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Suitability of *Vader* for Transposon-Mediated Mutagenesis in *Aspergillus niger*▽†

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The filamentous fungus *Aspergillus niger* is widely used in biotechnological applications. Strain CBS513.88 is known to harbor 21 copies of the nonautonomous transposon *Vader*. Upon selection of chlorate-resistant *A. niger* colonies, one *Vader* copy was found integrated in the *nirA* gene. This copy was used for vector construction and development of a transposon-tagging method. *Vader* showed an excision frequency of about 1 in 2.2×10^5 conidiospores. A total of 95 of 97 colonies analyzed exhibited an excision event at the DNA level, and *Vader* footprints were found. By employing thermal asymmetric interlaced (TAIL)-PCR, the reintegration sites of 21 independent excision events were determined. All reintegration events occurred within or very close to genes. Therefore, this method can be used for transposon mutagenesis in *A. niger*.

Transposons are ubiquitous mobile genetic elements and are present in all prokaryotic and eukaryotic genomes. While transposons were detected early on in bacteria, plants, animals, and yeasts, it was only in the 1990s that they were described for filamentous fungi (7, 15, 21). It became evident that fungi harbor the same types of transposable elements as other eukaryotes, i.e., class I and class II transposable elements. Of all the fungal transposons described so far, only one has been applied for transposon mutagenesis (8).

The genome sequencing project of *Aspergillus niger* CBS513.88 was completed a few years ago (20). As this species is of industrial significance, an unbiased random mutagenesis tool based on endogenous transposons and used to identify new genes and understand their function and to create new industrially important mutants would be of great value. In a recent survey, we characterized all transposable elements of *A. niger* CBS513.88 and analyzed them for activity during strain improvement cycles. In that study, only *Vader* transposons were found to be active (3). The *A. niger* CBS513.88 strain is known to harbor 21 copies of *Vader*, which is a nonautonomous transposon. It was originally detected in *A. niger* var. *awamori* (1, 19). The members of this transposon family share a very high degree of similarity, with lengths about 439 bp and terminal inverted repeats of 44 bp. *Vader* elements are believed to be transactivated by the *Tan1*-encoded transposase, which is also present in the *A. niger* CBS513.88 genome. With the present study, we show that *Vader* elements can be activated, as observed in the strain lineage (3), and we present a new method for transposon mutagenesis in *A. niger* based on the transactivation of a labeled *Vader* copy.

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MATERIALS AND METHODS

Strains and culture conditions. *Aspergillus niger* strain CBS513.88 was used in this study. For selection experiments, *A. niger* was grown on *Aspergillus* minimal medium (AMM; 6.9 mM KCl, 11.2 mM KH_2PO_4, 4 M KOH, 2.1 mM $MgSO_4 \cdot 7H_2O$, 50.5 mM glucose, 37 mM NH_4Cl, 0.1% (vol/vol) trace element solution I [76.5 mM $ZnSO_4 \cdot 7H_2O$, 178 mM H_3BO_3, 18 mM $FeSO_4 \cdot 7H_2O$, 7 mM $CoCl_2 \cdot 6H_2O$, 6.4 mM $CuSO_4 \cdot 5H_2O$, 25 mM $MnCl_2 \cdot 4H_2O$], 0.1% (vol/vol) trace element solution II [6.2 mM $Na_2MoO_4 \cdot 2H_2O$], and 1% (wt/vol) agarose). For excision selection, hygromycin B was added at a concentration of 125 μg/ml.

Transformation of *A. niger* was done according to published procedures (23), with the following modifications: 5×10^7 *A. niger* conidiospores were used to grow mycelia for protoplast preparation, mycelia were filtered through sterile Miracloth (Calbiochem, La Jolla, CA), and protoplast formation was performed using the lytic enzyme mixture Glucanex (Sigma, Steinheim, Germany).

Oligonucleotides and PCR amplification. Oligonucleotides used are given in Table S1 in the supplemental material and were synthesized by MWG-Biotech (Ebersberg, Germany). PCR was done as previously described (2–4).

Selection for excision of *Vader*. Mycelia of transformant AnT-6(3) carrying vector pIB635 were grown on solid medium, and conidiospores were obtained. A total of 10^7 conidiospores were placed on agarose plates containing minimal medium with 125 μg/ml hygromycin B. Conidiospores were then taken from hygromycin B-resistant colonies and streaked again on minimal medium with 125 μg/ml hygromycin B for 4 to 7 days in order to select against the formation of heterokaryons.

TAIL-PCR. Thermal asymmetric interlaced (TAIL)-PCR was based on published methods (17). Each 20-μl primary TAIL-PCR mixture contained 2 μl of 10× PCR buffer, 200 mM deoxynucleoside triphosphate (dNTP) mix, 250 nM oligonucleotide IB1345, 5 μM oligonucleotide IB1347, 0.02 to 0.025 U *Taq* DNA polymerase, and 20 to 60 ng DNA. For the secondary reaction, 1 μl of 40-fold-diluted primary TAIL-PCR product was used as the template, with 200 nM oligonucleotide EH1434 and 3 μM oligonucleotide IB1347. For the tertiary TAIL-PCR, again 1 μl of 40-fold-diluted secondary TAIL-PCR product was used as the template. A total of 100 nM oligonucleotide IB1336 and 3 μM oligonucleotide IB1347 were employed. Details are listed in Table 1. Specific products were gel purified and directly sequenced to confirm the identities of the integration sites.

Transposon trap experiments. The basic medium used for transposon trap experiments contained 4.2 mM $MgSO_4 \cdot 6H_2O$, 6.7 mM KCl, 0.65 mM $FeSO_4 \cdot 7H_2O$, 58 mM sucrose, and 0.143% (vol/vol) trace element solution (55.6 mM $ZnSO_4 \cdot 7H_2O$, 2.5 mM $CuSO_4 \cdot 5H_2O$, 6.5 mM $FeSO_4 \cdot 7H_2O$, 0.7 mM $MnSO_4 \cdot H_2O$, 1.6 mM boric acid, 0.4 mM $MoNa_2O_4 \cdot 2H_2O$). Three additional media were prepared by dissolving 35.3 mM $NaNO_3$ (solution 1), 7.3 mM hypoxanthine (solution 2), or 35.3 mM $NaNO_3$ and 225.5 mM $NaClO_3$ (solution 3) in 300 ml 75 mM K_2HPO_4/KH_2PO_4, pH 5.8. Solutions 1, 2, or 3 and 700 ml basic medium were autoclaved separately and mixed, and 2% agar was added. In the case of solution 3, 1% (vol/vol) of a 10% L-arginine solution was

TABLE 1. Thermal cycling conditions used for primary, secondary, and tertiary TAIL-PCR

TAIL-PCR	File no.	No. of cycles	Thermal setting(s) (time)
Primary	1	1	92°C (2 min), 95°C (1 min)
	2	4 high-stringency cycles	94°C (15 s), 55°C (1 min), 72°C (2 min)
	3	1 low-stringency cycle	94°C (15 s), 30°C (3 min), 30°C (+ 0.2°C/s) 18°C (+ 0.3°C/s), 72°C (2 min)
	4	9 reduced-stringency cycles	94°C (5 s), 37°C (1 min), 72°C (2 min)
	5	11 super cycles	94°C (5 s), 55°C (1 min), 72°C (2 min)
			94°C (5 s), 55°C (1 min), 72°C (2 min)
			94°C (5 s), 37°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)
Secondary	7	1	94°C (2 min)
	8	1	94°C (5 s), 55°C (1 min), 72°C (2 min)
	9	9 super cycles	94°C (5 s), 55°C (1 min), 72°C (2 min)
			94°C (5 s), 55°C (1 min), 72°C (2 min)
			94°C (5 s), 37°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)
Tertiary	10	1	94°C (2 min)
	11	19 normal cycles	94°C (30 s), 37°C (1 min), 72°C (2 min)
	6	1	72°C (5 min)

added after autoclaving. Primary selection for transposon trap experiments was done on solution 3-basic medium. Colonies obtained were then plated on solution 1-basic medium and solution 2-basic medium to exclude mutations in cofactor genes.

Vector construction. The 5′ and 3′ flanking regions of the *A. niger niaD* gene (An08g05610) were amplified from genomic DNA using primer pair IB0932 and IB0933 and primer pair IB0934 and IB0935, respectively. The *Aspergillus nidulans gpdA* promoter (Z32524) (22) was obtained using primer pair IB0910 and IB0911, and the *A. nidulans trpC* terminator (ENU24705) (18) was obtained using primer pair IB1041 and IB1042. Primer pair IB1039 and IB1040 served to amplify the *Escherichia coli hph* gene (V01499) (11). The sequence of the transposon *Vader* was not obtained via PCR but was synthesized by Sloning BioTechnology (Puchheim, Germany). This sequence contained flanking target site duplications and, within the transposon sequence, an AsiSI recognition site and an artificial anchor sequence for binding of a specific oligonucleotide in subsequent PCR analyses. The resulting vector pIB635 is shown here (see Fig. 2a).

RESULTS AND DISCUSSION

Transposon mutagenesis in general can be a powerful tool for generating mutation libraries (reviewed in references 6, 12, and 13). For this purpose, a heterologous transposon may be introduced in a new host (9), or endogenous transposons may be employed (14). To identify a *Vader* copy which can be transactivated, we used chlorate selection as a transposon trap. Chlorate-resistant *A. niger* colonies may be the result of mutations in either the *niaD* or *nirA* gene or in uptake or cofactor genes. After performing several physiological tests and molecular analyses on obtained isolates (data not shown), a *Vader* insertion event was observed in the *nirA* gene of one mutant (Fig. 1). Sequence analysis revealed the presence of the expected target site duplications.

Based on the sequence of the activated *Vader* element inserted in the *nirA* gene, a synthetic element was synthesized which contained a unique 20-bp anchor, 5′-GAGTCCCGTCG TACTTCTGC-3′, allowing for specific amplification of this synthetic *Vader* element. The synthetic *Vader* element was then cloned between the *gpdA* promoter and the open reading frame of the hygromycin B resistance gene (Fig. 2a). In this position, the element efficiently blocks the transcription of the

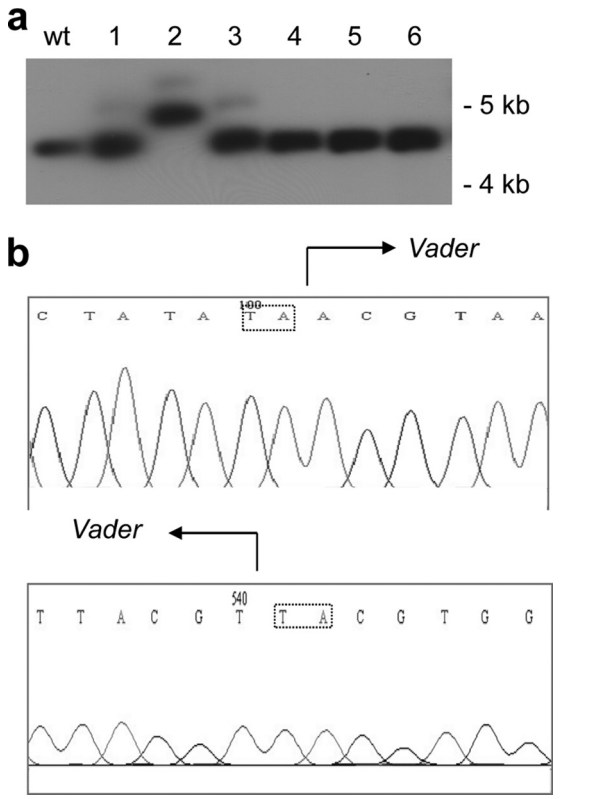

FIG. 1. Transposition of a *Vader* copy in the *A. niger nirA* gene. (a) Southern blot with genomic DNA from wild-type (wt) *A. niger* and six chlorate-resistant mutants (1 to 6). The DNA was digested with the restriction enzyme XhoI. ^{32}P[α-dCTP] was used to label *nirA* DNA. (b) Sequence analysis of the *Vader* insertion site in the *nirA* gene. The insertion site of *Vader* is indicated for both of the flanking sequences. The "TA" target site duplications are shown in boxes.

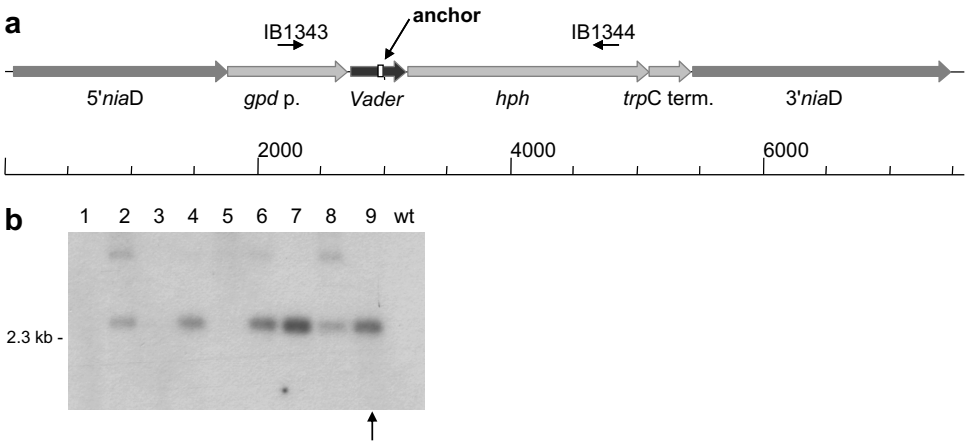

FIG. 2. Vector construction and *A. niger* transformation. (a) Vector pIB635 for transformation of *A. niger* strain CBS513.88. This vector contains a synthetic *Vader* element surrounded by its target site duplication. *Vader* was cloned between the *A. nidulans gpdA* promoter and the *E. coli hph* gene. The 5′ and 3′ flanking sequences from the *niaD* gene allow homologous recombination into the *A. niger niaD* sequence. (b) Southern blot hybridization of genomic DNA (lanes 1 to 9) from *A. niger* transformants selected on chlorate-containing plates and the wild-type (wt) strain CBS513.88 digested with the restriction enzyme XbaI. ^{32}P[α-dCTP] was used to label the probe. Six transformants were obtained. The arrow indicates transformant AnT-6(3), which was used for all further experiments.

resistance gene. The construct was targeted to the *A. niger niaD* locus using the 5′ and 3′ flanking sequences for homologous recombination. Several transformants which carried the synthetic *Vader* copy at the *niaD* locus were obtained (Fig. 2b). One transformant, AnT-6(3), was selected for all further experiments.

To select for mycelia in which a transposition event had occurred, 10^7 conidiospores each were plated on petri dishes containing minimal medium with 125 μg/ml hygromycin B. Under these conditions, transformant AnT-6(3) yielded 45 hygromycin B-resistant colonies, on average (Fig. 3a), indicating an excision frequency of 1 in 2.2×10^5 conidiospores. As a control, the same amount of conidiospores from the untransformed CBS513.88 strain was plated, but hygromycin B-resistant colonies were never observed (Fig. 3b).

The excision frequency of *Vader* is similar to that of the *impala* element from *Fusarium gramineum* (1 in 10^5 to 10^6 conidiospores) (10). The *impala* element has also been used in heterologous hosts with similar excision frequencies (8, 16, 25). The rather high excision frequency of *Vader* will facilitate to obtain large transposon mutant libraries of *A. niger* CBS513.88.

DNA was isolated from several hygromycin B-resistant isolates, and using oligonucleotides IB1343 and IB1344, part of the sequence flanking the synthetic *Vader* insertion site at the *niaD* locus was PCR amplified. Of 97 colonies examined, 95 gave evidence for excision of the *Vader* element from its original location (not shown). Sequence analysis was performed to confirm excision of transposon *Vader* from its donor site. *Vader* footprints are present in each excision event, as shown in Fig. 4. Each excision event is characterized by footprints of different types, such as ACGTTA, C, ACTA, ACCTA, AC, AGTTA, or ACGTA. One isolate exhibits the footprint GTTA, which was previously observed for *Vader* (3). While footprints like this are to be expected for most transposons, they are unusual for members of the *Fot1/pogo* subfamily, to which *Vader* belongs. Footprints of that subfamily typically contain a single copy of the target site duplication and the

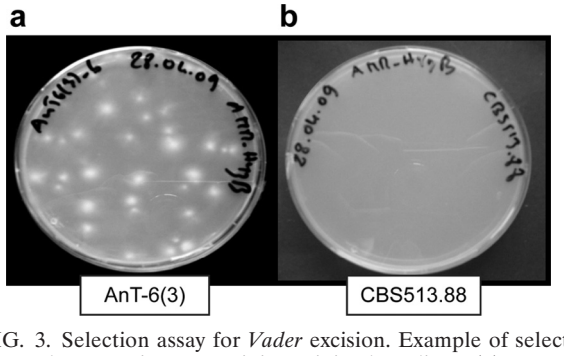

FIG. 3. Selection assay for *Vader* excision. Example of selection assay on hygromycin B containing minimal medium. (a) Transformant AnT-6(3) yields numerous hygromycin B-resistant colonies; (b) untransformed CBS513.88 strain never yields colonies on hygromycin B.

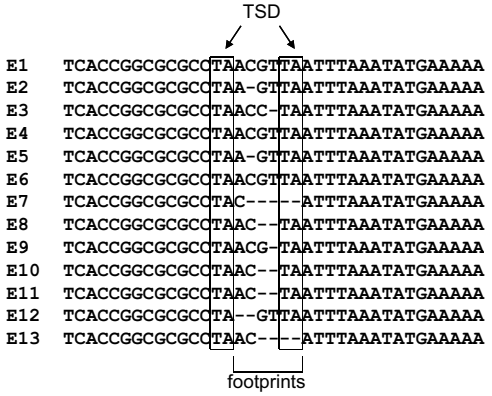

FIG. 4. Molecular analysis of *Vader* excision. Sequence analysis of 13 excision events, each exhibiting an individual footprint (TSD = target site duplication).

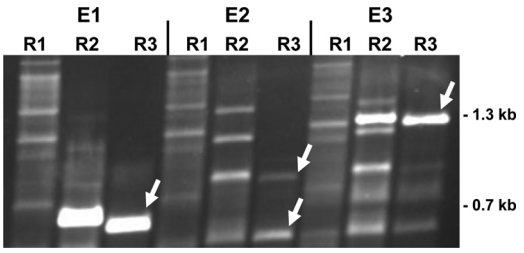

FIG. 5. *Vader* reintegration. Gel electrophoresis of three different TAIL-PCR products from excision events E1, E2, and E3. Lane designations R1, R2, and R3 indicate products of the primary, secondary, and tertiary TAIL-PCRs, respectively. The random oligonucleotide IB1347 and nested oligonucleotides IB1345, EH1434, and IB1336 were employed. The white arrows indicate PCR fragments which were sequenced and found to contain *A. niger* genomic sequences flanking the transposon *Vader* at its reintegration site.

first or last nucleotide of the transposon sequence (7). Even more distantly related transposons appear to have a preference for this type of footprint (5, 24), including the *mimp1* element (3, 9).

From the 95 analyzed *Vader* excision events, none showed loss of the element (data not shown). To identify the genomic reintegration sites following the individual excision events, we established a protocol for thermal asymmetric interlaced PCR (TAIL-PCR) (17). Three examples of successful TAIL-PCRs are shown in Fig. 5. Specific TAIL-PCR amplicons from the third round were eluted from agarose gels and subjected to sequence analysis. Reintegration sites were determined by comparison of the obtained DNA sequences with the *A. niger* genome sequence found in the database. To reconfirm the location of the integration sites, oligonucleotides flanking the integration sites were employed for PCR amplification (see Fig. S1 in the supplemental material). Only three reintegration sites were not found in the database, while all others were identified. The very high reintegration frequency of *Vader* is very promising for development as a mutagenesis tool, as comparable experiments with the *Restless* transposon did not lead to reintegration after excision (26).

Using the TAIL-PCR method, 21 different integration sites were found on chromosomes 1, 2, 3, 4, 5, and 8, indicating that the reintegration site is not limited by the proximity of the donor site, which is localized on chromosome 8. Instead, transposition reintegration appears to be random with respect to genomic location. This finding highlights the usability of the method described here.

In the assembled *A. niger* genome of 33.9 Mb, there are 14,165 protein-coding genes, with an average length of 1.572 bp. The gene density (number of genes/kb) is 0.42. Of the 21 reintegration events analyzed, 1 occurred in an exon, 12 in annotated introns (in some concerning hypothetical genes), 3 within 400 bp downstream of an open reading frame, and 2 within 500 bp upstream of an open reading frame. Another three integration sites were more than 500 bp upstream of an open reading frame (Table 2). A few reintegration sites were found two or three times. These duplicates were found only in mycelia from the same experiment.

Finally, it should be noted that it seems feasible to employ this method in other fungi as well, provided the transactivating transposase can be expressed in new hosts. The construction of vectors carrying both *Vader* and its transposase will aid this purpose and provide an additional tool for fungi relevant in biotechnology.

TABLE 2. Reintegration sites of the synthetic *Vader* element[a]

GenBank accession no.	Locus tag	Reintegration site location	Chromosome
XM_001390097	An03g02370	Intron[b]	5
XM_001395060	An12g00080	1,415 bp upstream	3
XM_001395339	An12g02970	378 bp downstream	3
XM_001390831	An06g00400	283 bp downstream	8
XM_001400379	An02g12850	Intron[b]	4
XM_001397721	An16g04470	Intron[b]	5
XM_001397940	An16g06750	489 bp upstream[b]	5
XM_001398488	An18g00560	Intron[b]	8
XM_001389142	An01g07540	Exon[c]	2
XM_001396557	An15g00180	1,295 bp upstream[b]	3
XM_001398918	An18g05010	Intron[c]	8
XM_001397938	An16g06730	Intron	5
XM_001397364	An06g00880	Intron	8
XM_001398449	An18g00160	Intron	8
XM_001397605	An16g03290	Intron	5
XM_001398419	An17g02280	556 bp upstream	5
XM_001400930	An14g03420	Intron	1
XM_001388909	An01g05210	Intron	2
XM_001395706	An12g06870	88 bp downstream	3
XM_001389436	An01g10550	319 bp upstream	2
XM_001389142	An01g07540	Intron[b]	2

[a] Three other reintegration sites were characterized, but the sequences were not included in the database.
[b] This reinsertion site was found twice.
[c] This reinsertion site was found three times.

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