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Impala, a transposon from *Fusarium oxysporum*, is active in the genome of *Penicillium griseoroseum*

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Abstract

An autonomous *impala* transposon trapped in *Fusarium oxysporum* by insertion within the *niaD* gene encoding nitrate reductase was introduced in the genome of the fungus *Penicillium griseoroseum*, a producer of pectinase enzymes. Through a phenotypic assay, we demonstrate that this element is able to excise from the *niaD* gene and to reinsert at new genomic positions. As in the original host, *impala* inserts into a TA site and footprints left by *impala* excisions are generally 5 bp. The fact that *impala* is able to transpose in *P. griseoroseum* offers the opportunity to develop a gene-tagging system based on this element with the objective to detect and clone genes related in pectinase production.
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Keywords: Transposon *impala*; Heterologous transposition; Filamentous fungus; *Penicillium griseoroseum*

1. Introduction

Transposable elements (TEs) are segments of DNA capable of moving from one location to another in the genome. They are ubiquitous in prokaryotic and eukaryotic genomes. They were recently detected in filamentous fungi by characterization of repeated sequences or their insertion into target genes [5–7,14] (see for a review). The whole spectrum of eukaryotic transposons is represented in fungi. Some species, exemplified by *Fusarium oxysporum* [1,7,10,23] and *Magnaporthe grisea* [8,9,12] appear to be very rich niches for these elements. Autonomous copies of TEs have been identified for a few groups such as *Fot1* and *impala*, two *Tc1-mariner*-like elements from *F. oxysporum* [11,20], *restless*, a member of the hAT family from *Tolypocladium inflatum* [13], *MAGGY*, a retrotransposon from *M. grisea* [22], and *Tad*, a LINE-like element from *Neurospora crassa* [4,15]. Currently, these elements are being tested for use as genetic tools in novel host fungi. Recently, *impala* was demonstrated to transpose in several heterologous hosts, *Fusarium moniliforme* [11], *M. grisea* [30] and *Aspergillus nidulans* [17]. Moreover, *impala* transposition was shown to be associated in the generation of different mutants impaired in pathogenicity in *F. oxysporum* f. sp. *melonis* [21] and demonstrated to have tagged a pathogenicity gene in *M. grisea* [30] and the *yB* gene in *A. nidulans* [17]. These results indicate that *impala* will be very useful for insertional mutagenesis in fungi because its transposition seems not to be dependent on host factors.

The aim of this work was to test the efficiency of *impala* transposition in the fungus *Penicillium griseoroseum* to develop a transposon-based tagging system in this species. The fungus *P. griseoroseum*, isolated in Viçosa (Brazil), produces a pectinolytic complex with low cellulases production, what turns it particularly interesting for the use in the textile industry [3]. Here we provide evidence for transposition of *impala* in *P. griseoroseum* on the basis of molecular analyses of excision and reinsertion events.

2. Materials and methods

2.1. Strains and culture conditions

The strain used in this work was a double mutant from *P. griseoroseum*, *nia yel*, impaired in nitrate reductase activity and producing yellow conidia. Complete medium

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was potato dextrose agar (PDA), and selective minimal medium (MMN) containing nitrate as the sole nitrogen source [24]. Hygromycin B-resistant (HygR) transformants were selected on PDA supplemented with 20% sucrose and 100 μg of hygromycin B (Sigma) per ml and purified by monospore isolation.

2.2. Plasmids

In the transformation experiments, plasmids pAN7.1 [25] and pNIL160 [30] were used. The pAN7.1 plasmid contains the hygromycin B phosphotransferase gene (*hph*) of *Escherichia coli* linked to a promoter and terminator of *A. nidulans*. The pNIL160 plasmid carries the *impala160* copy inserted in a 1.3-kb promoter of the *niaD* gene of *A. nidulans* [30]. The pGEM-T Easy (Promega) plasmid was used to clone the PCR products, according to the manufacturer's instructions. The pAN301 [19] plasmid, which contains the entire *A. nidulans niaD* gene, was cleaved with *Eco*RI and the 2.7-kb fragment containing the major part of the *niaD* open reading frame was used as a probe in hybridization experiments.

2.3. Transformation experiments

Protoplasts were produced using the protocol described in Queiroz et al. [26]. Transformation experiments were carried out according to the method described by Balance and Turner [2] with slight modifications.

2.4. Isolation of revertant colonies

Suspensions of approximately 10^7 conidia ml^{-1} of the co-transforming strains were produced and 10^6 conidia per Petri dish were plated on minimal medium. Twenty Petri dishes were used for each co-transformant. The dishes were incubated at 26°C during 30 days. After this period, the revertant colonies were transferred to new dishes with minimal medium and monosporic purification of the revertants was carried out.

2.5. DNA preparation and Southern blot analysis

DNA extraction for the Southern blot experiments was performed using the method described by Specht et al. [29]. About 5 μg of DNA was digested with *Eco*RI restriction enzyme and fractionated in agarose gel by standard methods [27]. Southern transfer was performed onto Hybond N nylon membranes (Amersham) and the DNA was fixed to the membranes by UV cross-linking. Membranes were probed with *impala* and *niaD* probes using standard procedures. The *impala* probe corresponded to an amplification product from plasmid pNIL160, and the *niaD* probe was isolated by fragment electroelution after *Eco*RI digestion of plasmid pAN301 [19]. These probes were ^{32}P-labeled with a 'T7 Quick prime' kit (Pharmacia Biotech). Hybridization and washing were performed using stringent conditions.

2.6. PCR amplification

For the traditional PCR and thermal asymmetric interlaced PCR (TAIL-PCR) experiments, the methods described by Hua-Van et al. [11] and Liu and Whittier [18], respectively, were applied. They were carried out in a Biomed 60 thermocycler (Braun) programmed to perform 30 cycles of 1 min at 92°C, 30 s at 60°C, 1 min and 30 s at 72°C, and a final extension of 10 min at 72°C for traditional PCR. The reactions were prepared for a volume of 50 μl, containing DNA (200–500 ng), 1 or 5μl of Appligene Taq buffer, 80 nM of each dNTP, 80 or 40 pmol of every oligonucleotide and 0.2 μl of Taq polymerase Appligene. The primers for the *impala* element amplification were: SPE5 (5′-AGAACACAACCCTGCCACGG-3′) and SPE3 (5′-TCCGGGCCGTATGCACAGAG-3′). The primers niaD144 (5′-GTTCATGCCGTGGTCGCTGCG-3′) and niaD145 (5′-CTTTCTTCCCGGCCAAAGCC-3′) were used to amplify the excision site of the element inside the *niaD* gene. The primers hph1 (5′-CAGCGAGAGCCTGACCTATTGC-3′) and hph2 (5′-GCCATCGGTCCAGACGGCCGCGC-3′) were used for the *hph* gene amplification. The amplification conditions and reactions used for the TAIL-PCR were exactly like described by Lin and Whittier [18]. In these experiments, the primers used were: AD1 (5′-TG(A/T)GNAG(A/T)ANCA(G/C)AGA-3′), Imp1 (5′-GGCGTTGTCAGGCGTTGTGA-3′), Imp2 (5′-GATGTGTGTGTGTGGAGGAGG-3′) and Div5 (5′-CGATCGGGTTAGGCCGGACCG-3′).

2.7. Sequencing

The DNA sequences of the excision sites of the *impala* element inside the *niaD* gene were realized according to the Sanger et al. method [28], using the automatic sequencer ABI 373 (Perkin Elmer – Applied Biosystem) and the 'Dye Terminator ABI Prism sequencing kit'. The oligonucleotide used in the sequencing was niaD144, described above.

3. Results and discussion

To determine if *impala* is able to transpose in the genome of *P. griseoroseum*, we followed a strategy identical to that detailed for *impala* in *M. grisea* [30]. We first demonstrated the absence of *impala*-related sequences in *P. griseoroseum* by a Southern blot experiment, using *impala* as a probe in low stringency conditions (results not shown). Plasmid pNIL160 containing the autonomous *imp160* copy inserted in the full-length promoter of the *niaD* gene of *A. nidulans* was introduced in a *nia*$^-$

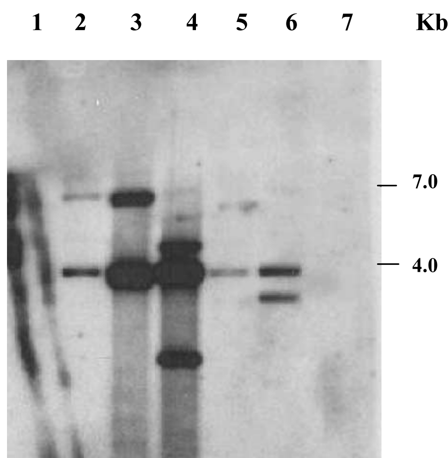

Fig. 1. Molecular analysis of the *P. griseoroseum* co-transformants. Genomic DNA of the co-transformants was digested with *Eco*RI and hybridized to *impala*. Lane 1: *nia yel* strain; lanes 2–7: T1, T2, T6, T7, T9, T10, respectively.

P. griseoroseum strain. This was achieved by co-transformation with the pAN7.1 plasmid conferring hygromycin B resistance. HygR transformants were detected after 5 days of growth on selective medium (PDA+hygromycin B), with a frequency ranging from 5 to 32 transformants per μg of pAN7.1. After transfer to new selective dishes, approximately 76% of these transformants did not develop, suggesting a high mitotic instability, and could represent abortive transformants. Ten HygR fast-growing transformants were selected for a PCR analysis by using specific primers for the *hph* gene and for the *impala* element. Six transformants were shown to contain the *impala* element and then they were analyzed by Southern blot to determine the pattern of integration of pNIL160 using an *impala niaD* probe (Fig. 1). Five co-transformants presented a 4-kb *Eco*RI fragment corresponding to the *niaD* gene interrupted by *imp160* and most exhibited extra bands suggesting multi-integration events, or rearrangements, some of them likely disrupting the 4-kb fragment. Only one transformant, T7, presented a simple pattern with only the 4-kb fragment.

In order to check if *impala* can excise from the *niaD* gene, five transformants were subjected to the phenotypic excision assay [11] by plating approximately 10^6 conidia of each one on 20 Petri dishes with MMN. After 4 weeks of incubation, 11 revertant colonies were detected from co-transformant T1.

Southern blot analysis of seven revertants from T1 digested with *Eco*RI and probed with *niaD* and *impala* (Fig. 2) confirmed the excision and reinsertion of *impala*. Revertants R2 to R7 displayed the 2.7-kb *niaD* fragment expected after *impala* excision. R3, R4, R6, R7 also showed a 4-kb fragment as in transformant T1 indicating the multi-copy integration of pNIL160. Absence of this band in R2 and R5 suggested plasmid deletions, which are frequently observed in tandem plasmid integrations. Some high-molecular mass bands were sometimes observed that could represent partial digestion or transgene rearrangements. Revertant R1 did not show the typical *impala* excision pattern since it presented a band of >2.7 kb and a 4-kb band, suggesting they may be internal rearrangements.

Hybridizations with an *impala* probe also revealed a complex pattern. *Impala* reinsertions were clearly detected in four revertants out of seven. This frequency is in the same range as that observed in *F. oxysporum*. No *impala* sequence was observed in R1 and R5. In R5 excised *impala* was likely lost, whereas in R1 that did not show the wild-type *niaD* sequence, *impala* loss might be the result of rearrangement. Some of the high-molecular mass *niaD* bands co-hybridized with *impala* and could result from rearrangements involving different copies of the construct. We can also observe in this area additional *impala*-hybridizing bands that might correspond to reinsertions.

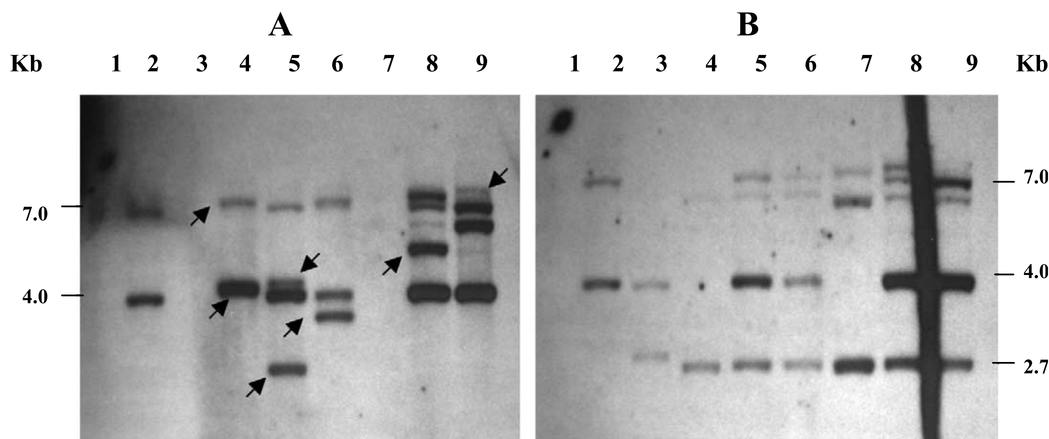

Fig. 2. Molecular analysis of T1 transformant and *niaD*+ revertants of *P. griseoroseum*. Genomic DNA was digested with *Eco*RI, submitted to agarose gel electrophoresis and transferred onto a nylon membrane. The membrane was hybridized with *impala* (A) and *niaD* (B). Lane 1: *nia yel* strain; lane 2, T1; lanes 3–9, R1, R2, R3, R4, R5, R6, R7. Arrows show reinserted *impala* elements.

Excision of *impala* usually leaves five additional base pairs in the *niaD* gene [16], which corresponds to the TA sequence duplicated in the insertion, and three nucleotides from either the 5′ or 3′ end of *impala* inverted terminal repeats (ITRs). For five revertants, the 2.7-kb *niaD* fragment amplified by PCR was cloned and sequenced to check for *impala* excision footprint. These footprints were 4 bp (CATA) in R2 and R7 and 5 bp (TACAG) in R3, R4 and CTGTA in R6. The sequence of one side of the *impala* new insertion site in the R2 genome amplified by TAIL-PCR showed that the insertion occurred into a TA dinucleotide (results not shown) as observed in *F. oxysporum* [16].

The results presented in this work clearly demonstrate the transposition of *impala* of *F. oxysporum* in *P. griseoroseum*. Using a phenotypic excision assay, we obtained excisions of the autonomous copy *imp160* from the *niaD* gene with typical excision footprints, as described in *F. oxysporum* [11,16] and other species [17,30]. Transposition certainly occurred through the action of the *impala* transposase since *impala*-homologous sequences were not detected in the genome of *P. griseoroseum*. However, the presence of highly divergent *impala* elements cannot be excluded. Checking the capacity of a defective marked element to transpose in this context could test this. Reinsertion of excised *impala* copies occurred in a high rate (approximately 70%) and appeared to be multiple in some revertants.

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