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# An inducible tool for random mutagenesis in *Aspergillus niger* based on the transposon *Vader*

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**Abstract** The ascomycete *Aspergillus niger* is widely used in the biotechnology, for instance in producing most of the world's citric acid. It is also known as a major food and feed contaminant. While generation of gene knockouts for functional genomics has become feasible in *ku70* mutants, analyzing gene functions or metabolic pathways remains a laborious task. An unbiased transposon-based mutagenesis approach may aid this process of analyzing gene functions by providing mutant libraries in a short time. The *Vader* transposon is a non-autonomous DNA-transposon, which is activated by the homologous *tan1*-transposase. However, in the most commonly used lab strain of *A. niger* (N400 strain and derivatives), we found that the transposase, encoded by the *tan1* gene, is mutated and inactive. To establish a *Vader* transposon-based mutagenesis system in the N400 background, we expressed the functional transposase of *A. niger* strain CBS 513.88 under the control of an inducible promoter based on the Tet-on system, which is activated in the presence of the antibiotic doxycycline (DOX). Increasing amounts of doxycycline lead to higher *Vader* excision frequencies, whereas little to none activity of *Vader* was observed without addition of doxycycline. Hence, this system appears to be suitable for producing stable mutants in the *A. niger* N400 background.

**Keywords** Transposon-based mutagenesis · *Vader* · *Aspergillus niger* · Tet-on promoter system

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## Introduction

Filamentous fungi are of utmost importance as model organisms and in biotechnology for instance as producers of heterologous enzymes or to generate new drug leads (Cragg and Newman 2013). The ascomycete *Aspergillus niger* is a producer of several secondary metabolites and mycotoxins (Nielsen et al. 2009) but especially its primary metabolites are used in biotechnology. The most famous example is probably the production of citric acid. Since 1919, strain ATCC 1015 is used for the fermentation of this acid, which is of high importance in the food industry (Schuster et al. 2002). However, *A. niger* strain CBS 513.88 is also biotechnologically used to produce several other important metabolites such as the industrial enzymes α-amylase, cellulase, and pectinase (Nielsen et al. 2009). In addition, some mycotoxins like ochratoxin A and fumonisins $B_2$ and $B_4$ are produced under distinct conditions; nevertheless, *A. niger* is regarded as safe production organism. The genome sequence of *A. niger* strain CBS 513.88 (Pel et al. 2007) and of *A. niger* strain ATCC 1015 (Andersen et al. 2011) have been established. Both sequences are used to provide the "*Aspergillus* Genome Database" with data (Cerqueira et al. 2014), giving the opportunity to study the whole organism in more detail. Additionally, the genome of strain N400 (NRRL3) was published just recently and is publicly available at the genome portal of the *Joint Genome Database* (Nordberg et al. 2014;

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Tsang et al. 2015) (http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html).

Good methods to analyze new genes, e.g., by performing targeted gene knockouts, are already available for *A. niger* (Carvalho et al. 2010). Yet, to discover gene functions, random mutagenesis is still an often-used method, for which transposons have proven to be highly useful (Paun and Kempken 2015). There are two different kinds of transposons: class I and class II. They differ in their mechanism to transpose. Whereas class I elements, also called retroelements, use an RNA-intermediate to transpose, class II elements, or DNA-transposons, directly transpose on DNA level via the cut and paste mechanism (Wicker et al. 2007). The different transposons are than subdivided into superfamilies and orders according to different structural features. The *Vader* element from *A. niger* belongs to the *Tc1*/*Mariner* superfamily of the class II elements (Amutan et al. 1996; Nyyssönen et al. 1996). It is defined by a short target site duplication (TSD) of the two base pairs (bp) "TA" upon integration and has a simple structure of terminal inverted repeats (TIR), in this case with a length of 44 bp. As the element does not code for a transposase, it is non-autonomous and believed to be activated by the *tan1* transposase. In strain CBS 513.88, 21 copies of *Vader* were found (Braumann et al. 2007). To follow *Vader* after excision, a synthetic element was developed (sVADER). This element carries an anker-sequence of 20 bp in the middle of the *Vader* sequence and was cloned into a vector between the *orf* of the *hph* gene and the *gpd*-promoter (Hihlal et al. 2011). If *Vader* is excised, the strain will get resistant against hygromycinB as the hygromycinB phosphotransferase (*hph*) gene will be transcribed (Punt et al. 1987). This phosphotransferase will inactivate the antibiotic by phosphorylation, so hygromycinB will not inhibit protein translation. The resulting vector pIB635 was transformed into strain CBS 513.88 and excision and selection of *Vader* excision was carried out on medium containing hygromycinB as a selection marker. Hihlal et al. (2011) showed that *Vader* is a useful tool for random mutagenesis in that particular strain.

In this study, we tested the synthetic *Vader* element in strain AB4.1, which is a derivate of strain N402 (van Hartingsveldt et al. 1987). Strain N402 was isolated as a mutant from strain N400 (NRRL3) that forms short and dense conidiophores, which makes the handling in the lab more easy (Bos et al. 1988). N402 and its derivatives are the most commonly used lab strain for fundamental studies. Additionally, it is very similar to the citric acid producing strain ATCC 1015, as recent sequencing analyses showed (Tsang et al. 2015). Between the genome of these two strains, only 34 single nucleotide polymorphisms (SNPs) occurred, which means a density of 1 SNP/Mb. In comparison, the strains CBS 513.88 and ATCC 1015 differ in 7.8 SNPs/kb (Andersen et al. 2011). This indicates that N400 and ATCC 1015 derive from the same isolate and therefore, it was desirable to use the *Vader* transposon for insertional mutagenesis in strain N400 as well. When transferring the synthetic *Vader* transposon to the N402 background (the often used derivate of N400), no *Vader* excision was observed. Considering this, we postulate, that this strain lacks an intact *tan1* transposase. Former studies showed that the Tet-on system nicely works in *A. niger* (Meyer et al. 2011). We therefore cloned the sequence of the *tan1* transposase from CBS513.88 behind a promoter based on the Tet-on system. This resulting construct pVG2.2_tanA was transformed into the *A. niger* strain AB4.1, resulting in the strain KG1.6. This strain was then transformed with the vector pIB635, which includes the synthetic *Vader* element (Hihlal et al. 2011). The Tet-on system consists of two expression modules, which ensure a tight expression of the gene of interest by adding the inducer doxycycline. As shown in this study, this system also is functional in the N402 background.

## Materials and methods

### Strains and culture conditions

In this study, we used *A. niger* strain AB4.1 (van Hartingsveldt et al. 1987). Protoplast transformation of this strain was done according to standard procedures (van den Brink et al. 1999; Hihlal et al. 2011). Vector pVG2.2_tanA was designed on the bases of pVG2.2. The *tan1* open reading frame was amplified from genomic DNA of strain CBS513.88 using the primers FW1 and RWV1 (see Table S1 in supplemental material), which include the recognition site for the restriction enzyme *Pme*I. The PCR product was cloned into pJet1.2 (CloneJet PCR Cloning Kit, Thermo Scientific) and sequenced before excision and cloning into pVG2.2, which was prior to that opened with *Pme*I. Vector pIB635 was used as previously described in Hihlal et al. (2011). Primary transformants were isolated on selective media and confirmed by Southern blot analysis following standard conditions using $\alpha P^{32}$-dCTP for detection (Southern 1975). Southern hybridization was also carried out for detection of the *Vader* copies in the different strains. The restriction enzyme *Eco*RI was used for digesting the genomic DNA. We used a 442-bp fragment of the *Vader* transposon, amplified with the oligonucleotides LP2652 and LP2653 from vector DNA, as the radioactively marked probe for hybridization. The strains were cultivated on different media, depending on the experiment either on the complete media BMM or on minimal medium (AMM) (Braumann et al. 2007; Hihlal et al. 2011). For excision experiments, 125 μg/mL hygromycinB (Roth, Karlsruhe) as well as 1 μg/mL to up to 200 μg/mL doxycycline (Sigma-Aldrich, Steinheim) was added to the media. For the wild-type strain AB4.1, 10 mM uridine (Sigma-Aldrich) was added to AMM.

### Analysis of the *tan1*-transposase

For comparing the sequences of the *tan1* transposase gene of strains CBS 513.88 and AB4.1, the *orf*s were amplified with the proofreading polymerase *pwo* (PeqLab, Erlangen) and the oligonucleotides LP2634 and LP2635. Different sequencing primer were used (see Table S1 in supplemental material). The software BioEdit was used to analyze the sequencing chromatograms of the gene (Hall 1999). DNA-Alignments were carried out with Clone-Manager Professional Suite Version 9 (Scientific & Educational Software), protein-alignments with ClustalW (Thompson et al. 1994). Translation from DNA to protein sequences was done with the online tool ExPASy (Gasteiger et al. 2003).

### Transposon excision experiments

For conidia isolation, the fungi were grown on solid BMM (Braumann et al. 2007) for 1 week at 25 °C at day-night cycle at 16 to 8 h, respectively. Conidia were isolated after standard procedures as previously described using 0.9 % NaCl solution with 0.1 % Tween20 (Kramer et al. 2014). Depending on the strain, different amounts of conidia were plated out on solid AMM medium. For the control strain KG1.6, $10^6$ conidia were plated out, for BN59.13 and BN59.20, we used $10^6$ to $10^3$ conidia depending on the doxycycline concentration in the medium. The plates were incubated at 25 °C for 4–5 days. Single colonies were isolated after streaking on AMM plates containing 125 μg/mL hygromycinB without doxycycline, and these mutants were grown for five more days.

### Analysis of the mutant strains

For DNA and RNA isolation, the mutants were grown in liquid BMM for 4 days at 150 rpm and 25 °C. Nucleic acid isolations were done according to standard procedures using phenol-chloroform-isolation (Borges et al. 1990; Kollath-Leiß et al. 2014). Confirmation of the excision of *Vader* was done with a PCR as previously described (Hihlal et al. 2011). We used the oligonucleotides IB1343 and IB1344 (see Table S1 in supplemental material), which bind in the promoter region and in the *hph* gene, respectively, generating a 1.7-kb fragment including *Vader* and a 1.2-kb fragment without *Vader*. The PCR products were then sequenced according to Sanger (Sanger et al. 1977) at the Institute of Clinical Molecular Biology (Christian-Albrechts-University, Kiel) to confirm the excision and to analyze the footprints. For finding the reintegration sites, we used the TAIL-PCR (Liu and Whittier 1995). This method was used as previously described with the random primer IB1348 and the three distinct primers IB1345, IB1336, and IB1346. The products of the third PCR cycle were sequenced and analyzed with the online database EnsemblFungi (Kersey et al. 2009).

For the confirmation of the *Vader* excision on RNA level, we used a RT-PCR-Kit (Qiagen). After DNase I treatment and control PCR, we used the RNA to confirm whether a hygromycinB signal is detectable. The oligonucleotides IB1039 and IB1040 were used under standard procedures giving a 1000-bp fragment, if binding at the *hph* gene sequence.

## Results

### The Tan1 transposase from lab strain AB4.1

For the *Vader* insertional mutagenesis, we used lab strain AB4.1, which is a derivate from lab strain N402. Figure 1 shows the strain designations and derivatives, and Table S2 in the supplemental material shows a listing of all strains used and produced in this study. N402 contains at least six copies of *Vader* compared to 21 in the CBS 513.88 strain (Fig. 2). Plasmid pIB635, which contains the tagged *Vader* transposon between the *gpdA* promoter and the hygromycinB selection marker, thereby preventing expression of the hygromycinB resistance gene, was targeted to the *niaD* locus (see Hihlal et al. 2011 for further details). The correct integration of the tagged *Vader* transposon resulted in two N402-derived transformants (SP12.12 and L1) and was confirmed by Southern blot analysis (data not shown). Despite several attempts, no hygromycinB resistant colonies were obtained after plating out $1 \times 10^6$ spores of either SP12.12 or L1 on AMM containing hygromycinB. Hihlal et al. (2011) previously showed a frequency of *Vader* excision of about 1 in $2.2 \times 10^5$ spores, but in the N402 derivatives, no hygromycinB-resistant colonies were obtained when $1 \times 10^6$ spores were plated out (data not shown).

A possible explanation for the inability for *Vader* excision in N402 could be the loss of an active transposase. However, Southern blot analysis revealed that there are at least two

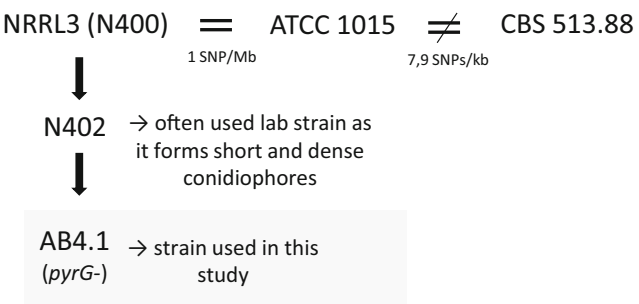


**Fig. 1** Strain designation of three *A. niger* strains. We used the *A. niger* strain AB4.1 which is the *pyrG*-mutant from strain N402 (van Hartingsveldt et al. 1987). Furthermore, this strain is derived from strain NRRL3 (also known as N400) (Bos et al. 1988). The genome of N400 is almost similar to the genome of strain ATTC 1015 differing in only 1 SNP/Mb (Tsang et al. 2015) whereas the genome of strain CBS 513.88 is quite different to the other two with 7.9 SNPs/kb (Andersen et al. 2011)

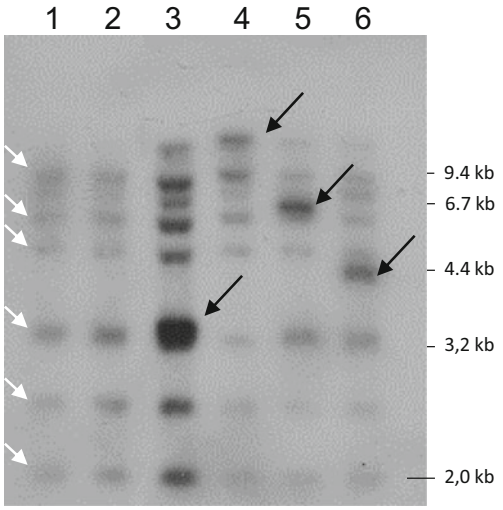


**Fig. 2** Southern blot analysis of *Vader* copies. Three different mutant strains and strains AB4.1, KG1.6, and BN59.20 were used to identify how many *Vader* copies are present in the lab strain N402 from which they derive. A DNA-Probe of 442 bp from *Vader*, which was amplified from the vector DNA pIB635 with the oligonucleotides LP2652 and LP2653, was used for hybridization. The genomic DNA was hydrolyzed with the restriction enzyme *Eco*RI. At least six different *Vader* copies (*white arrows*) are visible with an additional one in the strain BN59.20 and the mutant strains (*black arrows*). *1*: AB4.1; *2*: KG1.6; *3*: BN59.20; *4–6*: three transposon mutants

hybridizing bands (see Fig. 3a; data shown for the derivate AB4.1 and the transformant strains), as well as a third somewhat less intense band present in N402. This indicates at least two copies of the *tan1* transposase gene being present in N402 and derivatives (Fig. 3a). This assumption was confirmed by comparing these data with the genomic sequences of N400. There are two nearly identical copies of *tan1* present in N400 and one less identical (Fig. S1). When digesting the genomic

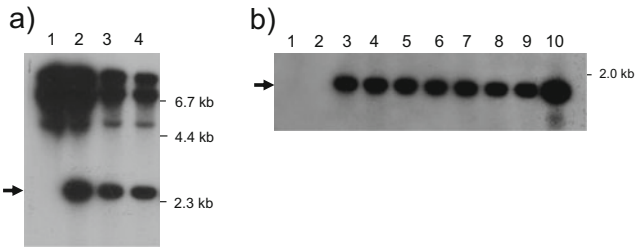


**Fig. 3** Southern blot analysis of the integration of the different vectors. **a** The vector pVG2.2_tanA was transformed into the strain AB4.1. For the Southern blot, a DNA-Probe of 1000 bp from the *tan1* gene was used, which was amplified from the genomic DNA of AB4.1 with the oligonucleotides LP2554 and L52555. The genomic DNA was hydrolyzed with the restriction enzyme *Sac*I. Four copies of the *tan1* gene are visible in the KG-strains whereas the wt strain lacks the integrated one (*arrows*). *1*: wt AB4.1; *2*: KG1.4; *3*: KG1.6; *4*: KG1.9. **b** Strain KG1.6 was transformed with the *Vader*-vector pIB635. For the Southern blot, a DNA-Probe of 700 bp from the *hph* gene was used, which was amplified from the vector DNA with the oligonucleotides LP2343 and IB1040. The genomic DNA was hydrolyzed with the restriction enzyme *Eco*RI. It only hybridized with the BN59-strains (*arrow*). *1*: AB4.1; *2*: KG1.6; *3–10*: BN59.11–16, 18, 20

DNA with *Sac*I, three different hybridizing bands with the size of 20.5, 8.4, and 4.8 kb should arise in a Southern hybridization as was shown in Fig. 3a.

Direct sequencing of a PCR amplicon of the full-length transposase from the derivate strain AB4.1 (for strain designation see Fig. 1) led to the detection of dozens of mutations compared to the transposase gene from strain CBS 513.88. The latter has already been proven to be functional by Hihlal et al. (2011). Hence, we compared the genomic sequences of *tan1* from strain CBS 513.88 with ATCC 1015 and the recently available N400 (Fig. S2). These two strains each carry two *tan1* copies with one having 32 single nucleotide polymorphisms (SNPs) compared to the *tan1* sequence in strain CBS 513.88 and the other having 44 SNPs. An alignment of the protein sequence of the two transposases in N400 with the one in CBS 513.88 shows that the mutations of the *tan1* copies in N400 cause severe changes in the protein (Fig. 4). The 32 SNPs of the first *tan1* copy lead to 21 changes on protein level including four stop codons. The second copy exhibits 31 amino acid changes with three of them being stop codons. These differences almost certainly are causing the dysfunction of *tan1* in AB4.1.

## Inducible expression of *tan1* from CBS 513.88 is used for VADER excision in AB4.1

We reasoned that if the mutation in *tan1* is responsible for the inability to excise the *Vader* transposon from the N402-derived strains SP12.12 and L1, reintroduction of an active *tan1* would restore *Vader* transposition. We therefore cloned the complete open reading frame of the *tan1* transposase from strain CBS 513.88 and placed it under the control of a promoter based on the Tet-on system (Meyer et al. 2011). The construct pTet-tanA–pyrG* (pVG2.2_tanA) was transformed to AB4.1 to give rise to KG1.4, KG1.6, and KG1.9 (Fig. S3). The vector DNA was integrated via homologous recombination at the *pyrG* locus (orotidine-5′-phosphate decarboxylase), and we selected for this integration event on medium without uridine (van Hartingsveldt et al. 1987). Integration of the pVG2.2_tanA was confirmed by Southern blot analysis, giving an additional signal at 2.4 kb (Fig. 3a for KG strains). KG1.6 was subsequently selected to be transformed with the plasmid pIB635 using the chlorate resistance as a selection marker to identify transformants with targeted integration of the tagged *Vader* transposon at the nitrate reductase gene (*niaD*) locus (Unkles et al. 1989). As shown in Fig. 3b, several transformants containing the tagged *Vader* transposon with the predicted integration pattern were identified and strains BN59.13 and BN59.20 were used in further experiments.

To determine whether induced expression of *tan1* would activate excision of the synthetic *Vader* transposon, $1 \times 10^7$ to $1 \times 10^4$ spores of the strains containing the pTet-Tan1 and sVADER (BN59.13 and BN59.20) were plated out on

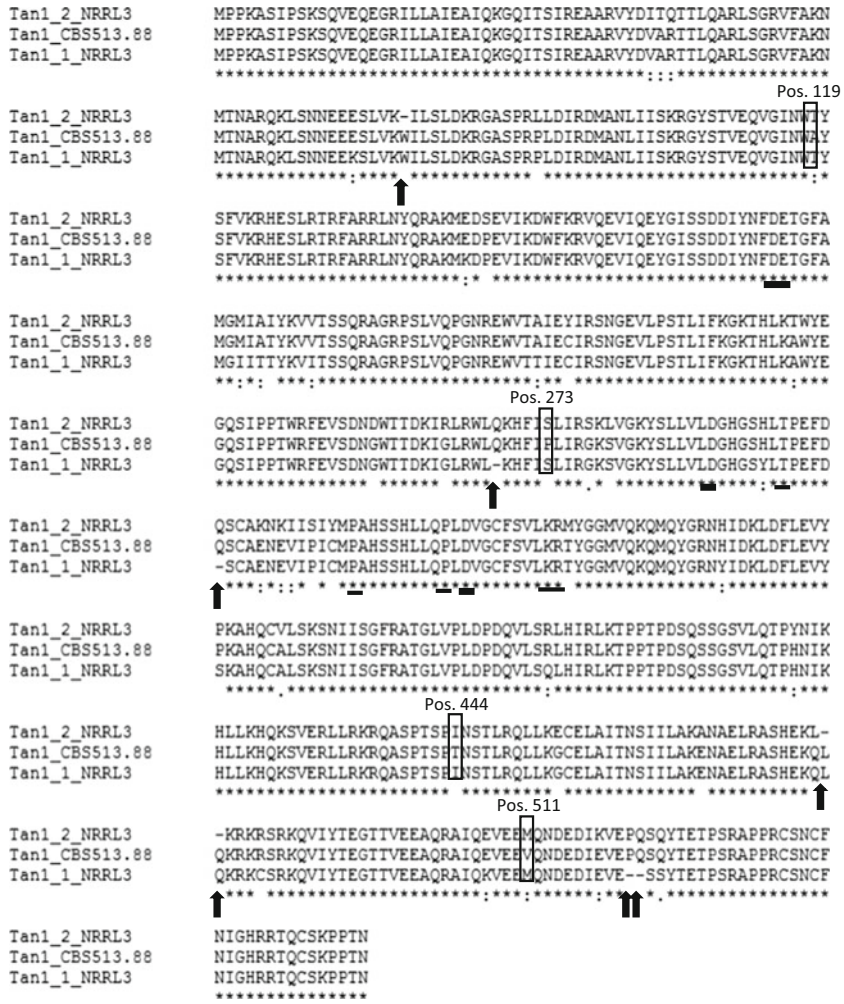


**Fig. 4** Alignment of the transposases from CBS513.88 and NRRL3 (N400). The mutations within the *orf* of the *tan1*-transposases from NRRL3 (N400) lead to amino acid exchanges. Indicated in the *boxes* are the four changes identified with the pool-sequencing of *tan1* from AB4.1. The conserved regions (DE dipeptide and the D(35)D motif), which are underlined, are not affected (Nyyssönen et al. 1996). The mutations on DNA-level lead to four new stop-codons in tan1-copy 1 and three in tan1-copy 2 (*arrows*). Figure generated with ClustalW (Thompson et al. 1994)

AMM + 125 μg/mL hygromycinB plates containing increasing concentrations of doxycycline or no doxycycline. As a negative control, we used KG1.6. Figure S4 (supplemental material) shows AMM plates with different doxycycline concentrations. Strain KG1.6 does not show any growth, whereas strains BN59.13 and BN59.20 gave rise to distinct hygromycinB-resistant fungal colonies. As an additional control, spores of the strains containing the sVADER, but not containing the pTet-TanA construct (SP12 and L1), were plated out on AMM-hygromycinB plates again, and as in former controls, these strains did not yield any hygromycinB-resistant colonies (Table 1). The number of hygromycinB-resistant colonies in BN59.13 and BN59.20 was found to be DOX concentration dependent. Table 1 shows the excision frequencies calculated from the number of hygromycinB-resistant colonies obtained after plating out $1 \times 10^7$ to $1 \times 10^4$ spores of each of the strain at different DOX concentrations. Additionally, Fig. 5 shows the graphical illustration of these results. The more DOX is present in the medium, the more excision events of *Vader* occur.

## Molecular analysis of hygromycinB-resistant colonies

We isolated RNA from two excision mutants and the control strains to confirm expression of the *hph* gene in the excision mutants. As shown in Fig. S5 (supplemental material), the *hph* transcript was detected in the mutant strains with *Vader* excision only. Strains AB4.1 and KG1.6, which do not carry the hygromycinB-resistance gene and in strain BN59.13, in which the transcription of the gene is blocked by the *Vader* sequence, did not show expression of the hygromycinB-resistant gene.

Several hygromycinB-resistant colonies, which were isolated from AMM, were analyzed regarding the footprints *Vader* has left upon excision as well as the reintegration sites of the *Vader* element after reinsertion. Whenever a transposon gets excised from its original position, it will leave a footprint, as the excision process is imprecise (Feschotte and Pritham 2007). From four independent experiments, 30 excision mutants were identified. Figure 6 shows nine different footprints, which were found in these 30 mutants. As such some excisions are identical. With the highest frequency the sequence

**Table 1** Excision frequencies on AMM + 125 μg/mL HygB + different concentrations of doxycycline

| | – DOX | 1 μg/mL DOX | 5 μg/mL DOX | 10 μg/mL DOX | 20 μg/mL DOX | 50 μg/mL DOX | 80 μg/mL DOX | 100 μg/mL DOX | 200 μg/mL DOX |
|---|---|---|---|---|---|---|---|---|---|
| SP12 | – | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| L1 | – | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| KG1.6 | – | – | – | – | – | – | – | – | – |
| BN59.13 | $4.7 \times 10^5$ | $5.2 \times 10^5$ | $9.8 \times 10^4$ | $4.2 \times 10^4$ | $1.0 \times 10^4$ | $8.0 \times 10^3$ | $2.7 \times 10^3$ | $3.3 \times 10^3$ | $6.9 \times 10^2$ |
| BN59.20 | $5.8 \times 10^5$ | $9.5 \times 10^4$ | $3.0 \times 10^4$ | $2.3 \times 10^4$ | $1.8 \times 10^4$ | $6.8 \times 10^3$ | $5.6 \times 10^3$ | $5.3 \times 10^3$ | $1.2 \times 10^3$ |

*n.d.* not determined

"5′ - TAAC-TA - 3" was observed (11 times). The second most sequence was "5′ - TA-GTTA - 3′" for seven times and the sequence "5′ - TA-ACGTTA - 3′" was observed for four times.

Furthermore, we analyzed the genomic reintegration sites, as it was shown before, that not all transposons necessarily reintegrate into the genome (Windhofer et al. 2002). *Vader* was found to be reintegrated in 27 of 30 tested mutants (not shown). For analyzing the genomic reintegration sites, we employed TAIL-PCR, as described earlier (Hihlal et al. 2011). The PCR products from the third round of the TAIL-PCR were sequenced and analyzed on EnsembleFungi. The original position of the *Vader* element is on chromosome VIII. We analyzed reintegration events from three mutant strains and found integration sites of *Vader* on chromosomes V, VI, and VIII (Fig. 7).

## Discussion

Transposons, earlier regarded as junk DNA, now are seen as forces to enable genetic diversity (Janicki et al. 2011). Yet in many if not most filamentous fungi, repetitive sequences and

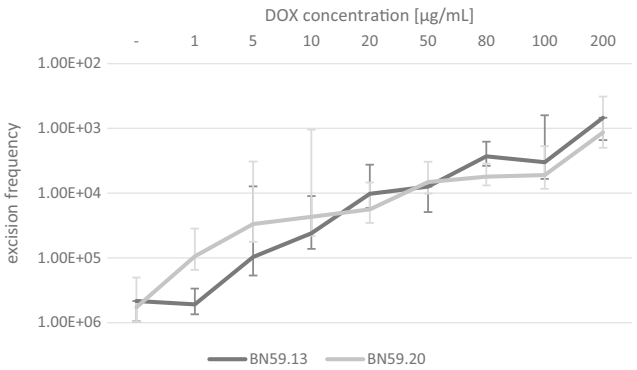


**Fig. 5** Doxycycline-dependent excision frequency on AMM. The promoter based on the Tet-on system is activated by doxycycline. Different concentrations of doxycycline were tested. Control strain KG1.6 did not show any growth on AMM at all. Strains BN59.13 and BN59.20 showed distinct growth and a raise of the excision frequency by adding more doxycycline. The *bars* are indicating the standard deviation. Ordinate in logarithmic scale. Up to 5 different experiments; $n = 3–19$

therefore transposons are inactivated by a mechanism called RIP (repeat-induced point mutation) (Clutterbuck 2011). This is particularly true for the model organism *Neurospora crassa*, where different mechanisms led to the inactivation of almost all transposable elements (Selker et al. 2003; Wang et al. 2015). Therefore, synthetic-introduced transposons can function as a powerful tool to establish mutant libraries in filamentous fungi. *Vader* proved to be a useful transposon-mediated mutagenesis tool in the *A. niger* strain CBS 513.88. Based on the previous experience, we set out to employ *Vader* in the *A. niger* lab strain N402. This strain is a good candidate to use the *Vader* transposon for insertional mutagenesis, as it is the preferred strain for many tasks. However, no *Vader* excision was observed in strains SP12.12 and L1, which carry the sVADER element in the N402 background. As *Vader* is non-autonomous, it needs to be activated in *trans* by a transposase encoded by an autonomous transposon, which is believed to be the *tan1* transposase (An07g09460) from the *tan* transposon of *A. niger* (Amutan et al. 1996; Nyyssönen et al. 1996). Analyzing the transposase, we found that N400 harbors three different *tan1* sequences just as strain ATCC 1015. The three copies of *tan1* are identical in N400 and ATCC 1015, confirming the observation that these two strains are closely related (Tsang et al. 2015). Comparing the *tan1*-sequences of the three different *A. niger* strains, we found severe changes. The both somewhat more identical copies of the transposase in N400 and ATCC 1015 harbor 32 and 44 differences compared to the functional copy of CBS 513.88. This also explains the findings of the different SNPs in AB4.1 (derivate from N402) as both *tan1* copies were sequenced in pool. These differences on DNA-level result in severe changes in the amino acid sequences of Tan1. Not only amino acid exchanges but also new stop codons arose. Because of the lack of transposon excision, we suspected that these mutations affect the transposase activity in AB4.1. Furthermore, it is possible that the transposase in AB4.1 is not properly expressed under laboratory conditions. As in strain CBS513.88, the excision frequency of *Vader* was low compared to the induced one in BN59.13 and BN59.20, we think that the efficiency of transposons might also be affected by the insufficient expression of the transposase. This might function as a natural

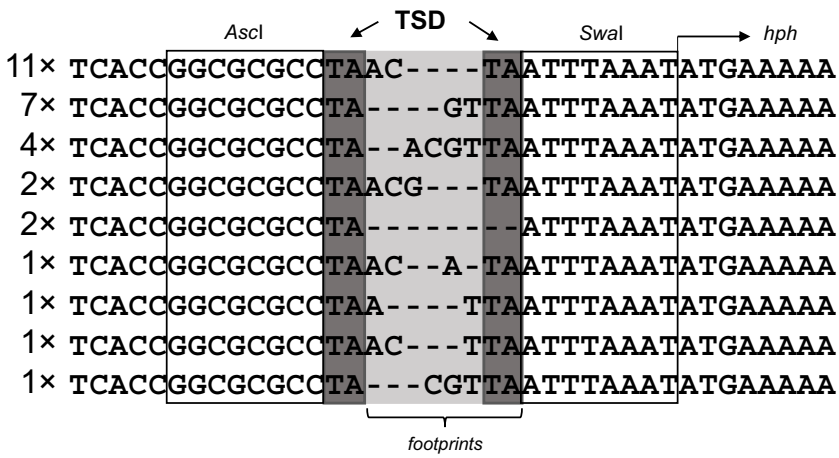


**Fig. 6** Footprints of the *Vader* excision. When a DNA-transposon is cut out of its original position, it will leave a footprint of several base pairs. The synthetic *Vader*-element is flanked by the two target sites for the restriction enzymes *Asc*I and *Swa*I followed by the *hph*-gene. Additionally, the transposon is flanked by the TSD of "TA." Nine different footprints with changing frequencies were identified

regulation of transposition. Our results also indicate that there is no other transposase in *A. niger* which is able to activate *Vader*.

To produce stable mutants for the lab strain N402, we cloned the *tan1* transposase from strain CBS 513.88 under the control of the inducible promoter based on the Tet-on system. This promoter is activated by the inducer doxycycline. By using the Tet-on system, we established an inducible tool to produce large amounts of potentially stable mutants. The results clearly indicate an increase in the number of excisions at increasing DOX concentrations suggesting that the level of *tan1* expression directly correlates with the excision. The observation that some excision takes place in the absence of DOX in strains BN59.13 and BN59.20 suggests that the pTet-promoter is not completely tight. In a previous paper, the expression system was tested with the *mluc* gene as a reporter system and repression was found to be 99.5 % (Meyer et al. 2011). This would mean 1 from 200 spores or 500 from $10^6$ spores, which could account for the excision events we observed in the absence of DOX.

Previous data showed inhibition of the *A. niger* growth rate at doxycycline concentrations above 125 μg/mL. However, in our experiments, a doxycycline concentration of 200 μg/mL does not seem to inhibit growth, as the excision frequency is rising. This argues against any growth inhibition of doxycycline.

Even at the lowest DOX concentration tested the efficiency of excision is somewhat higher than compared to the excision frequencies obtained by the endogenous tan1 promoter in CBS513.88. The frequencies reported previously ($2.2 \times 10^5$) by Hihlal et al. (2011) are comparable with the frequency obtained without DOX induction in BN59.20 indicating that the endogenous *tan1* in CBS 513.88 gene is very lowly expressed indeed.

*Vader* footprints were found in all tested mutants. A total of nine different footprints were found. As these were identified in independent experiments, the occurrence of each type of footprint must be an indication for a preference of the transposase enzyme when generating a footprint. Six to none base pairs are left after excision leaving behind different sequences. These data are similar to those shown in Hihlal et al. (2011), indicating that overexpression of the *tan1* transposase had no effect on the excision process. Additionally, we

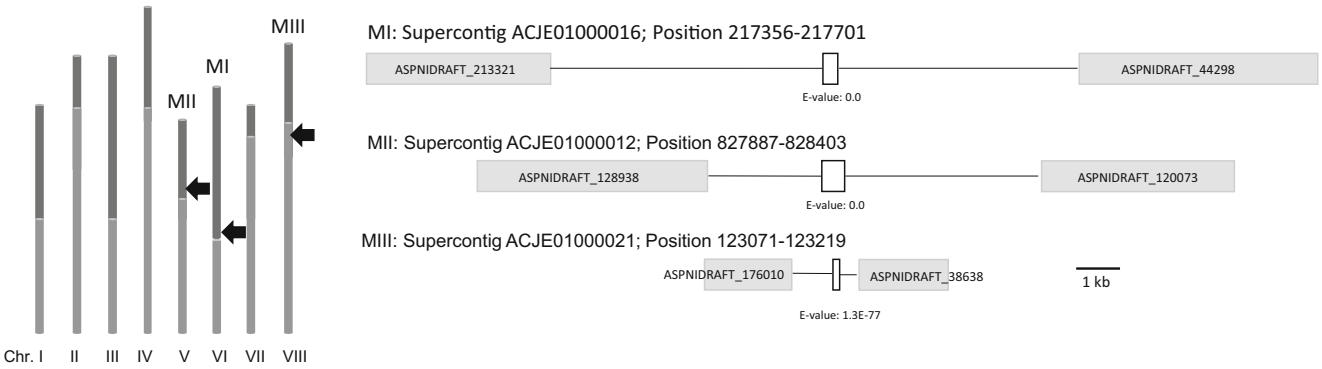


**Fig. 7** Schematic diagram of the reintegration sites of *Vader*. Three reintegration sites were analyzed. We found that *Vader* was relocated to the chromosomes V, VI, and VIII. The original position of the synthetic *Vader*-element is at the *niaD* locus on chromosome VIII. In all three cases, *Vader* was translocated between two gene-coding regions (GenBank ID is given), implicating, that *Vader* integrates randomly throughout the genome (*arrows*). The *white rectangle* represents the TAIL-PCR-amplicons which were sequenced and analyzed via blastn. The *bar* indicates 1 kb

analyzed the reintegration frequency. From analyzing 30 excision mutants, we found reintegration of *Vader* in 27 mutants. This is in good correspondence with data shown for the two other *Tc1*/*mariner*-elements *Fot1* and *Impala* where a loss frequency of up to 16 % was found (Evangelinos et al. 2015; Li Destri Nicosia et al. 2001). This might be due to the fact that during the excision-process, the DNA-transposon might be present in the cell as a free intermediate (Kempken and Kück 1998; Li Destri Nicosia et al. 2001). The integration sites of *Vader* were analyzed via TAIL-PCR. The reintegration sites were distributed on three different chromosomes. In all cases, we found *Vader* reintegrated between gene coding regions. In one case, an integration site on supercontig ACJE01000021, the integration was close to a gene (ASPNIDRAFT_38638) confirming data from a previous paper (Hihlal et al. 2011). As such, we did not analyze further integration sites at this time. However, as minimal medium was used for selection, and well growing colonies only were analyzed, we performed a negative selection against insertions in open reading frames essential for growth on minimal medium.

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**Compliance with ethical standards** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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