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Fungal Genetics and Biology 43 (2006) 439–445
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Evidence of RIP (repeat-induced point mutation) in transposase sequences of *Aspergillus oryzae*

Maria D. Montiel, Heather A. Lee *, David B. Archer

Institute of Genetics, School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Received 11 October 2005; accepted 26 January 2006
Available online 13 March 2006

Abstract

A DNA methyl-binding column was used to isolate genomic fragments enriched for DNA-methylation from *Aspergillus parasiticus*. One of the isolated sequences presented 67% identity at the protein level with the transposase from the transposable element *Tan1* of *Aspergillus niger* var. *awamori*, and was found to be present in at least 20 copies in the *Aspergillus oryzae* database. Analysis of four copies showed evidence of C:G to T:A transitions in at least 98.2% of the mutations found over a 1032–1180 bp region spanning a large part of the transposase sequence. Using copy specific primers three sequences were amplified from a different strain of *A. oryzae* and a similar pattern of C:G to T:A transitions was found. These transitions are similar to those observed in RIP, in *Neurospora crassa*, where cytosine-methylation is believed to be involved. Using methylation-sensitive Southern blotting, no evidence of methylation was found in the transposase sequences in these two *A. oryzae* strains as well as one *A. parasiticus* and one *Aspergillus flavus* strain.

Keywords: Repeat-induced point mutation; Transposase; *Aspergillus oryzae*; DNA methylation

1. Introduction

Repeat-induced point mutation (RIP) is an irreversible genome defence system that detects DNA duplications greater than 400 base pairs (bp) and introduces C:G to T:A mutations. Originally identified in *Neurospora crassa*, RIP acts at a precise stage in the sexual cycle, between fertilization and meiosis, with a significant preference for CpA contexts, and typically leads to dense cytosine methylation (Cambareri et al., 1989; Selker et al., 1987). Evidence of RIP has been demonstrated after several rounds of sexual reproduction on a homologously integrated cosmid in *Podospera anserina* (Graia et al., 2001), and also on the *Pat* element in *P. anserina* (Hamman et al., 2000), which is a close relative of *N. crassa*. RIP-like mutations leading to degenerate fungal transposons have also been reported in the sequences of transposable elements in several phylogenetically more distant filamentous fungi. The transposable element *Fot1* in *Fusarium* species (Daboussi et al., 2002); the *impala* element in *Fusarium oxysporum* (Hua-Van et al., 2001); the retrotransposon MAGGY in *Magnaporthe grisea* (Nakayashiki et al., 1999; Ikeda et al., 2002); the *copia*-type and *Helitron*-like elements in *Microbotryum violaceum* (Hood et al., 2005); the *Pholy*-like retrotransposon in *Leptosphaeria maculans* (Attard et al., 2005) all display RIP-like mutations, with C:G to T:A transitions, on some copies. Whilst *M. violaceum* is sexual and *P. anserina* can propagate via a sexual cycle, *Fusarium* species are asexual and *M. grisea* has a sexual phase only observed under laboratory conditions. Among *Aspergillus* species these C:G to T:A transitions have been observed in the retrotransposons *Dane1* and *Dane2* (Nielsen et al., 2001), and the MATE elements (Clutterbuck, 2004) in *A. nidulans*, and also in the *Afut1* retrotransposon in *Aspergillus fumigatus* (Neuveglise et al., 1996). *A. nidulans* has a sexual cycle but *A. fumigatus* is believed to propagate asexually, despite evidence for sexual potential (Dyer et al., 2003; Paoletti et al., 2005).

* Corresponding author. Fax: +44 115 951 3251.
E-mail address: heather.lee@nottingham.ac.uk (H.A. Lee).

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doi:10.1016/j.fgb.2006.01.011

During our search for DNA methylation in the *Aspergillus* section *Flavi* complex (Montiel et al., 2003) we isolated a sequence from *A. parasiticus* that has several copies with homology in the *Aspergillus oryzae* genome. These sequences show evidence of RIP-like transitions, which we report here.

2. Materials and methods

2.1. Strains and DNA isolation

For this study *A. parasiticus* (ATCC 56775), *A. flavus* (ATCC 6474), and *A. oryzae* (ATCC 42149 and ATCC 14895) strains were used. Cultures were maintained on AMMN slopes (1% w/v glucose, 60 mM KNO_3, 0.04 μM biotin, 2% v/v ACM salts; Morrice et al., 1998), and stored as spore stocks on silica gel. Cultures (100 ml) of minimal medium (MM; containing, per litre, 10 g of glucose, 6 g of KNO_3, 0.52 g of KCl, 0.52 g of $MgSO_4 \cdot 7H_2O$, 1.52 g of KH_2PO_4, trace amounts of $Na_2B_4O_7 \cdot 10H_2O$, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot 2H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and $ZnSO_4 \cdot 7H_2O$) were inoculated with 1×10^5 spores ml^{-1} in 250 ml shake flasks and grown at 25 °C, 150 rpm for 3 days. The mycelia were harvested through Miracloth, freeze-ground in liquid nitrogen and freeze-dried for 16 h.

Freeze-dried mycelia were resuspended in 10 ml DNA extraction buffer (50 mM Tris–HCl, 25 mM EDTA, 500 mM NaCl, and 1% SDS, pH 8.0), heated at 65 °C for 0.5 h and vortexed with an equal volume of phenol:chloroform (1:1 by vol.). Following centrifugation, the aqueous phase was removed to a fresh tube and a second phenol:chloroform extraction performed. Isopropanol (0.6 vol.) was added to the recovered aqueous phase, and the DNA precipitated immediately by centrifugation. After washing with 70% ethanol the precipitated DNA was air-dried and resuspended in 40μl TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6). RNase (10 μg in 10 μl) was added and the DNA incubated at 37 °C for 2 h to degrade any contaminating RNA.

2.2. Nucleic acid analysis

Protein sequences were aligned with CLUSTALW on the Infobiogen web site (http://www.infobiogen.fr) and with Vector NTI suite 7. Accession numbers for the sequences used: RID (*N. crassa*), AF500227; Masc1 (*Ascobolus immersus*), AAC49849; DmtA (*A. nidulans*) AF428247; Dim-2 (*N. crassa*), AF348971. The *A. fumigatus* DmtAf sequence was obtained from TIGR (http://tigrblast.tigr.org/ufmg/). The website used to interrogate the *A. oryzae* genome by BLAST was http://tigrblast.tigr.org/er-blast/index.cgi?project=asp. In addition, the *A. oryzae* genome sequence has been submitted to GenBank with Accession Nos. AP007150–AP007177 (Machida et al., 2005).

2.3. Enrichment of methylated DNA on MBD columns

To enrich for the methylated component of the *A. parasiticus* genome, genomic DNA was first restricted with *Mse*I (AATT), which cuts DNA into small fragments (100–200 bp) but leaves CpG sequences relatively intact. A methyl-CpG binding (MBD) column was prepared according to Cross (2002) using the methyl-CpG-binding domain of MeCp2 (kindly donated by Sally Cross). The *Mse*I-digested DNA was passed over the MBD column, which fractionates DNA according to the degree of CpG methylation. To assess the column, plasmid pUC19 was linearised and methylated with *Sss*I methylase at all 169 CpGs. A mixture of the unmethylated and methylated plasmid was applied to the column and eluted with a salt gradient. The eluted fractions were precipitated with ethanol including Pellet Paint® Co-Precipitant carrier (Novagen). The DNA was resuspended in 10 μl of TE and fractionated on an agarose gel. DNA was found in the fractions eluted at 0.4 M and at 0.8 M NaCl. Subsequently, methylated plasmid only was passed down the column and was eluted in the 0.8 M NaCl fraction, indicating that the unmethylated and methylated versions of pUC19 were separated by the MBD column. When *Mse*I-digested *A. parasiticus* DNA was applied to the MBD column, most of the DNA eluted in the 0.4 M salt fraction with only a small proportion-binding tightly under those conditions. This was subsequently eluted in the 0.8 M salt fraction.

2.4. Cloning and identification of methylated sequences

DNA fragments from the 0.8 M salt fraction of the MBD column were ligated to an *Mse*I adapter (5′-GAC GATGAGTCCTGAG-3′; 3′-TACTCAGGACTCAT-5′; originally designed for AFLP analysis; Montiel et al., 2003) and then amplified by PCR using a primer corresponding to the *Mse*I adapter sequence (5′-GACGATGAGTCCT GAGTAA-3′). The amplified sequences were cloned using pGEM(R)-T Easy vector (Promega) and sequenced.

The resulting sequences were used to search the databases using BLASTN, BLASTX or TBLASTN programs (Altschul et al., 1997).

Transposase homologues in *A. oryzae* ATCC 14895 of three of the transposase sequences from *A. oryzae* ATCC 42149 identified in the *A. oryzae* database (http://tigrblast.tigr.org/er-blast/index.cgi?project=asp; sequences A, B and D) were amplified by PCR using the primers in Table 1. The PCR products were cloned directly into the pGEM(R)-T Easy vector (Promega) and sequenced.

Table 1
PCR primers for different copies of the transposase sequence

Sequence	Oigonucleotide sequence 5′–3′
A	AACATCAAGCGGTTAAAGCTT (F)
	CCTGAGCTCAAAGCCTGCTT (R)
B	AATGGCAATATTAAGCGATT (F)
	CTTAAATTCTGCTTTGCACG (R)
D	CCTTTATACGAGTCTTTTAATAGTA (F)
	TGCTTTATATATCGCTATAACTATTAATGT (R)

(F, forward; R, reverse.)