicillin (100 μg mL^{-1}) and tetracycline (15 μg mL^{-1}) were added as required. Agar was added for the solid media at a concentration of 20 g L^{-1}.

The *Rhizopus* strains used in this study are summarized in Table 1. *Rhizopus oryzae* strain PYR-17 (*pyr 181*) was generously provided by Dr. Skory from the Agricultural Research Service USDA. This uracil auxotroph was derived from the NRRL 395 wild-type strain. PYR-17 is optimal for transformation experiments because it rarely reverts to prototrophy, due to a mutation in the splice junction of the *pyrG* gene (Skory 2002). Potato dextrose agar (PDA; Himedia, India) and RZ (Skory 2000) were used as growth and minimal media, respectively. Both were supplemented with 20 g L^{-1} glucose. To isolate genomic DNA, spores were grown in liquid yeast extract peptone dextrose (YPD) medium (3 g L^{-1} yeast extract, 5 g L^{-1} peptone, and 20 g L^{-1} glucose). Spores were collected by scraping the sporulated mycelium and filtering it through sterile glass wool. To obtain homogeneous spore suspensions, 0.1 % (v/v) Tween 80 (Panreac, Spain) was added to the solutions and liquid media. Spores were suspended in 15 % (v/v) glycerol for storage at −20 °C. For single colony isolation and quantification, 0.02 % (v/v) Triton X-100 (Serva, Germany) was added to solid media. Media were supplemented with 0.2 g L^{-1} of uridine as required. All *Rhizopus* strains were cultivated at 30 °C.

Retrieval of repetitive elements from the genome sequence of Rhizopus oryzae *RA 99-880*

Repetitive elements were detected by searching the genome sequence against itself and filtering for non-degenerate alignment sequences longer than 200 bp by using the REPuter software (Kurtz *et al.* 2001). The BlastN program available through the Fungal Genome Initiative (http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative) or National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) online services was used to align selected repeats and estimate the number of copies in the genome of *Rhizopus oryzae* RA 99-880.

Construction of recombinant plasmids

DNA manipulations were performed according to Sambrook *et al.* (1989). Enzymes for molecular manipulations were purchased from Thermo Scientific (Lithuania) except AccuTaq DNA polymerase (Sigma–Aldrich, USA) that was used to amplify flanking sequences of integrated plasmids. Oligonucleotide primers are listed in Table 2. The plasmid bearing the *Rhizopus oryzae pyrG* gene was constructed as follows: The *pyrG* locus was amplified as a 2268-bp fragment by using *Pfu* DNA polymerase, the PyrG-F/PyrG-R primer pair, and total genomic DNA from strain NRRL 395. This fragment was blunt-end ligated into *Sma*I-digested pUC19, generating the 4954-bp pPyrG. RptH was amplified by PCR using HF-1/HR-1 primers and NRRL 395 DNA as template. The 2069-bp product was digested with *Nhe*I and cloned into pPyrG digested with *Xba*I and treated with calf intestinal alkaline phosphatase (CIAP). The resultant plasmid, pPyrG-H, was 7013 bp in length. In a similar manner, a 589-bp fragment bearing rptZ was amplified from NRRL 395 DNA with primers ZF-1/ZR-1. The resultant product was digested with *Sac*I and cloned into pPyrG treated with *Sac*I and CIAP. The cloned PCR products were sequenced on both strands. Sequences of isolated rptZ and rptH are

Table 1 – *Rhizopus* strains used in this study.

Strain	Species	Product[a]
NRRL 395	*R. oryzae*	Lactate
PYR-17[b]	*R. oryzae*	Lactate
VKPM F-815	*R. oryzae*	Lactate
VKPM F-159	*R. oryzae*	Lactate
ATCC 34612	*R. oryzae*	Fumarate, Lactate, Ethanol, Lipase, Glucoamylase
ATCC 22581	*R. oryzae*	Fumarate, Ethanol, Glycerol
DSM 853	*R. oryzae*	Lipase
VKPM F-909	*R. oryzae*	Lipase
NRRL 2710	*R. microsporus*	Lipase, Fumarate, Ethanol

a Information regarding products is available from the microbial culture collection that provided the strains.
b PYR-17 is a uracil auxotroph derivative of NRRL 395 (see Materials and Methods section).

available from GenBank by accession numbers KP071934 and KP071935, respectively.

Biolistic transformation

Transformation of the *Rhizopus oryzae* PYR-17 mutant was performed by microprojectile particle bombardment using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, USA), as described by Skory (2002) with minor modifications. M5 tungsten particles (Bio-Rad) were washed three times in 96 % ethanol and twice in deionized water before they were stored as a 60 mg mL^{-1} aqueous suspension at −20 °C. To prepare the particles for experiments, 50 μL of the particle suspension was mixed with 5 μg of undigested plasmid DNA. Next, 50 μL of 3.75 M $CaCl_2$ and 5 μl of 1 M spermidine (free base, Sigma–Aldrich) were added. Particles were vigorously shaken for 2 min and incubated in an ice bath for 10 min. Particles were collected by brief centrifugation and the pellet was rinsed with 96 % ethanol, then loaded in equal amounts onto five macrocarriers. The pressure was set by using an 1100-psi rupture disk. The distance between the rupture disk-retaining cap and the macrocarrier cover lid was set to 3.2 mm. The stopping screen support was placed in the bottom position. Approximately 1×10^8 spores were spread on RZ agar plates prior to bombardment. The distance between the stopping screen support and the target was 7.5 cm. The chamber vacuum reached 1.1 mmHg by using two successively assembled high-output pumps (Millipore, USA).

Extraction of fungal genomic DNA

A 500-mL flask containing 100 mL YPD medium was inoculated with approximately 1×10^8 spores and incubated for 12 h at 30 °C with shaking at 200 rpm. The mycelium was harvested by centrifugation, washed once with 0.05 M EDTA (pH 7.8), and resuspended in a minimal volume of buffer (0.05 M Tris–HCl, 0.15 M NaCl, and 0.05 M EDTA, pH 7.8). Using a pestle and mortar, the suspension was ground to a fine powder in liquid nitrogen. After thawing, 100 μg mL^{-1} proteinase K and 0.5 % (w/v) sodium dodecyl sulfate were added and the extract was incubated for 2 h at 55 °C. An equal volume of warmed solution containing 2 % (w/v) cetyltrimethylammonium bromide and 1.4 M NaCl was added. The resulting mixture was incubated at 65 °C for 1 h with occasional inversion, and then at 37 °C for 15 min. Standard phenol-chloroform extraction and ethanol precipitation were performed (Sambrook *et al.* 1989).

Southern blot analysis

Genomic DNA prepared from transformed cells was digested using restriction endonucleases, separated by electrophoresis in a 1 % (w/v) agarose gel, and transferred onto a charge-modified nylon membrane (Sigma–Aldrich). DNA fragments were fixed on the membrane by exposing it to UV irradiation for 1 min. Probes were labeled with [^{32}P]dATP using the Hexa-Label DNA labeling kit (Thermo Scientific) according to the manufacturer's instructions. The membrane was hybridized under strong conditions (Sambrook *et al.* 1989) and exposed to X-ray film (Kodak, USA) for 12–24 h. The number of vector copies was estimated from the radiograms by using densitometric evaluation in ImageJ 1.4 software (National Institute of Health, USA). It should be mentioned that for high-copy

Table 2 – Primers used in this study.

Primer name	Purpose	Primer sequence (5′–3′)[a]
PyrG-F	Cloning of *pyrG* gene	**CCC GGG** GAA TTC TTC TTT TAG GTT AAA AAC TC
PyrG-R	Cloning of *pyrG* gene	**GGA TCC** GAA TTC CAA AGC TTT TCA TAT ATT G
GF-1	Amplification of rptG	**GTC GAC** AAG AAC ACA CTG ATG CAT TC
GR-1	Amplification of rptG	**GTC GAC** ATC CTC CCC CAG ACT TGA C
HF-1	Amplification and cloning of rptH	ATG **CTA GC**G TTG AAA CGT CTT ACT GCT GC
HR-1	Amplification and cloning of rptH	ATG **CTA GC**T GCT TGA ATC TCA TAA TGT TGG
JF-1	Amplification of rptJ	**CCC GGG** CTT TAC CAC TCT TGA TCT TCG
JR-1	Amplification of rptJ	**CCC GGG** ATA CTT CCT GGG AAA TAA CC
ZF-1	Amplification and cloning of rptZ	T**GA GCT C**TG TTG TAG AAC AGA GTC TGA T
ZR-1	Amplification and cloning of rptZ	T**GA GCT C**TG TTA TGT TCT GGT AAA GTA AAA G
H-up	Left flank verification of integrated pPyrG-H	GTG TCT TCA GCT CTG GTG AC
M13-R	Left flank verification of integrated pPyrG-H	GAG CGG ATA ACA ATT TCA CAC AGG
PyrG-end	Right flank verification of integrated pPyrG-H	TTA GGT GCA CCT TGA GTC C
H-dw	Right flank verification of integrated pPyrG-H	TTC CCA AGT GTA ATC CAT AGG

a Bold letters correspond to introduced restriction sites.

Table 3 – Summary of *R. oryzae* RA 99-880 genomic repetitive elements.

Name	Full length (bp)	LTR length (bp)	Copy number per genome[a]	Length of selected region (bp)[b]	Similarity of selected region (%)[c]	Reference sequence (nucleotides position)[d]
Element-G	6070	462	15 (16)	2274	99–100	AACW02000235.1 (282242–288311)
Element-H	6271	453	15 (16)	2053	99–100	AACW02000295.1 (132620–138890)
Element-J	6021	345	14 (17)	2172	99–100	AACW02000030.1 (665–4480)
Element-Z	–	575	34 (39)	575	94–100 (99–100)[e]	AACW02000381.1 (2164–1589)

a The number of full-length copies of the element in the genome of *R. oryzae* RA 99-880. The total number including truncated copies is given in parentheses.
b The length of the selected conserved regions (rptG, rptH, rptJ, and rptZ, see Results section).
c The minimum and maximum similarity estimated for the full-length copies within selected region of repeats with the consensus sequence.
d The GenBank accession number.
e The minimum and maximum similarity estimated for the 16 most conservative genomic sequences is shown in parentheses.

number transformants the values could be understated due to the signal saturation.

Results

Distribution of repetitive genomic sequences in Rhizopus *strains*

We examined the *Rhizopus oryzae* RA 99-880 genome and identified numerous non-degenerate repeat pairs exceeding 200 bp in length. The distribution of these sequences, each denoted by a letter of the alphabet, was analyzed with three main criteria to select appropriate sequences: length, copy number, and similarity. The majority of repetitive sequences selected in this manner were identified as LTR-type retro-elements or solo-LTR sequences (McCarthy & McDonald 2003). Four repetitive sequences were selected for further analysis (Table 3).

Element-G, element-H, and element-J represent LTR-type retro-elements approximately 6 kb in length. In addition to these full-length elements, the *R. oryzae* RA 99-880 genome contained numerous solo-LTRs. Elements H and J each include a single open reading frame (ORF), while element-G contains

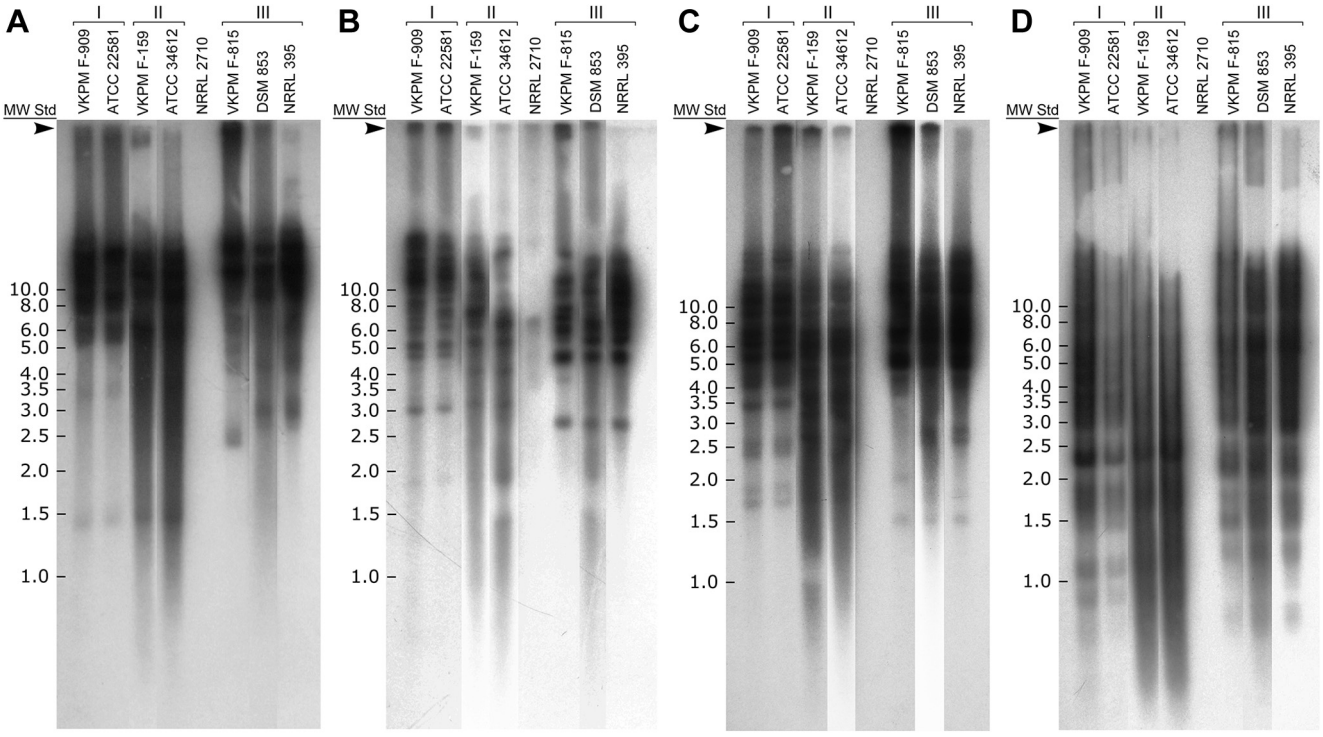

Fig 1 – Southern hybridization analysis of total genomic DNA from seven *Rhizopus oryzae* strains. *Rhizopus microsporus* NRRL 2710 was tested as an out-group strain. Genomic DNA was digested with *ScaI*. RptG, rptH, rptJ and rptZ and the entire sequence of element-H and element-G lack *ScaI* recognition sites, whereas element-J contains one *ScaI* site in an ORF upstream of the rptJ sequence. The following probes were used: (A) rptG generated using primers GF-1 and GR-1; (B) rptH generated using primers HF-1 and HR-1; (C) rptJ generated using primers JF-1 and JR-1; and (D) rptZ generated using primers ZF-1 and ZR-1. The four probes were amplified from a mixture of genomic DNA from the eight *Rhizopus* strains analyzed on the blot. The GeneRuler 1-kb DNA ladder (Thermo Scientific) was used as a molecular weight standard. The arrows at the top of the blot mark the relative position of the wells. Roman numerals designated the group number (see Results section).

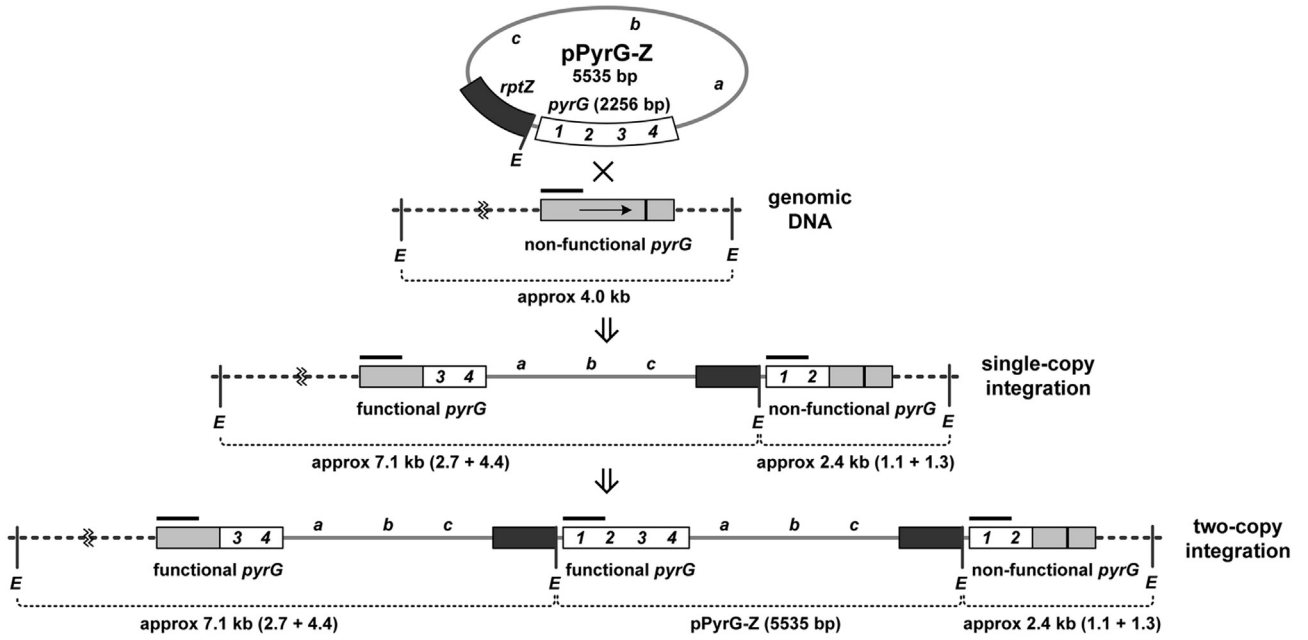

Fig 2 – Proposed mode of integration of pPyrG-Z into the genomic locus of *pyrG* gene. Two-copy integration as a particular case of multicopy integration of the plasmid is shown. *Eco*88I recognition sites are designated as *E*. The regions used as the probes for Southern blot analysis of transformed cells are depicted as black bars. Drawing not to scale.

two ORFs. For each retro-element, we selected approximately 2 kb of the most conserved region within the ORF for subsequent analysis. These regions were named rptG, rptH, and rptJ.

Element-Z (herein, rptZ) is 575 bp in length and widely distributed in the *R. oryzae* RA 99-880 genome, comprising more than 34 copies. Initially, rptZ appeared to be a solo-LTR because it contains TG dinucleotides on both 5′-ends, a TATA-box, and a polyadenylation signal. We failed to find the full-length sequence of rptZ, suggesting it had been lost in this strain. Approximately a half of the rptZ elements appeared as part of another repetitive genomic sequence that extended

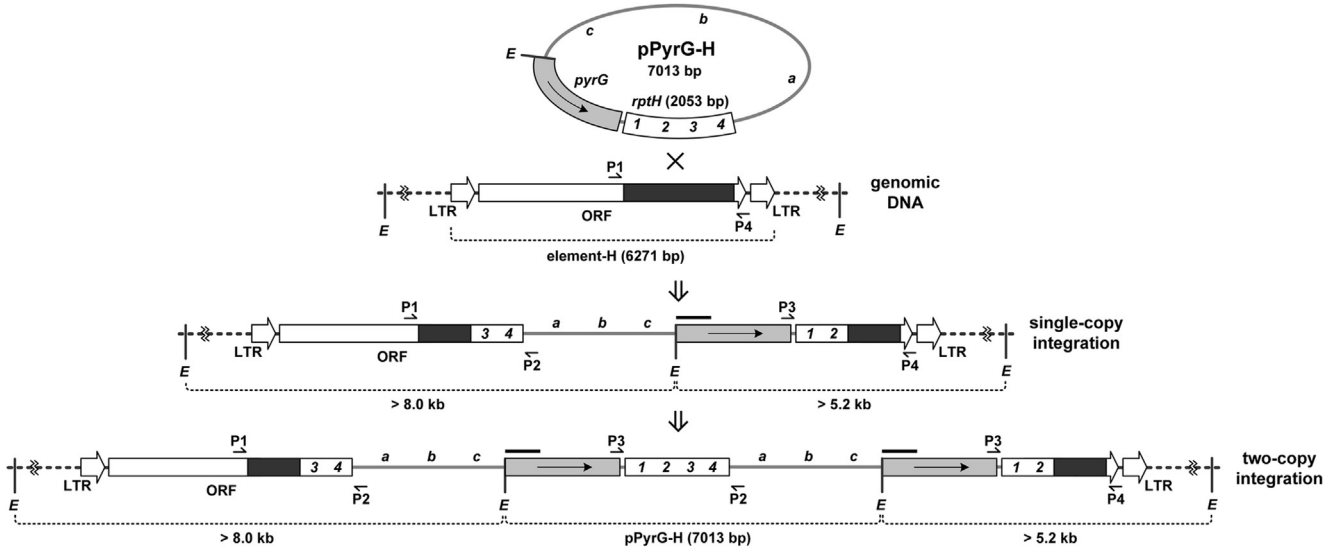

Fig 3 – Proposed mode of integration of pPyrG-H into one of the chromosomal copies of element-H. Two-copy integration as a particular case of multicopy integration of the plasmid is shown. *Eco*88I recognition sites are designated as *E*. P1, P2, P3, and P4 are designated as the annealing sites for primers H-up, M13-R, PyrG-end, and H-dw, respectively. The minimal sizes of left and right flanking *Eco*88I fragments were estimated for integration into a full-length copy of element-H. The regions used as the probes for Southern blot analysis of transformed cells are depicted as black bars. Drawing not to scale.

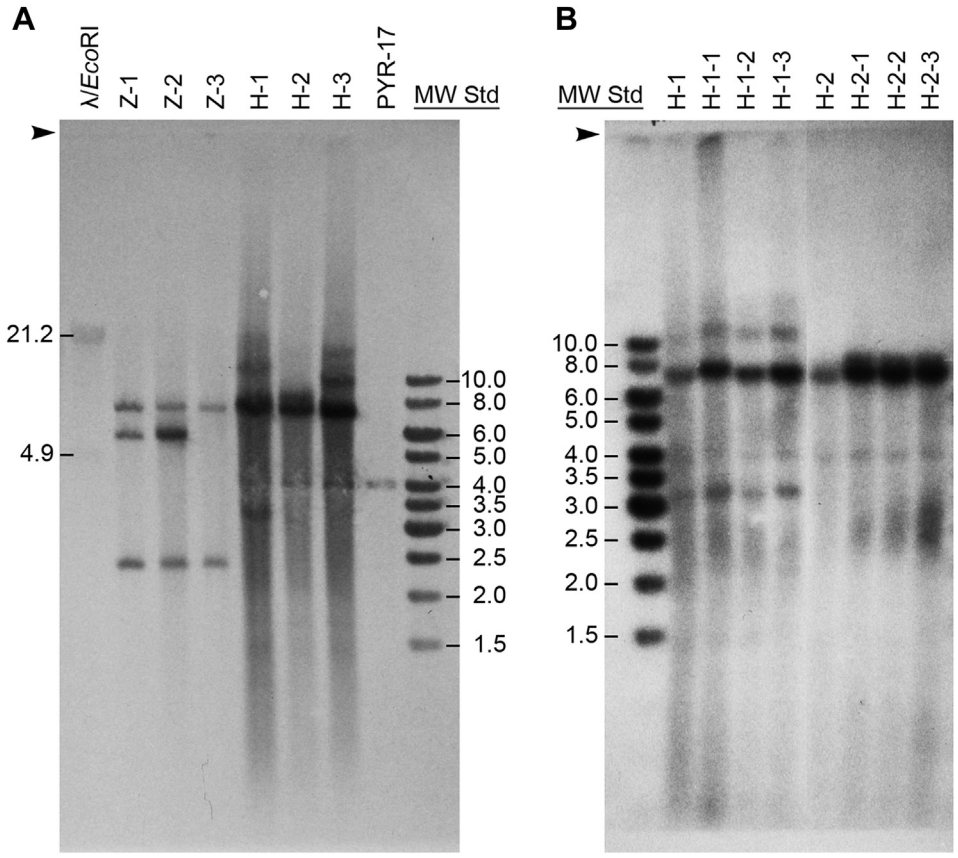

Fig 4 – Southern hybridization of total genomic DNA from *R. oryzae* PYR-17 strain transformants. (A) Isolates Z-1, Z-2, and Z-3 were obtained using transformation with pPyrG-Z, while H-1, H-2, and H-3 were transformed with pPyrG-H. DNA from untransformed strain PYR-17 serves as the control. (B) Strains H-1-1, H-1-2, H-1-3, H-2-1, H-2-2, and H-2-3 were independently obtained by ten rounds of sporulation from transformants H-1 and H-2, respectively. Genomic DNA was digested using *Eco*88I. RptH and rptZ, and the full-length sequence of element-H lack *Eco*88I recognition sites. The probe was a 718-bp *Eco*RI-*Eco*RI fragment of pPyrG that binds upstream of the ORF in the *pyrG* locus (see Figs 2 and 3). Lambda DNA/*Eco*RI marker and GeneRuler 1-kb DNA ladder are used as molecular weight standards. The arrows at the top of the blot show the relative positions of the wells.

for an additional 1.5 kb on each side. None of these sequences encoded a reverse transcriptase/integrase or possessed an ORF longer than 201 bp.

Several *R. oryzae* genomes were analyzed for the presence of rptG, rptH, rptJ, and rptZ by Southern blotting. These strains are preferable recipients for transformation because they accumulate valuable industrial products, as summarized in Table 1. As shown in Fig 1, all *R. oryzae* strains contain multiple copies of rptG, rptH, rptJ, and rptZ. In contrast, DNA from *Rhizopus microsporus* NRRL 2710 (formerly *Rhizopus oligosporus*) showed only limited homology to rptH. Patterns obtained from seven *R. oryzae* strains divided them into three distinguishable groups that evidently reflected their relatedness. Notably, the two best lactate producers, VKPM F-815 and NRRL 395, were clustered in group III together with DSM 853, a well-known producer of the *R. oryzae* lipase. We failed to find any information about lactic acid production by this strain. These repeats were not found in the genomes of other mucoralean fungi, including *Mucor circinelloides* ATCC 8541, *Absidia griseola* ATCC 20430, *Cunninghamella homothallica* ATCC 16161, and *Blakeslea trispora* NRRL 2457 (data not shown).

Transformation of Rhizopus oryzae *with plasmids bearing repetitive genomic sequences*

Two repetitive sequences (rptH and rptZ) were used in transformation experiments. Both sequences were cloned into the pPyrG vector containing the *pyrG* gene as a selectable marker. The *Rhizopus oryzae* PYR-17 strain were transformed with the resultant plasmids, pPyrG-H and pPyrG-Z, and pPyrG by using a biolistic method. A series of ten transformation experiments were performed using each construct. Because of the multinucleate state of *R. oryzae* spores, an additional sporulation round was required to isolate homokaryotic clones. At the same time, the transformation method we used does not facilitate isolation of progeny from a single transformant. Therefore, spores obtained from each bombarded plate were harvested and analyzed separately after sporulation.

Transformation with pPyrG and pPyrG-Z resulted in well growing and sporulating mycelium. In the second generation, single clones showed growth from slow to wild-type. However, in the transformation experiments with pPyrG-H aerial hyphae did not form on two of the ten plates. In the second

generation, three of eight remaining transformations produced only small prototrophic clones that stopped growing. Thus, only derivatives of five successful transformation experiments with pPyrG-H were further analyzed.

Several well-growing prototrophic clones that were isolated from each transformation were transferred to a separate PDA plate with uridine and allowed to sporulate. The percentage of prototrophic clones obtained through this procedure was assessed by plating spores on minimal media in the presence or absence of uridine. A transformant was considered mitotically stable if the numbers of clones on both media were approximately equal. Stable clones were obtained in approximately half of the ten experiments, regardless of which construct was applied. Notably, under selective conditions, four additional rounds of sporulation without isolation of single clones did not alter the number of experiments in which stable transformants were isolated.

Experiments with pPyrG-Z and pPyrG-H yielded independent stable transformants, six of which were selected for Southern hybridization analysis (Z-1, Z-2, Z-3, H-1, H-2, and H-3). Proposed modes of integration of pPyrG-Z and pPyrG-H are shown in Fig 2 and Fig 3, respectively. The original 4-kb *pyrG* fragment in all three pPyrG-Z transformants was replaced by fragments of about 2.4 kb and 7 kb in length (Fig 4A). Transformants Z-1 and Z-2 contained one and three additional plasmid copies (5535 bp), respectively. Thus, pPyrG-Z integrated in all cases additively into *pyrG* locus, showing accidental insertion of one to four copies arranged in tandem.

Stable transformants obtained using pPyrG-H carried the intact 4-kb *pyrG* locus, whereas the plasmid could present as either an integrated tandem array or autonomously replicating high-molecular-weight concatemer (Fig 4A). The major band, corresponding to the entire plasmid size (7013 bp), comprised approximately 7–10 copies. Minor bands that were visible in all transformants could represent right flanking sequences of additively integrated pPyrG-H in one of the genomic copies of element-H. The predicted size of these fragments exceeded 5 kb (see Fig 3). However, in one case the smaller fragment was observed (see H-1 in Fig 4A) that indicated the presence of a rearrangement, as previously reported (Yanai *et al.* 1990; Skory 2002; Michielse *et al.* 2004; Skory 2004). Amplification of the left and right flanking regions with primers specific to the junctions revealed that pPyrG-H integrated in the element-H sequence in all cases (Fig 5). The only exception was the right flank of the H-1 transformant, for which the expected 2.2-kb PCR product was not obtained. These data support the hypothesis that rearrangement was occurred in this particular case.

To exclude the possibility that a high-molecular-weight concatemer coexisted with the integrated plasmid, the hybridization patterns were examined in the progeny of isolates H-1 and H-2. Three identical clones obtained from each of the two transformants were independently passed through ten rounds of sporulation. After each sporulation, a single clone was isolated and used to obtain a subsequent generation of spores. To eliminate the selective pressure facilitating maintenance of the plasmid, the PDA medium used for propagation was supplemented with uridine. Under these conditions, clones H-1-1, H-1-2, H-1-3, H-2-1, H-2-2, and H-2-3 were found to be prototrophic. No changes in band number and position were observed in the Southern hybridization (Fig 4B). However, in most cases, the intensity of the main band (7013 bp) increased by at least 2-fold, corresponding to 15–25 copies of the plasmid.

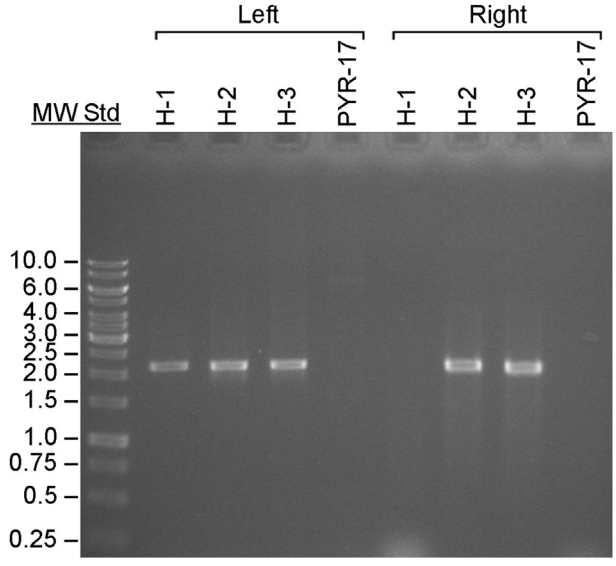

Fig 5 – PCR verification of the left and right flanking regions after integration of pPyrG-H. Genomic DNA from transformants H-1, H-2, H-3, and recipient strain PYR-17 were used as templates. The sequence specific to the left junction was amplified as a 2184-bp fragment by using primers H-up and M13-R. The right flanking junction was amplified as a 2210-bp fragment with primers PyrG-end and H-dw (see Fig 3). GeneRuler 1-kb DNA ladder was used as molecular weight standard.

Discussion

In this study, we demonstrated that a 575-bp region of homology is relatively short for fulfilling the requirements of the crossover pathway of homologous recombination in *Rhizopus oryzae*. About 34 homologous partners of rptZ existed in the genome; however, the recombination occurred solely through the single copy of the 2256-bp *pyrG* locus. This finding was consistent with previous data. Homologous regions used successfully for integrative transformation of *R. oryzae* have all exceeded 2 kb (Horiuchi *et al.* 1995; Skory 2002; Skory & Ibrahim 2007). Reducing the homologous region of the *pyrG* locus to 1535 bp decreased the efficiency of transformation by 20-fold (Skory 2005). In contrast, 250 bp or less in yeast and mammalian cells is sufficient for reciprocal exchange (Rubnitz & Subramani 1984; Jinks-Robertson *et al.* 1993).

Another phenomenon we observed was the inability of introduced DNA to integrate once it had been selected as a mitotically unstable extrachromosomal concatemer. We could not isolate mitotically stable clones by prolonged cultivation and sporulation under selective pressure if the clone was virtually absent among transformants in the second generation. Several processes can induce integration of tandemly arranged copies of the plasmid. Consecutive additive integrations of

single copies of plasmid can lead to this arrangement. However, such events are unlikely due to the low efficiency of crossover-type homologous recombination between the plasmid and chromosomes of *R. oryzae*. Alternatively, concatenated molecules might be formed before integration. Such molecules could be assembled through one of three processes: ligation of broken ends, homologous recombination between circular copies, or rolling-circle replication. As nonlinearized plasmids were used in our study, the stochastic disruptions and re-ligations cannot result in correct head-to-tail assemblage, thus excluding the participation of the first process. We cannot entirely eliminate the possibility that intermolecular homologous recombination contributes to the build-up of concatemers. However, these processes alone lead to the formation of covalently closed circular structures. Rolling-circle replication results in linear molecules with free ends, which are obligatory for the homologous integration process. If the ends of this linear molecule recombine or ligate through non-homologous end joining, the plasmid will be maintained extrachromosomally as a high-molecular-weight concatemer. Such structures rarely integrate into the genome.

Application of the longer but less prevalent rptH reduced the transformation efficiency. Five of ten experiments failed to generate prototrophic clones. However, the total number of experiments that yielded mitotically stable transformants did not change. Stable transformants contained multiple copies of the pPyrG-H plasmid integrated within genomic rptH sequences in a tandem array. One possible explanation for the reduced transformation efficiency of pPyrG-H is that the presence of rptH repressed transcription of the selectable marker *pyrG*. In this scenario, the majority of transformants bearing few copies of the plasmid would not be visible, whereas selection is still valid relative to multicopy transformants. This would also explain the elevation in plasmid copy number that is observed upon continuous cultivation of stable transformants (Fig 4B). Although the medium we used contained exogenous uridine, the selective pressure allowed prototrophic clones to grow and sporulate more quickly than auxotrophs on this medium (data not shown). Similar approaches based on defective selectable markers have been used for multiple-copy transformation in a number of other species (Lopes *et al.* 1989; Bergkamp *et al.* 1992; Le Dall *et al.* 1994). As a result, the constructed vector pPyrG-H ensures multicopy integration in a number of homologous sequences distributed throughout the genome. This is advantageous for high-level expression of recombinant proteins.

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