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ORIGINAL ARTICLE

A highly efficient gene-targeting system for *Aspergillus parasiticus*

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Abstract

Aims: To establish a system that greatly increases the gene-targeting frequency in *Aspergillus parasiticus*.
Methods and Results: The *ku70* gene, a gene of the nonhomologous end-joining (NHEJ) pathway, was replaced by the nitrate reductase gene (*niaD*) in *A. parasiticus* RHN1 that accumulates *O*-methylsterigmatocystin (OMST). The NHEJ-deficient strain, RHΔku70, produced conidia, sclerotia and OMST normally. It had identical sensitivity as RHN1 to the DNA-topoisomerase I complex inhibitor, camptothecin, and the DNA-damaging agent, melphalan. For targeting an aflatoxin biosynthetic pathway gene, *adhA*, partial restriction enzyme recognition sequences in its flanking regions were manipulated to create homologous ends for integration. Using a linearized DNA fragment that contained *Aspergillus oryzae* pyrithiamine resistance gene (*ptr*) marker the *adhA*-targeting frequency in RHΔku70 reached 96%.
Conclusions: The homologous recombination pathway is primarily responsible for repair of DNA damages in *A. parasiticus*. The NHEJ-deficient RHΔku70, easy creation of homologous ends for integration, and the *ptr*-based selection form a highly efficient gene-targeting system. It substantially reduces the time and workload necessary to obtain knockout strains for functional studies.
Significance and Impact of the Study: The developed system not only streamlines targeted gene replacement and disruption but also can be used to target specific chromosomal locations like promoters or intergenic regions. It will expedite the progresses in the functional genomic studies of *A. parasiticus* and *Aspergilllus flavus*.

Introduction

Aspergillus parasiticus produces aflatoxins and is a pathogen of agricultural commodities such as corn, cotton, peanut and tree nuts. Much of the research of *A. parasiticus* has centred on elucidating the biochemical and regulatory mechanisms of aflatoxin biosynthesis and pathogen–host interactions (Bhatnagar *et al.* 2006). The available *A. flavus* genomic resources, such as expressed sequence tags and whole genome sequence, are having an increasing impact on *A. parasiticus* research due to the close relatedness of the two species (Payne *et al.* 2006; Yu *et al.* 2007). The *A. flavus* genome is 36·8 Mb and contains about 12 000 genes. The majority of these genes encode hypothetical proteins, whose functions are yet to be determined. Although DNA microarray is an efficient tool for examining genome-wide differences in gene expression (Wilkinson *et al.* 2007), gene-targeting through fungal transformation is still the preferable approach for elucidating gene function.

Homologous integration is the most efficient method of targeting a gene. Although homologous integration of exogenous DNA into the genome occurs readily in yeast, it occurs in only about 1–10% of transformants of animals, plants, or fungi even when a long stretch of homologous sequence is present. The integration of transforming DNA into a genome occurs through repair mechanisms of DNA double-strand breaks (DSB). DSB

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can occur *via* DNA lesions, such as inhibition of DNA topoisomerase I that generates transition single-strand breaks during DNA replication or binding of DNA by intercalating agents (Adachi *et al.* 2004). Two types of mechanisms, namely, the homologous recombination (HR) pathway and the NHEJ pathway are involved in DSB repairs (Kanaar *et al.* 1998). The HR pathway mediates the integration of exogenous DNA into a chromosome through regions that are homologous to the sequence of exogenous DNA. The NHEJ pathway mediates the integration of exogenous DNA irrespective of sequence homology. These two mechanisms act independently and function competitively (Van Dyck *et al.* 1999). The competitive nature of the two mechanisms makes it possible to inactivate gene(s) of one pathway to favour another pathway. In this study, an NHEJ-deficient *A. parasiticus* strain was generated by deleting its *ku70,* a key gene in the NHEJ pathway. The gene-targeting frequency of *adhA*, a gene involved in aflatoxin biosynthesis, in this strain increased significantly to 96%.

Materials and methods

Fungal strains

Aspergillus parasiticus RHN1 is a *niaD*-deficient strain. It does not produce aflatoxins but accumulates OMST as the end product due to a defect in the *ordA* gene that is necessary for aflatoxin biosynthesis. RHΔku70 is an NHEJ-deficient strain generated in this study.

Construction of gene-targeting vectors of *ku70* and *adhA*

Orthologous *A. parasiticus* genes share approximately 98–99% identity to *A. flavus* genes at the nucleotide level. Therefore, *A. flavus ku70* gene, including its flanking regions, was first identified from the 5X draft genome sequence of *A. flavus* (http://www.aspergillusflavus.org/genomics/). A three-step procedure was used to construct the *ku70*-targeting vector. A 1·4-kb 5′-untranslated region (UTR) and a 1·7-kb 3′UTR of *ku70* were generated by polymerase chain reaction (PCR) using primer pairs of ku5Sm/ku5X and ku3X/ku3S (Table 1), respectively. The fragments were cloned sequentially into pUC19 followed by the insertion of a 6·7-kb *Xba*I fragment that contains *A. parasiticus niaD* selectable marker (Fig. 1a). The two *Hin*dIII and one *Sal*I sites on the 6·7-kb DNA fragment had been removed by standard recombinant techniques (P.-K. Chang, unpublished results) prior to the insertion. The vector, pAΔku70, was linearized by *Sma*I and *Sal*I digestion prior to transformation. Similarly, for the construction of the *adhA*-targeting vector, a 0·7-kb 5′UTR plus coding region and a 0·8-kb 3′UTR plus coding region of *A. parasiticus adhA* were generated using adh5H/adh5P and adh3P/adhSm (Table 1) respectively. The PCR fragments were cloned into pUC19 followed by the insertion of the *ptr* selectable marker (Kubodera *et al.* 2002) amplified from pPTR1 (Takara, Japan) with

Table 1 Oligonucleotide primers used in this study

Primers	Sequence
ku900	TCAAGATCTGTCCCACGG
ku1500	TGACGGACGTCATCAGCG
ku5C	TCGCTTAACTCCCGGGGAATTGGCTTCT
ku3C	TACCAATCGCCAGTGAACGCTC
xxv1	CGTTTTCGGACTCTCTTCTG
xxv2	CTCTTCCACTGTGCTATCCA
ku5Sm	CTTCGCCCGGGTACGGGTCACCTAATC
ku5X	TATCTAGAGTCGTAAGTCATGAATTGCGT
ku3X	ATTCTAGACAACGCTAGTATTGGTTACGAG
ku3S	CTAGAACGAATTCGTGTCGACACTGA
ptr730P	ATACTGCAGACGGGCAATTGATTACGG
ptr1230P	TTACTGCAGCCGCTCTTGCATCTTTG
adh5H	AGACAAGCTTAGTAACATCCTGA
adh5P	CGAGGTCACATCGCTGCAGACG
adh3P	ATTCTGCAGCACTCGGGCTCGCACG
adh3Sm	ATACCCGGGCGGTGCTCAGGACTCCT
adh140	GCTGACATCCAACCTCTAGA
adh720	CGATTCCTCGAGCAGCCATA

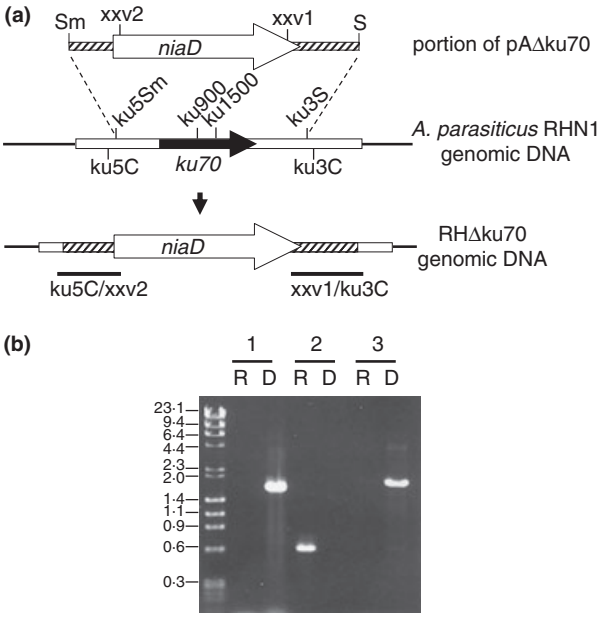

Figure 1 Targeted replacement of *ku70* by the *niaD* selectable marker. (a) The expected genomic pattern of a transformant after double-crossover recombination. (b) PCR analysis of genomic DNA of the recipient strain (R) and its *ku70* deletion mutant (D). The primers used were 1: xxv1 and ku3C, 2: ku900 and ku1500 and 3: xxv2 and ku5C respectively.

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ptr730P and ptr1230P (Table 1). The vector was linearized by *Hin*dIII and *Sma*I digestion prior to transformation.

Creation of homologous DNA ends from partial restriction enzyme recognition sequences

Restriction sites not present in a selectable marker (*niaD* or *ptr*) and in the pCU19 vector but present in the targeting regions are not always available for vector construction. The linearized gene-targeting DNA fragment, after restriction enzyme digestion to separate it from the pCU19 portion, must contain ends homologous to the fungal genomic sequence in order to increase the frequency of targeted integration. Manipulation of partial restriction enzyme recognition sequences was carried out to circumvent the dependence on complete recognition sequences that are commonly used by many researchers. As an example, the sequence 5′-CTTCGGCCGGGTAC…-3′ is located in the upstream of *ku70* and contains a partial *Sma*I recognition sequence. The ku5Sm primer (5′-CTTCGCCCGG-GTAC…-3′, Table 1) was designed so that the G was replaced by a C to create a complete *Sma*I site. The nonhomologous sequence 5′-CTTCGCCC-3′ was removed after *Sma*I digestion leaving the homologous end, 5′-GGGTAC…-3′ for homologous integration. The same principle was applied to the design of other primers.

Preparation of protoplasts and fungal transformation

Approximately 10^8 conidia were inoculated into 100 ml Czepak–Dox Broth (CZ, Becton, Dickinson and Company, Sparks, MA, USA) supplemented with 0·5% casamino acids. The culture was shaken at 150 rev min^{-1} for 11–12 h at 30°C. Mycelia were collected on a 100 μm nylon cell strainer, transferred to a 50 ml tube and resuspended in 20 ml filter-sterilized enzyme solution that contained 200 mg lysing enzymes (Sigma, St Louis, MO, USA), 50 mg driselase (Sigma), 200 mg β-D-glucanase G (InterSpex Products, San Mateo, CA, USA) and 40 mg cell-wall digesting enzyme (Applied Plant Research, Wageningen, The Netherlands) in 0·55 mol l^{-1} KC1, 0·05 mol l^{-1} citric acid, pH 5·8. The digestion was allowed to progress for 2–3 h at 30°C with shaking (60 rpm). Protoplasts were harvested by filtering through a 40 μm nylon cell strainer and pelleted. The protoplasts were washed twice with a solution of 0·6 mol l^{-1} KC1, 50 mmol l^{-1} $CaC1_2$ and 10 mmol l^{-1} Tris-HCl, pH 8·0. The polyethylene glycol (PEG) solution used consisted of 40% (w/v) PEG4000 (Fluka, Seelze, Germany), 0·6 mol l^{-1} KCl, 50 mmol l^{-1} $CaC1_2$ and 10 mmol l^{-1} Tris-HC1, pH 8·0. The fungal transformation and regeneration procedures described previously were used (Chang *et al.* 2004). Pyrithiamine (PT) was added to CZ regeneration agar medium as a selective agent at a concentration of 0·1 μg ml^{-1} for *prt*-based transformation. Agar plates from the *niaD*- and *prt*-based transformation experiments were incubated at 30°C for up to 7 and 12 days, respectively.

Drug sensitivity test

Sensitivity of *A. parasiticus* RHΔku70 to camptothecin and melphalan were compared with that of RHN1. Potato dextrose agar medium (PDA) plates (60 × 15 mm) containing 2·5 or 5·0 μmol l^{-1} of camptothecin (Sigma) were made using a 10 mmol l^{-1} stock solution prepared by dissolving 34·8 mg camptothecin in 10 ml dimethyl sulfoxide (DMSO). PDA containing 2·5 or 5·0 μmol l^{-1} of melphalan (Sigma) were made using an 8·2 mmol l^{-1} stock solution prepared by dissolving 5 mg melphalan in 2 ml 0·09% hydrochloric acid (w/v). Four replicate plates were used for each concentration and the control containing either DMSO or hydrochloric acid only. The plates were incubated at 30 °C for 4 days.

Thin layer chromatography of aflatoxin intermediates

Two PDA agar plugs were cored from each 5-day-old transformant culture plate with a Transfertube®, placed into a 2-ml microfuge tube and extracted with 0·4 ml of 100% methanol for 3 h. After tubes were spun at the maximum speed in a microfuge for 10 min, 200 μl of each supernatant was transferred to a new 0·5 ml tube for complete evaporation to dryness overnight. The pigment residues were dissolved in 20 μl chloroform and 10 μl was spotted onto a silica gel thin layer chromatography (TLC) plate. TLC was carried out using a toluene–ethyl acetate-acetic acid (50 : 30 : 4, v/v/v) solvent system as previously described (McCormick *et al.* 1987).

Results

Generation of *ku70*-deleted *Aspergillus parasiticus* recipient strain for transformation

Two of 25 nitrate-utilizing transformants were *ku70*-deleted strains. Deletion of *ku70* in the two transformants (RHΔku70) was confirmed by PCR with primers specific to the *ku70*-coding region, downstream and upstream regions of *niaD* and regions beyond the expected homologous integration sites (Fig. 1a). No PCR products were generated from the RHΔku70 genomic DNA when the primer pair ku900/ku1500 was used. In contrast, a 0·6-kb PCR fragment was amplified from the RHN1 genomic DNA (Fig. 1b). The primer pair xxv1/kuC3, which

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amplifies a small region beyond the ku3S position and the downstream region of *niaD* (Fig. 1a), generated a 1·8-kb PCR fragment from the RHΔku70 genomic DNA (Fig. 1b). Likewise, kuC5/xxv2 yielded a 1·9-kb PCR fragment, expected to be present in another flanking region after *ku70* was replaced by *niaD* through homologous integration. These two PCR fragments were not produced from the parental RHN1 genomic DNA.

Culture morphology and drug sensitivity of *ku70*-deleted *A. parasiticus*

Production of conidia, sclerotia and OMST were normal for RHΔku70 (data not shown). A comparison of the averages of colony diameters showed that the radial growth of RHΔku70 was about 94% that of RHN1 (45 mm *vs* 48 mm). Camptothecin inhibited growth and delayed conidial formation of RHΔku70 and RHN1 equally and the effects were concentration-dependent. Melphalan had no discernible effects on either strain.

Increase in gene-targeting frequency in *Aspergillus parasiticus* RHΔku70

The *adhA* gene, chosen to test if the frequency of homologous integration in RHΔku70 would increase, encodes an alcohol dehydrogenase which converts 5′-hydroxyaverantin (HAVN) to averufin (Yu *et al.* 2004). Impairment of *adhA* yields colonies of an intense yellowish orange colour resulting from the accumulation of HAVN and other aflatoxin intermediates in the mycelia. Fifty of 80 PT-resistant transformants were examined on PDA plates. Forty-eight transformants exhibited a yellowish orange colour. In contrast, none of the 18 RHN1 control transformants had colour. Figure 2a shows that all coloured transformants accumulated predominantly HAVN and significantly decreased amounts of OMST. The two non-coloured transformants predominantly accumulated OMST. PCR analyses with primers specific to the resulting flanking regions after homologous integration (data not shown) and with primers adh140/adh720 (Fig. 2b) indicated that these coloured transformants were genuine *adhA* disruptants. The 2·1-kb PCR fragment indicated that the 2·0-kb *ptr* marker had replaced the 0·6-kb region in *adhA* after double-crossover homologous integration at the *adhA* locus. The gene-targeting frequency was 96%.

Discussion

The *A. parasiticus* RHΔku70 produces conidia, sclerotia and OMST normally, and thus can be used as a recipient strain for elucidating functions of genes involved in the regulation of aflatoxin biosynthesis, fungal development and pathogen–host interactions. Slight decreases in colony growth were also observed for *ku70*-deleted *Aspergillus sojae* and *A. oryzae* strains (Takahashi *et al.* 2006), *akuB*-deleted *Aspergillus fumigatus* (da Silva Ferreira *et al.* 2006) and *mus51*-deleted *Neurospora crassa* (Ninomiya *et al.* 2004). This appears not to have adverse effects on studies of gene functions. Camptothecin traps topoisomerase I complex by inhibiting the religation step (Adachi *et al.* 2004). As observed in this study, camptothecin sensitivity of NHEJ-deficient yeast is essentially the same as that of the wild type (Vance and Wilson 2002). The growth inhibition on RHN1 and RHΔku70 by camptothecin is likely due to a direct effect on replication resulting from decreased amounts of available topoisomerase I. Sensitivity to DNA-damaging agents such as phleomycin, 4-nitroquinoline oxide, methane methyl sulfonate (da Silva Ferreira *et al.* 2006; Takahashi *et al.* 2006) and melphalan (this study) varies among *Aspergillus* species, which suggests that HR and NHEJ mechanisms may act somewhat differently on DSB repairs in each species. The insensitivity of *A. parasiticus* strains to melphalan implies that HR is primarily responsible for repair of DNA damages in *A. parasiticus*.

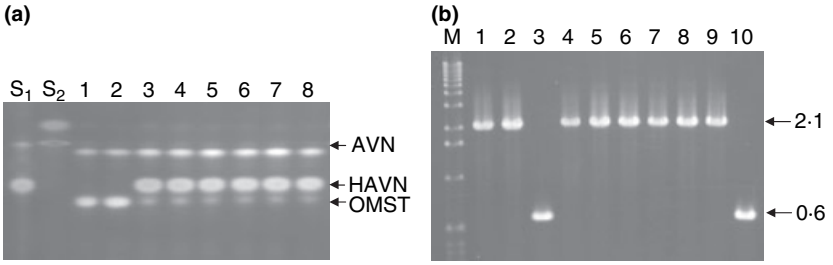

Figure 2 Characterization of *adhA* disruptants. (a) Thin layer chromatography analysis of aflatoxin intermediates produced by PT-resistant transformants. Lanes 1 and 2, non-coloured transformants morphologically identical to RHΔku70. Lanes 3–8, transformants morphologically identical to RHΔku70 except for having a yellowish orange colour on PDA. S_1 and S_2 are aflatoxin intermediate references. HAVN, 5′-hydroxyaverantin; AVN, averantin; and OMST, *O*-methylsterigmatocystin. (b) Genomic patterns of transformants revealed by PCR using primers adh140 and adh720; M, DNA 1-kb ladder. Transformants 3 and 10 are same as 1 and 2 in panel A; Others, coloured transformants.

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Gene-targeting frequencies in NHEJ-proficient *A. parasiticus* strains are commonly 1–10% (Cary *et al.* 1999; Chang *et al.* 2004; Wen *et al.* 2005). The *adhA*-targeting frequency in *A. parasiticus* RHΔku70 reaches 96%. Gene-targeting frequencies of *crzA*, a homologue of *Saccharomyces cerevisiae crz1* (Cyert 2003), in RHΔku70 and *nadA*, a putative aflatoxin biosynthetic gene, in aflatoxigenic *A. parasiticus* BN9Δku70 also reach 98% and 100% respectively (P.-K. Chang, unpublished results). Therefore, consistent with studies on other fungi (Ninomiya *et al.* 2004; da Silva Ferreira *et al.* 2006; Takahashi *et al.* 2006) an NHEJ-deficient pathway in *A. parasiticus* greatly facilitates homologous integration and reduces necessary time and workload because knockout mutants are readily obtained in a single transformation experiment. When using a NHEJ-proficient recipient strain sometimes several attempts are necessary before a knockout mutant is obtained.

The gene-targeting frequencies in closely related *A. sojae* and *A. oryzae ku70*-deleted strains range from 60% to 75% (Takahashi *et al.* 2006). An increase in the length of flanking regions to 2·0 kb increases the gene-targeting frequency to 87% but still is lower than that achieved in *A. parasiticus* RHΔku70. In *akuB*(*ku80*)-deleted *A. fumigatus*, targeting-vectors containing flanking regions of 1·5–2·0 kb yield about 80% homologous integration (da Silva Ferreira *et al.* 2006). For *A. parasiticus*, the length of flanking regions seems to play a less role in homologous integration because for *adhA* (this study) *crzA* and *nadA* (P.-K. Chang unpublished results) the length of flanking regions is within 0·6–1·0 kb. In *N. crassa mus-51*(*ku70*) or *mus-52*(*ku80*)-deleted strain (Ninomiya *et al.* 2004), flanking regions of 0·5–1·0 kb give frequencies of homologous integration comparable with RHΔku70. The reason for some fungi requiring longer flanking regions to achieve the same gene-targeting frequency is not clear. It is probably related to the activity of the restriction system of each species, which degrades transforming DNA. Studies have shown that flanking regions smaller than 0·5 kb consistently give low frequencies of homologous integration even in an NHEJ-deficient background (Ninomiya *et al.* 2004; da Silva Ferreira *et al.* 2006; Takahashi *et al.* 2006). A different threshold length of flanking regions might be required for the HR pathway to operate properly and efficiently in each fungus.

The use of *ptr* as a selectable marker provides several advantages. First, generation of mutants is not necessary. Second, growth of untransformed strains is inhibited by low PT concentrations. Third, its size is small and does not have restriction sites, *Bgl*I, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sma*I and *Xho*I that are also not present in pUC19 vector. Partial sequences of the aforementioned restriction sites can be utilized in the directional cloning and in the creation of homologous DNA ends (see *Materials and methods*). The use of short partial recognition sequences such as *Sma*I (5′-CCC-3′ or 5′-GGG-3′) gives even greater flexibility, which makes targeting any genomic regions possible because of its wide presence. This system also makes it possible to insert a DNA fragment into a specific chromosomal location, such as a promoter region, for studying gene expression. In addition, it may be used to investigate synergism of two related genes by generating double knockout mutants, such as *norA* and *norB* (Yu *et al.* 2004). These genes are assumed to have overlapping functions in aflatoxin biosynthesis but have not been resolved conclusively in NHEJ-proficient *A. parasiticus* due to the extremely low frequency of targeting both genes by cotransformation.

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