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Tools and Techniques

Minos as a novel Tc1/mariner-type transposable element for functional genomic analysis in *Aspergillus nidulans*

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ABSTRACT

Transposons constitute powerful genetic tools for gene inactivation, exon or promoter trapping and genome analyses. The *Minos* element from *Drosophila hydei*, a Tc1/mariner-like transposon, has proved as a very efficient tool for heterologous transposition in several metazoa. In filamentous fungi, only a handful of fungal-specific transposable elements have been exploited as genetic tools, with the *impala* Tc1/mariner element from *Fusarium oxysporum* being the most successful. Here, we developed a two-component transposition system to manipulate *Minos* transposition in *Aspergillus nidulans* (*AnMinos*). Our system allows direct selection of transposition events based on re-activation of *niaD*, a gene necessary for growth on nitrate as a nitrogen source. On average, among 10^8 conidiospores, we obtain up to $\sim 0.8 \times 10^2$ transposition events leading to the expected revertant phenotype (*niaD*$^+$), while ~16% of excision events lead to *AnMinos* loss. Characterized excision footprints consisted of the four terminal bases of the transposon flanked by the TA target duplication and led to no major DNA rearrangements. *AnMinos* transposition depends on the presence of its homologous transposase. Its frequency was not significantly affected by temperature, UV irradiation or the transcription status of the original integration locus (*niaD*). Importantly, transposition is dependent on *nkuA*, encoding an enzyme essential for non-homologous end joining of DNA in double-strand break repair. *AnMinos* proved to be an efficient tool for functional analysis as it seems to transpose in different genomic loci positions in all chromosomes, including a high proportion of integration events within or close to genes. We have used *Minos* to obtain morphological and toxic analogue resistant mutants. Interestingly, among morphological mutants some seem to be due to *Minos*-elicited over-expression of specific genes, rather than gene inactivation.

1. Introduction

The availability of transposon-based genetic methodologies for functional genomic analysis, such as insertional mutagenesis and gene or enhancer trapping, is very useful to study gene function and genome organization (Ryder and Russell, 2003; Ivics and Izsvák, 2010; Muñoz-López and García-Pérez, 2010; Joly-Lopez and Bureau, 2014). Transposons belonging to the Tc1/mariner cut-and-paste type superfamily (Class II) have been shown to be functional in several organisms, such as fungi, animal and plant genomes, and constitute the most widely used mobile elements for gene-tagging and functional analysis (Plasterk et al., 1999; Pavlopoulos et al., 2007; Muñoz-López and García-Pérez, 2010). The short target sites of most Tc1/mariner cut-and-paste transposable elements allow, in principle, a single transposon to screen an entire genome with mutagenic insertions. In practice, however, every transposon has a variable insertional bias for certain genomic loci, making the entire-genome mutagenesis difficult.

Among Tc1/mariner-like transposons, Minos, a mobile element isolated from Drosophila hydei, has been shown to be active in several insect species and mammalian model organisms and human cells in culture, where it can be used for genome wide, transposon-mediated mutagenesis (Franz and Savakis, 1991; Arcà et al., 1997; Zagoraiou et al., 2001); for a recent review see Pavlopoulos et al. (2007). Because of the ability of *Minos* to transpose at high frequencies and the fact that 60% of all *Minos* insertions were in or close to genes, the *Minos* system has become one of the

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most powerful mutagenic molecular tools in metazoa (Bellen et al., 2004). An important advantage of the *Minos* element as a tool in insertional mutagenesis, compared to other Tc1/*mariner* elements, is the distribution of the integration sites, with insertion incidents being almost perfectly random, despite a slight intron preference (Bellen et al., 2011). *Minos* has two terminal inverted repeats of 255 bp, which flank a single open reading frame of 1.8 kb encoding the Minos transposase (Arcà et al., 1997; Pavlopoulos et al., 2007). The Minos transposase (MnTpase) open reading frame (ORF) is interrupted by a 60 bp long intron and encodes a 361 amino acid protein, showing homology with mobile elements of the Tc1/*mariner* superfamily (Franz and Savakis, 1991; Franz et al., 1994). The N-terminal region (amino-terminus) of the enzyme contains a putative DNA-binding domain resembling the paired domain, an evolutionarily conserved feature of the Pax protein family. The C-terminal region (carboxyl-terminus) contains an Asp-Asp-Glu catalytic triad strongly related to all Tc1 transposases (Sasakura et al., 2003).

The transposition of *Minos* takes place into a TA dinucleotide of the insertion site that is duplicated upon an insertion event (Franz and Savakis, 1991). After the excision, a footprint is left, characteristic of the transposon presence on this genome site. In most cases, the footprint is 6 bp long and consists of the four terminal nucleotides of the inverted repeat plus the duplicated TA dinucleotide. The footprint pattern suggests a potential excision mechanism, involving transposase-related staggered cuts at the ends of the inverted repeats. Minos transposase possibly creates single strand overhangs of the four terminal bases of each end. During reinsertion, the overhangs of the mobilized *Minos* element join the TA overhangs of the target integration site. Finally, a polymerase-mediated fill-in of the single stranded DNA takes place. This leads to regeneration of the transposon ends and duplication of the target TA. This model predicts that a heteroduplex flanked by the TA duplication is formed at the site of excision (Arcà et al., 1997).

Active Class II transposition events have been described for cut-and-paste elements in several fungi, including the plant pathogen *Fusarium oxysporum* (Daboussi et al., 1991; Daboussi and Langin, 1994; Langin et al., 1995), *Aspergillus niger* (Glayzer et al., 1995; Nyyssönen et al., 1996) (Amutan et al., 1996), *Aspergillus fumigatus* (Hey et al., 2008) and industrial *Aspergillus oryzae* strains (Ogasawara et al., 2009). Several Tc1/mariner transposons exist in fungi, but their copy numbers are however lower compared to other eukaryotes (Miskey et al., 2005; Kempken and Kück, 1998; Daboussi and Capy, 2003). In some model fungi, such as the filamentous ascomycete *Aspergillus nidulans*, many transposons are present, but are inactive. This might be due to continuous selection for stability, which would favor fungal lineages with less active transposable elements (Daboussi and Capy, 2003).

In *A. nidulans*, where no endogenous transposition has been detected, two heterologous transposons from *F. oxysporum* have been tested, *Fot1* and *impala*. The *impala* element belongs to the *Tc1-mariner* family (Daboussi et al., 1992; Daboussi, 1997). *Impala* has proved to be an efficient tool for gene-tagging and functional analysis, mainly because of its relatively high frequency (approximately 10^{-5}) of transposition and little host specificity within ascomycetes (Li Destri Nicosia et al., 2001; Villalba et al., 2001; Firon et al., 2003; de Queiroz and Daboussi, 2003; Carr et al., 2010; Cecchetto et al., 2004). However, *impala* also shows a trend for transposition within introns or 5′ regions, rather than in ORFs or essential regulatory regions (Deschamps et al., 1999).

In this work, we develop and evaluate a novel, *Minos*-based, two-component, transposon system, called *AnMinos*, for genomic and gene analysis in *A. nidulans*. *AnMinos* transposition frequency proved comparable to that of *impala* (up to 10^{-5}) and its randomness sufficient to be used as a tool for gene tagging. We show that *AnMinos* transposition is not affected by temperature, UV irradiation or the transcription status of the original integration locus, but requires an intact non-homologous DNA repair system (i.e. a functional NkuA protein). We experimentally tested *Minos* usefulness as a gene-tagging tool by characterizing morphological or toxic analogue resistant mutants.

2. Materials and methods

2.1. A. nidulans media, strains, transformation and standard genetic and molecular techniques

Standard complete (CM) and minimal media (MM) for *A. nidulans* were used. Media and supplemented auxotrophies were at the concentrations given in http://www.fgsc.net. Nitrogen sources and analogues were used at final concentrations: urea 5 mM, sodium nitrate 10 mM, 5-fluorouracil (5-FU) 50 μM, 5-fluorouridine (5-FUd) 10 μM and 5-fluorocytosine (5-FC) 50–100 μM. Media and chemical reagents were obtained from Sigma–Aldrich (Life Science Chemilab SA, Hellas) or AppliChem (Bioline Scientific SA, Hellas). *A. nidulans* strains used in this work are listed in Supplementary Table S1. Derivatives of mutant strains were made with standard genetic crossing using auxotrophic markers for heterokaryon establishment. The *pabaA1*, *riboB2*, *pyroA4*, *pyrG89*, and *argB2* are genetic mutations resulting in auxotrophies for p-aminobenzoic acid, riboflavine, pyridoxin, uracil/uridine and arginine respectively (http://www.fgsc.net/Aspergillus/gene_list/supplement.html). *A. nidulans* transformation was performed as described previously (Koukaki et al., 2003). DNA from *A. nidulans* was extracted as described in http://www.fgsc.net. Standard cloning techniques were as described in Sambrook et al., (1989).

2.2. Construction of a yA inoB double deleted strain

The deletion of the *yA* gene, encoding a conidial laccase responsible for the green pigment in conidia, (ΔyA::pyr4) has been previously described (Li Destri Nicosia et al., 2001). Deletion of *inoB* (EU233789; EU233790/AN7625), encoding myo-inositol-1-phosphate synthase, necessary for inositol biosynthesis was performed using the relevant fungal genetics stock center knock-out cassette for *A. nidulans*. Amplification of the *inoB* knock-out cassette was performed as described in the fgsc.net webpage, using the supplied specific primers and KAPA HiFi HotStart polymerase (Kapa Biosytems, London). The PCR program used, included an initial denaturation step of 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 5 min at 72 °C, and a final extension step of 10 min at 72 °C. The *ΔinoB* cassette, including a $pyrG^{Af}$ selection marker, encoding orotidine 5′-monophosphate decarboxylase from *Aspergillus fumigatus*, was used to transform a *pyrG89 pyroA4 ΔnkuA::argB strain*. *ΔinoB::*$pyrG^{Af}$ transformants carrying the *ΔinoB::*$pyrG^{Af}$ genetic construct were selected by complementation of the *pyrG89* auxotrophy (lack of pyrimidine biosynthesis). A purified selected transformant was crossed with strain *ΔyA::pyr4 riboB2 pabaA1* and progeny with the *ΔinoB::*$pyrG^{Af}$ *ΔyA::pyr4 pyroA4 ΔnkuA::argB* genotype was selected as the recipient strain for introducing and mobilizing the *AnMinos* (Section 2.3).

2.3. Construction of a Minos Cassette (AnMinos) and a vector expressing Minos transposase

The *yA* and *inoB* genes were amplified from genomic wild-type *A. nidulans* DNA using primer pairs 3–4 for *yA* and 5–6 for *inoB* (see Supplementary Table S2 and Fig. 1) generating ClaI/KpnI restriction sites. The plasmid pMi3xP3DsRed (Pavlopoulos et al., 2004) was double digested with ClaI/KpnI removing the DsRed gene and one of the Minos inverted repeats (IRs). This vector was used to clone the *yA* gene in such a direction where the IR would be

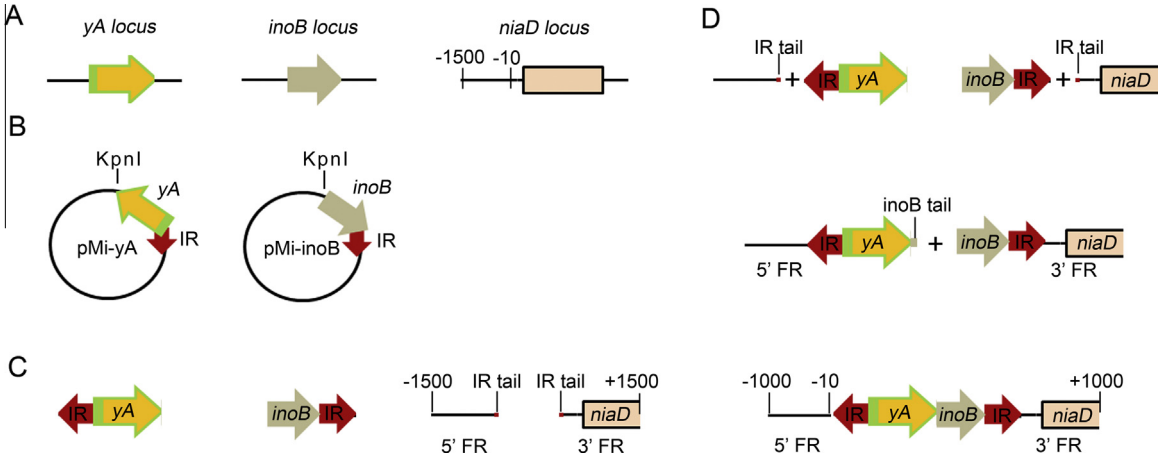

Fig. 1. Strategy for the construction of a *AnMinos* cassette. (A) Fragments containing the *yA* and *inoB* genes were amplified from genomic DNA using primers carrying unique restriction enzyme adaptors, and (B) subsequently cloned in vectors containing the 255 bp of the *Minos* inverted repeat (IR). The *yA* fragment was cloned in such a direction where the IR would be upstream (left) of *yA* whereas the *inoB* fragment was cloned in a direction where the IR would be downstream (right) of *inoB*. (C) Fragments IR-*yA* and *inoB*-IR were amplified from plasmids pMi-yA and pMi-inoB respectively. An upstream fragment corresponding to the region −1500 to −11 (left flanking region) and a fragment corresponding to the region −10 till +1500 (right flanking region) of the *niaD* gene were amplified using primers carrying 25–27 bp of the Minos IR. This overlap with the IR is necessary for the following joining steps. (D) The linear fragments of the left flanking of *niaD* plus IR-yA and the inoB-IR plus the right flanking of *niaD* are joined by two independent single-joint PCRs using nested primers. The reverse nested primer for the *yA* introduced 28 bp of the *inoB* fragment after the end of *yA* as required for the final joining step. The two joint fragments underwent a final single-joint PCR using nested primers.

upstream (left) of *yA* (pMi-yA), whereas the *inoB* fragment was cloned in a direction where the IR would be downstream (right) of *inoB* (pMi-inoB) (Fig. 1). Using primer pairs 7, 4 and 5, 7 fragments IR-*yA* and *inoB*-IR were amplified respectively. A left flanking region of the *niaD* locus, from −1500 bp to −11 bp upstream from the translation initiation codon of the *niaD* gene (5′ flanking region, 5′FR), was amplified from genomic wild-type DNA using primers 8–9. A right flanking region of the *niaD* locus, from −10 bp to +1500 bp (3′ flanking region, 3′ FR), was amplified using primers 10–11. These flanking regions enable the homologous integration of the final *AnMinos* cassette in the −10 site of the *niaD* locus. The reverse primer for 5′FR and the forward primer for 3′FR add 25–27 base pairs of the *AnMinos* IR to the amplified FRs of *niaD*, necessary for the joint PCR step to follow. DNA fragments of 5′FR and IR-*yA* were used as templates in a single-joint PCR (Yu et al., 2004a) using nested primers for *niaD* and *yA* (primers 12–13), resulting in a joint 5′FR-IR-*yA* fragment with a tail of 28 bp of the *inoB* sequence, required for the next joining step. Following the same strategy a joint *inoB*-IR-3′FR fragment was amplified using primers 14–15. A final joining step between the two fragments was performed using LA Taq polymerase (Takara, Kyoto) for long amplicons and primers 16–17, generating the final *AnMinos* cassette (5′FR-IR-*yA*-*inoB*-IR-3′FR). This cassette is used for introducing *AnMinos* into niaD locus in an appropriate recipient strain (see Section 2.4). The above strategy is depicted in Fig. 1. All primers used are shown in Supplementary Table S2. For the construction of a vector expressing the Minos transposase (*MnTpase*), the intronless ORF of the relevant gene was amplified from plasmid pBS(SK)MimRNA (Loukeris et al., 1995) using primers 1–2, (see Supplementary Table S2) generating restriction sites NcoI/BamHI and was cloned in frame with the $gpdA^{mini}$ promoter and the 3′ UTR of *trpC* gene, in a plasmid also carrying the *argB* gene, encoding the ornithine carbamoyltransferase needed for arginine biogenesis. The new vector was used to construct a strain expressing MnTpase (see Section 2.5).

2.4. Integration of AnMinos in the niaD promoter

The *AnMinos* cassette was integrated, through homologous recombination, in a specific site of the *niaD* promoter, corresponding to position −10 from the translation initiation codon. Targeted recombination was achieved by transforming a *ΔyA ΔinoB ΔnkuA* recipient stain (described in Section 2.2) with a linear DNA cassette including *AnMinos*, constructed as described in Section 2.3. The *ΔnkuA* mutation permits the direct selection of transformants through homologous recombination in the *niaD* locus, which should also lead to the genetic complementation of the *ΔinoB* and *ΔyA* markers. In the initial transformation experiments several $niaD^{-}$ $inoB^{+}$ transformants were purified, but unexpectedly they scored as yA^{-} mutants (yellow conidiospores). Subsequent analysis through PCR, Southern blotting and sequencing showed that the transformants characterized contained the proper integration of a single *AnMinos* cassette at the expected −10 site of *niaD* (see Fig. 4), but a missense mutation has been generated within the *yA* gene copy within the integrated cassette (see also Section 3.2). As this fortuitous inactivation of the *yA* marker must not affect *AnMinos* mobilization, we used a selected transformant, hereafter called *AnMinos-yA*$^{-}$, to test mobilization of *AnMinos* (see Section 2.6). We subsequently also reconstructed strains carrying genetically intact *AnMinos* cassettes, scoring as $niaD^{-}$ $inoB^{+}$ yA^{+} (*AnMinos-yA*$^{+}$). As it will be shown later, the functionality of the *yA* gene within the *AnMinos* cassettes did not affect transposition frequency (see Table 1).

2.5. Testing AnMinos transposition

A strain expressing the intronless Minos transposase was constructed by isolating an $argB^{+}$ transformant generated upon heterologous integration of the relative plasmid, described in Section 2.3 (see Fig. 2B). This strain, called *MnTpase*, was genetically crossed with strains carrying *AnMinos* cassettes (*AnMinos-yA*$^{+}$ or *AnMinos-yA*$^{-}$). Progeny selected for further work contained the *AnMinos* cassettes and the gene for MnTpase, as well as, the genomic knock-out versions of *inoB* and *yA*. This allowed the follow-up of *AnMinos* transpositions or simple excision events. In addition, isogenic versions differing only in respect to the functionality of the *nkuA* gene ($nkuA^{+}$ *versus ΔnkuA*), were isolated. Finally, for strains containing *AnMinos-yA*$^{-}$ two $nkuA^{+}$ strains, A and B, differing only in their auxotrophic requirements for pyridoxine (prototroph *versus pyroA4* auxotroph) were selected.

CM agar plates were inoculated with different strains carrying *AnMinos* elements and Minos transposase gene, and incubated for

Table 1
Frequency of *AnMinos* transposition estimated as colonies growing on media containing nitrate as sole nitrogen source (*niaD*$^+$).

Strain (10^8 conidiospores)	Growth medium	Selection medium	Selection temperature	Transposition frequency
I				
AnMinos-yA$^-$ (A)	CM	MM, NO_3	37 °C	0.21×10^{-6}
AnMinos-yA$^-$ (B)	CM	MM, NO_3	37 °C	0.49×10^{-6}
AnMinos-yA$^+$	CM	MM, NO_3	37 °C	1.10×10^{-6}
AnMinos-yA$^-$ *ΔnkuA::argB*	CM	MM, NO_3	37 °C	0
AnMinos-yA$^+$ *ΔnkuA::argB*	CM	MM, NO_3	37 °C	0
II				
AnMinos-yA$^-$ (A)	CM	MM, NO_3, ino	37 °C	0.34×10^{-6}
AnMinos-yA$^-$ (A)	MM, NH_4^+	MM, NO_3	37 °C	0.61×10^{-6}
AnMinos-yA$^-$ (A)	MM, urea, NO_3	MM, NO_3	37 °C	0.61×10^{-6}
AnMinos-yA$^-$ (A)	CM	MM, NO_3	18 °C	5.60×10^{-6}
AnMinos-yA$^-$ (A)	CM	MM, NO_3	25 °C	5.09×10^{-6}
AnMinos-yA$^-$ (A)	CM	MM, NO_3	37 °C	4.89×10^{-6}
AnMinos-yA$^-$ (A)	CM	MM, NO_3	42 °C	4.42×10^{-6}
AnMinos-yA$^-$ (B)	CM	MM, NO3	4 °C/37 °C	$0.43–0.73 \times 10^{-6}$
AnMinos-yA$^-$ (B)	CM	UV > MM, NO_3	37 °C	$3.01–4.65 \times 10^{-6}$

(I) Two strains harboring *AnMinos-yA*$^-$ (yellow conidiospores), one prototroph (A) and one carrying the *pyroA4* auxotrophic mutation (B), and a strain harboring *AnMinos-yA*$^+$ (green conidiospores) were tested for *AnMinos* transposition. In addition, two strains, isogenic to *AnMinos-yA*$^-$ and *AnMinos-yA*$^+$, also carrying the *ΔnkuA* null mutation were also tested. (II) Frequencies of *AnMinos-yA*$^-$(A) transposition obtained at various physiological or stress conditions. The effect of cold temperature (4 °C) was carried out as described in Supplementary Table S3. The effect of UV irradiation was carried out as described in Supplementary Table S4. CM and MM stand for Complete and Minimal medium, respectively. NO_3 is 10 mM $NaNO_3$ as sole nitrogen source. Inositol (ino) is used to detect frequency of excision events that led to *AnMinos-yA*$^-$ loss. For this and other explanations on the rationale of different conditions used, see text. Results shown represent averages of at least 2 independent experiments with standard deviation <50%.

4–5 days at 37 °C. Conidiospores from 1/4 of a fully grown plate were harvested in 25 ml of sterile distilled water. The liquid solution was filtered through sterile miracloth and the filtered spore solution was obtained by centrifugation at 4000 rpm, for 10 min, at room temperature. After discarding the supernatant, the spore pellet was resuspended in 5 ml sterile distilled water. The spore concentration was estimated by both Colony-Forming Unit (CFU) and Hemocytometer plate (0.1 mm depth, 1/400 mm^2) counting. 100 µl of the initial spore stock was added in a tube containing 900 µl sterile distilled water (10^{-1} dilution). Each spore suspension was serially diluted up to final 10^{-6} dilution. 100 µl of the 10^{-4}, 10^{-5} and 10^{-6} spore suspensions was plated on CM plates and incubated at 37 °C for 3 days, and colonies were counted. For Hemocytometer plate counting, the initial spore suspension was serially diluted up to 10^{-2} dilution and 20 µl was counted on the plate. Based on the concentration of the initial spore suspension, a specific volume corresponding to 10^8 spores, was transferred into sterile 25 ml falcons containing 3–4 ml of melted cooled (50 °C) top MM (0.4% agar), carefully mixed, and quickly used to inoculate MM plates containing nitrate as a sole nitrogen source and appropriate supplements. *niaD*$^+$ revertants were isolated after incubation at 37 °C for 3 days, and eventually purified by streaking on the same medium that colonies were selected.

2.6. AnMinos transposition mobilization under different conditions

For specific experiments, conidiospores were obtained from CM or MM agar plates, supplemented with vitamins, and ammonium tartrate or nitrate, and urea as nitrogen sources, for 7 days at 37 °C. The number of spores was estimated, as described in Section 2.5. 10^8 spores were then plated either directly, or after specific treatment (see below), on a single MM-sodium nitrate agar plates. In all cases, plating was performed using molten MM-sodium nitrate agar cooled to 50 °C. Emerging colonies were counted. The mean frequency of transposition for each strain was

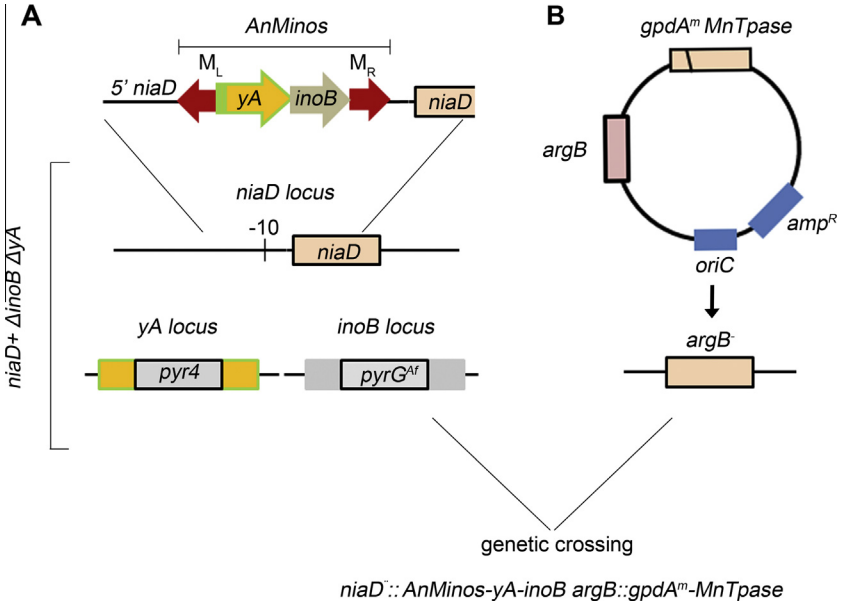

Fig. 2. Strategy for the development of a two-component Minos transposition system in *A. nidulans*. (A) Schematic representation of *AnMinos* homologous integration in the −10 of *niaD* locus of a *ΔyA::pyr4 ΔinoB::pyrG*^{Af} *niaD*$^+$ strain. *AnMinos*, consisting of the left (M_L) and right (M_R) inverted repeats of the *Minos* element and the *yA* (green pigment in conidia) and *inoB* (inositol biosynthesis) genes, is flanked by ~1000 bp of upstream and downstream sequences from the integration site (−10). (B) Schematic representation of *MnTpase* vector and integration into a *argB*$^-$ auxotroph strain (lack of arginine biosynthesis). The intronless ORF of Minos transposase was cloned under the expression of a constitutive promoter *gpdA*^{mini} in a vector carrying the *argB* selection marker (biosynthesis of arginine), the ampicillin resistance gene (amp^R) and an *Escherichia coli* origin of replication. A selected transformant, called *MnTpase*, was genetically crossed with strains carrying *AnMinos* cassettes. Progeny selected for further work contained the *AnMinos* cassettes and the gene for MnTpase, as well as, the genomic knock-out versions of *inoB* and *yA*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

determined by scoring colonies able to use nitrate as the sole nitrogen source and in MM lacking inositol, indicating excision of the AnMinos transposon from the niaD marker gene and successful re-insertion in a new genome locus (see also Section 3.3). The basal transposition rate was determined by incubating plates at 37 °C for 5 days. Several variables were assessed with respect to their effect on transposition rates. In one set of experiments, *niaD*$^{+}$ revertants from a single stock suspension of *AnMinos-yA*$^{-}$ were selected at various temperatures (18, 25, 37 and 42 °C). For assessment of the effect of low temperature on transposition, aliquots of the spore stock of *AnMinos-yA*$^{-}$ were kept for 1–12 days at 4 °C and then plated at 37 °C. For the assessment of the effect of UV, aliquots of 10^{8} spores of *AnMinos-yA*$^{-}$ were exposed to irradiation in open Petri dishes for various time periods (0, 15, 30, 60 and 120 s), collected and plated on MM-NO_3 agar plates.

2.7. Isolation of AnMinos morphological and 5-FU mutants

Morphological mutants were collected visually in the course of establishing the frequency of transposition of *AnMinos-yA*$^{-}$. On average we detected 2–5 morphological mutants per ~300–500 transposition events obtained from 10^{8} conidiospores. To obtain 5-FU mutants, we plated an amount of spores (~1.5×10^{10}) that will, in principle, lead to ~15,000 *niaD*$^{+}$ revertants. This was based on the concentration of conidiospores and the mean frequency of transposition as estimated in this work (~10^{-6}). Spores were plated, as described in Section 2.5, in 10 MM-nitrate plates also containing appropriate supplements and 100 μM 5-fluorouracil (5-FU). Plates were incubated at 37 °C for 4–5 days. 5-FU resistant colonies were isolated and purified on selective media. We obtained a total of seven 5-FU resistant colonies. The flanking sequences of six transposon-dependent 5-FU mutants are described in Section 3.8.

2.8. Transposon flanking sequence determination

Flanking sequences corresponding to genomic sequences bordering the 5′ or 3′ ends of *AnMinos* were determined by adaptation of a two-step PCR strategy developed by Chun et al., (1997). In brief, 1 μg genomic DNA was digested with restriction endonuclease enzymes that do not cut within the *AnMinos* cassette, diluted 10-fold and re-ligated overnight. Each ligation product was purified by Phenol:Chloroform:Isoamyl alcohol and ethanol precipitated and DNA was diluted in 30 μl distilled water. 10 μl (200 ng) ligated DNA was used in a 50 μl PCR with primers 18 and 22 and KAPA Taq DNA Polymerase (Kapa Biosystems, London), using the following program: An initial denaturation step at 95 °C for 2 min, 30 cycles of a denaturation step at 95 °C for 30 s, an annealing step at Tm-5 for 30 s and an extension step at 72 °C for 4 min, and a final extension step at 72 °C for 5 min. Each PCR product was ethanol precipitated and diluted in 20 μl and 1 μl was used in a second 50 μl PCR as template DNA with primers 18 and 22 and KAPA Taq DNA Polymerase, using the program including: an initial denaturation step at 94 °C for 3 min, 10 cycles of a denaturation step at 94 °C for 30 s, an annealing step at Tm-5 for 40 s and an extension step at 72 °C for 4 min, followed by 20 cycles of a denaturation step at 94 °C for 30 s, an annealing step at Tm-5 for 40 s and an extension step at 72 °C for 4 min adding 20 more seconds at the end of each cycle and a final extension step at 72 °C for 5–10 min. The PCR products were analyzed by agarose gel electrophoresis and purified by gel extraction. Each sample was sequenced using primer 20. The location of the transposon insertion was defined by comparing the sequence tags to the publicly available *A. nidulans* genome database (AspGD) using the blastn. The regions identified by the sequence tags were subjected to similarity searches using various Blast algorithms at AspGD in order to identify putative coding sequences affected by the transposition event. Footprints left by transposon excision were analyzed by sequencing the PCR product obtained using primers complementary to niaD regions flanking the transposon insertion site (primers 8 and 11). Primers used are listed in Supplementary Table S2.

2.9. Microscopy

Cultures and observation of the asexual compartments (conidiophores) of *A. nidulans* morphological mutants were performed according to Galanopoulou et al., (2014). Ethanol 70% v/v was added onto the coverslip prior to microscopic observation.

3. Results

3.1. Development of a Minos-based transposition system in A. nidulans

To detect whether *Minos* transposes in *A. nidulans* we followed a strategy previously employed for the *impala* element (Li Destri Nicosia et al., 2001; Dufresne and Daboussi, 2010). The general idea is to introduce, by homologous recombination, a modified *Minos* element hosting a direct selection marker, in the promoter of the *niaD* gene, encoding nitrate reductase. The selection marker used is the *inoB* gene, encoding myo-inositol-1-phosphate synthase, necessary for inositol biosynthesis. The endogenous *inoB* gene was deleted in the transformation recipient strain used (*ΔinoB*) and the *inoB*$^{+}$ transformants would also be *niaD*$^{-}$, and consequently unable to grow on nitrate as sole nitrogen source due to the absence of nitrate reductase transcription. Upon activation of transposition, *via* the *in trans* expression of Minos transposase (see Section 3.4), the excision of *Minos* would, in principle, restore the *niaD* promoter, since the expected short excision footprint would not affect transcription, as shown for *impala* excision from this site (Li Destri Nicosia et al., 2001). In that case, transposition events could be directly selected by plating conidiospores in minimal media supplemented with nitrate as the sole nitrogen source.

3.2. Construction of a modified Minos element for A. nidulans

A modified *Minos* transposable element (*AnMinos*) was made by cloning the *A. nidulans inoB*$^{+}$ and *yA*$^{+}$ genes between the two terminal inverted repeats of *Minos* (for details see Section 2.3, Figs. 1 and 2A). *inoB*$^{+}$ is the gene marker to select the original transformant carrying the *Minos* element by complementation of the *ΔinoB* mutation present in the recipient strain, and for subsequent follow-up of transposition events. The *yA*$^{+}$ gene (necessary for the green color of conidiospores) was included as a subsidiary visual marker to follow directly transposon loss (appearance of yellow colonies), given that the recipient strain is deleted for the *yA* gene (*ΔyA*). For targeted insertion of the *AnMinos* element in a specific site of the *niaD* promoter, we also joined *niaD*-specific external flanking sequences in *AnMinos* (see Section 2.3, Figs. 1 and 2A). The specific site chosen for the integration of *AnMinos* is at −10 bp upstream from the translation initiation codon of the *niaD* ORF. This is based on previous observations showing that the excision of the *impala* transposable element from exactly this site functionally restores the *niaD* promoter (Li Destri Nicosia et al., 2001). A linear DNA fragment containing *AnMinos* flanked externally by the *niaD* sequences was used for transforming a *ΔinoB ΔyA ΔnkuA* recipient strain. The *ΔnkuA* null mutation allows efficient gene-targeting of linear DNA cassettes by homologous recombination (Nayak et al., 2006).

Transformants harboring the *AnMinos* element were isolated (phenotypically *inoB*$^{+}$ *niaD*$^{-}$) and single-copy integration of the *AnMinos* in the *niaD* promoter was confirmed by PCR, Southern

analysis and sequencing. Unexpectedly, original transformants produced yellow conidiospores, suggesting the absence of a functional *yA* gene. This is due to a G to A transition in the acceptor site of the 3rd intron in the yA^+ gene present in the *AnMinos* element, apparently produced during PCR amplification of the relevant DNA fragment. We re-constructed the entire *AnMinos* and eventually obtained a strain harboring a non-mutated *AnMinos* integrated in the *niaD* promoter ($inoB^+$ $niaD^-$ yA^+). As shown later, both elements, named *AnMinos-yA*$^-$ and *AnMinos-yA*$^+$, transpose equally well (see Table 1). All transformants harboring *AnMinos* elements proved phenotypically stable. A similar observation has been made with strains harboring modified *impala* transposable elements lacking their transpose gene (Li Destri Nicosia et al., 2001).

3.3. Construction of a two-component AnMinos transposition system

We constructed a strain expressing the Minos transposase (MnTpase) from the medium-strength constitutive $gpdA^m$ promoter (for details see Section 2.3 and Fig. 2B). In brief, a plasmid containing $gpdA^m$-*MnTpase-trpC*$^{3'}$ and the *argB* gene as a selection marker, was used to transform an $argB^-$ recipient strain. All $argB^+$ transformants analyzed were stable and showed similar wild type-like morphology. One transformant arising from heterologous plasmid integration conserving an intact $gpdA^m$-*MnTpase-trpC*$^{3'}$ construct, as judged by Southern analysis and PCR (not shown), was selected for further work (named *MnTpase*). This transformant was crossed with the strain(s) carrying the *AnMinos* element(s) and appropriate progeny harboring both the *AnMinos* element and $gpdA^m$-*MnTpase-trpC*$^{3'}$ cassette were selected. In order to be able to follow the presence of the *AnMinos* elements ($inoB^+$ and yA^+), the progeny selected needs to be both *ΔinoB* and *ΔyA*. In addition, we selected progeny that was either $nkuA^+$ or *ΔnkuA*, in order to investigate the role of NkuA in *AnMinos* transposition. NkuA is the orthologue of the Ku70 protein, a component of the non-homologous end joint repair complex or NHEJ (Chapman et al., 2012).

3.4. AnMinos transposes in the nkuA$^+$ strain only upon expression of its own transposase

Approximately 10^8 conidiospores of *AnMinos-yA*$^-$*/MnTpase* or *AnMinos-yA*$^+$*/MnTpase* strains ($nkuA^+$ or *ΔnkuA*) were collected from freshly made complete media petri dishes and plated on minimal media containing nitrate as the sole nitrogen source. In several independent experiments, approximately 100 to 1000 $niaD^+$ $inoB^+$ revertants were obtained only in the $nkuA^+$ genetic background (Fig. 3 and Table 1-part I). This was seen in two independent *nkuAΔ* strains for each construct, which strongly suggested that *AnMinos* was mobilized only when a functional NkuA protein is present. Our system further allows distinguishing whether the absence of NkuA affects excision or re-integration. If it affects only re-integration, we should obtain at least colonies having lost the transposon, able to grow on NO_3^- as nitrogen source, which are nevertheless $inoB^-$, and in the case of the *AnMinos-yA*$^+$ also yA^-. Thus, if *nkuA* was necessary only for re-integration we should obtain NO_3^- utilizing colonies in the presence of inositol, but not in its absence. We did not obtain any colonies either in the presence or in the absence of inositol, which shows that NkuA is necessary for the primary excision event or its repair.

The frequency of *AnMinos* transposition ranged from 0.21 to 5.6×10^{-6}, which is comparable to that of *impala* (Li Destri Nicosia et al., 2001). Upon replica plating of 200 $niaD^+$ revertants isolated in minimal media supplemented with inositol, 32 (16%) scored as $inoB^-$ when replica plated on minimal media without inositol. This showed that the rate of *AnMinos* loss by excision is similar to the one obtained with *impala* (Li Destri Nicosia et al., 2001).

3.5. Excision footprints and AnMinos loss

We sequenced excision sites in the *niaD* locus from five strains, four of which exhibited phenotypes compatible with *AnMinos* transposition ($niaD^+$ $inoB^+$), and one from a strain that has probably lost *AnMinos* ($niaD^+$ $inoB^-$). Results, presented in Fig. 4, show that excision footprints consisted of the four terminal bases of the transposon flanked by the TA target duplication and led to no major DNA rearrangements. This result further confirmed the *AnMinos* has been successfully mobilized.

3.6. AnMinos rate of transposition is not affected by temperature, UV irradiation or niaD transcription

We repeated the experiments leading to *AnMinos* transposition under conditions where the $niaD^+$ revertants were selected at various temperatures (18, 25, 37 or 42 °C), or conidiospores were kept at low temperature (4 °C) for various periods of time (0–12 days) before selection. The latter was based on a recent report showing that transposition of *impala* in *A. fumigatus* is highly activated by prolonged exposure to low temperatures (Carr et al., 2010). We have not detected any major effect on the rate of *AnMinos*

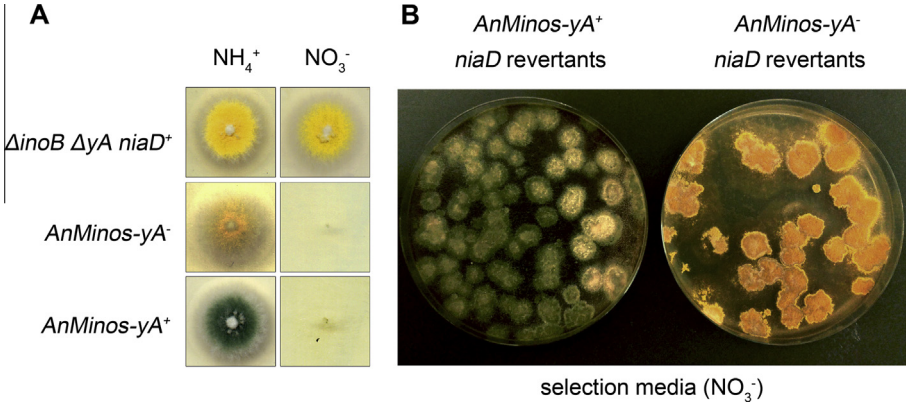

Fig. 3. *AnMinos* transposition as evidenced by direct selection for $niaD^+$ revertants. (A) Growth test on Minimal Media (MM) containing 10 mM ammonium tartrate or sodium nitrate as sole nitrogen sources of strains harboring either *AnMinos-yA*$^-$ (yellow) or *AnMinos-yA*$^+$ (green) and a control recipient strain without *AnMinos*. (B) Selection of colonies carrying *AnMinos* transposition events on MM-nitrate plates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transposition (or loss) under any of the conditions tested, including exposure to low temperatures (Table 1-part II and Supplementary Table S3).

We also repeated the experiment leading to *AnMinos* transpositions after short exposure to UV irradiation or in conditions where the conidiospores harvested were collected from minimal media containing either nitrate plus urea or ammonium tartrate, as nitrogen sources. Nitrate induces *niaD* transcription, whereas ammonium ions repress *niaD* transcription. The rationale of the latter test was to investigate whether the transcription status of the locus where An*Minos* was originally integrated has an effect on its mobilization. None of these treatments had a significant effect on An*Minos* transposition (Table 1-part II and Supplementary Table S4).

3.7. Direct genetic selection of morphological mutants

Among transposition events, we visually screened for those that have altered colony morphology. The frequency of such events was approximately 1%. Fig. 5 shows the growth phenotypes and microscopic morphology of a number of these mutants. We sequenced the flanking regions of nine transposition sites leading to morphological mutants (Table 2). In four cases *AnMinos* transposed into the ORF of the *brlA* gene (AN0973), in three distinct integration sites (679, 1052 and 1125 bp from the translation start codon). The remaining five events concerned disruption of the ORF of *atmA* (AN0038), modification of sequences making part of the promoter of the *nsdD* (AN3152) or the 3′ sequences of the AN1030, or integration in the 5′ UTR of the *oefA* gene (AN9087) or 3′ UTR of AN0225 gene. BrlA is a well-studied C_2H_2 zinc finger transcription factor and regulator of conidiophore development (Chang and Timberlake, 1993). AtmA is a putative 1-phosphatidylinositol-3-kinase involved in DNA damage response and *atmA* mutants are known to display defects in cell polarity and in microtubule organization at the hyphal tip (Malavazi et al., 2006). OefA is a LisH domain-containing protein and *oefA* null mutants display a "fluffy" phenotype (Lee et al., 2005). NsdD is a GATA-type zinc-finger transcription factor required for sexual development (Han et al., 2001). AN1030 and AN0225 correspond to uncharacterized proteins. AN1030 has domains with predicted nucleotide binding capacity and is similar to putative oxidoreductase/transferases, while AN0225 is a putative ATPase (http://www.aspgd.org/). These results confirm that *AnMinos* is an appropriate tool to tag genes involved in *A. nidulans* developmental differentiation. More interestingly, however, is the observation that one of the mutants obtained is most probably the result of transcriptional up-regulation of the tagged ORF, rather than its down-regulation. This is the case of *nsdD*. The morphology of the mutant obtained, which is characterized by over-production of cleistothecia (see Fig. 5B) is compatible with the phenotype of NsdD over-expression, rather than that of null alleles, which fail to produce cleistothecia or any sexual reproductive organs (Han et al., 2001) (Fig. 5B).

3.8. Direct genetic selection of 5-FU resistant mutants

We used *AnMinos* to obtain mutants resistant to 5-fluorouracil (5-FU), a powerful antifungal and anticancer agent. We obtained seven (~0.05%) *niaD*$^+$ *inoB*$^+$ 5-FU resistant mutants in an estimated population of ~15,000 transpositions events (Fig. 6). Table 3 summarizes the identity of the relevant *AnMinos* flanking regions. Six mutants analyzed (Fur1–6) arise from different transposition events. Among the six transposition events, two concerned integration into the ORF of known genes. The first (Fur-5) is in the *strA/fsrA* gene (AN8071), encoding a putative scaffold protein with similarity to mammalian striatin required for normal sexual development and conidiation (Wang et al., 2010). The second (Fur-6) is in *gltA* (AN5134), a gene encoding a NAD(+)-dependent (GOGAT) glutamate synthase, with a predicted role in glutamate and glutamine metabolism (Macheda et al., 1999). How the activity of these proteins is related to 5-FU toxicity is unknown and will need further investigation. One 5-FU resistant mutant is due to a transposition in an intron sequence of AN7502 (Fur-4), a putative uridine kinase with a predicted role in pyrimidine metabolism; thus, the knock out of this gene can be easily related physiologically to 5-FU resistance.

Three distinct transpositions events occur in the 5′ region of AN2746 (Fur-1, Fur-2 and Fur-3) an uncharacterized transporter. We do not know whether these insertions result in down- or up-transcription of the transporter, leading to exclusion of the analogues either by defective uptake or by active export respectively. The fact that three independent and different transposition events lead to the same phenotype establishes the causal link between the transposon insertion and the resistance to the toxic analogues.

We further tested the 5-FU resistant mutants with respect to cross-resistance to other pyrimidine toxic analogues (see Fig. 6). *strA/fsrA* (Fur-5) and *gltA* (Fur-6) mutants showed little or no

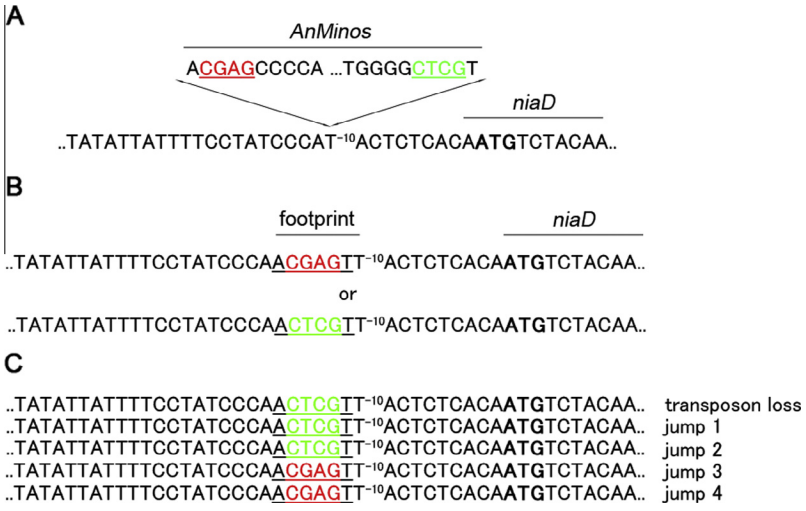

Fig. 4. *AnMinos* excision footprints. (A) Initial integration of *AnMinos* at the *niaD* locus. (B) Two possible excision footprints (underlined sequence) are left in the *niaD* locus after the excision of *AnMinos*. (C) Sequence footprints of 4 transposition events, denoted as "jumps" (excision and successful re-integration in a new locus), and of one case of transposon loss.

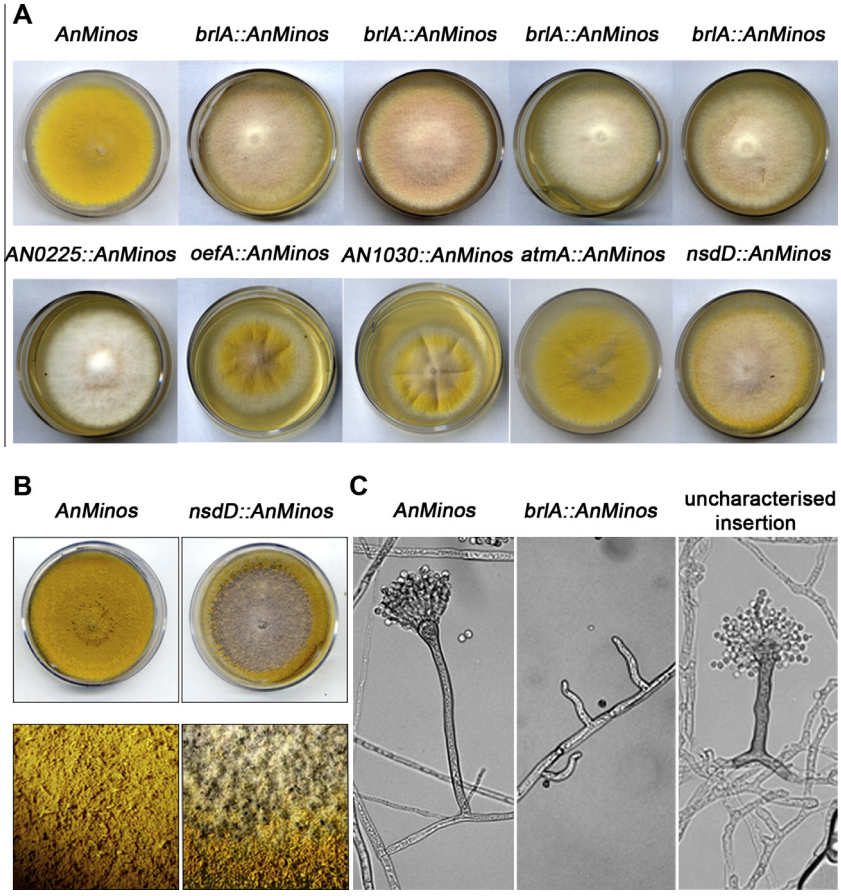

Fig. 5. Morphological mutants obtained through *AnMinos* transposition. (A) Growth phenotypes in CM after 3 day incubation at 37 °C (upper panel). (B) The phenotype of a strain harboring an *AnMinos* transposition linked to the *nsdA* gene grown for 8 days, which leads to the hyper-production of cleistothecia. The lowest panel is a picture from a stereoscopic view at 3× magnification. Cleistothecia are apparent as brownish spherical bodies. (C) Microscopic phenotypes (lower right panel) of asexually differentiated hyphae from an *AnMinos*-based *blrA*$^{-}$ mutant, a wild-type control and an uncharacterized insertion. All other characterized morphological mutants shown in 4A, other than *ΔblrA*, show asexual differentiation similar to that of a wild-type.

Table 2
Molecular characterization of morphological mutants obtained through *AnMinos* transposition.

Strain	ANID	Chromosome	Activity	Region	Insertion site
Mor-1	AN0973	VIII	***brlA***, C2H2 zinc finger transcription factor	ORF (+678, +679)	ATCAT/ATACA
Mor-2	AN0973	VIII	As above	ORF (+1051, +1052)	CATGT/ATGTT
Mor-3	AN0973	VIII	As above	ORF (+1051, +1052)	CATGT/ATGTT
Mor-4	AN0973	VIII	As above	ORF (+1124, +1125)	CCCAT/AGCAA
Mor-5	AN3152	VI	***nsdD***, Predicted GATA-type zinc-finger transcription factor required for sexual development	Upstream[a] (−1323, −1322)	GGCCT/ACCAG
Mor-6	AN9087	VI	***oefA***, LisH domain-containing protein	5′UTR-1st exon (−165, −164)	CTCAT/AATAT
Mor-7	AN0038	VIII	***atmA***, Putative 1-phosphatidylinositol-3-kinase	ORF-5th exon (+5780, +5781)	CTAGT/AGATG
Mor-8	AN0225	VIII	Uncharacterized – Orthologues have bile acid-export ATPase activity	3′UTR-3rd exon (+5188, +5189)	TACAT/ACATA
Mor-9	AN1030	VIII	Uncharacterized – Putative oxidoreductase	72 bp downstream from AN1030	CAACT/AAACA

Colonies showing abnormal morphology were selected and isolated. Integration site of *AnMinos* was defined by sequencing flanking sequences and search of these sequences, using blastn, in AspGD database (see Section 2). Relevant genes affected were identified through the AspGD database.
[a] Transcription starts at −1172.

resistance to 5-fluorocytosine (5-FC) and 5-fluorouridine (5-FUd), similar to the original strain before transposition. AN2746 (Fur1–3) and AN7502 (Fur-4) mutants were more resistant to 5-FC than original strain, whereas AN7502 (Fur-4) and at least one of the integration events in AN2746 (Fur-1), also acquired increased resistance to 5-FUd.

The AN2746 putative transporter has several transmembrane domains characteristic of members of the Major Facilitator Superfamily (MFS) and shows similarity (30% identity) with transporters specific for riboflavin, hexoses or monocarboxylates in fungi or mammals. This level of similarity is however insufficient to reveal the specificity of AN2746, but we are tempted to propose that among its physiological substrates might be uracil, cytosine and pyrimidine nucleosides. Interestingly, the major uracil (and 5-FU), cytosine (and 5-FC) and nucleoside (and 5-FUd) transporters have been characterized and shown to belong to two different transporter families, known as Nucleobase Cation Symporter 1 family (NCS1) or the Concentrative Nucleoside Transporters (CNT), respectively (Pantazopoulou and Diallinas, 2007; Hamari et al., 2009; Krypotou et al., 2015).

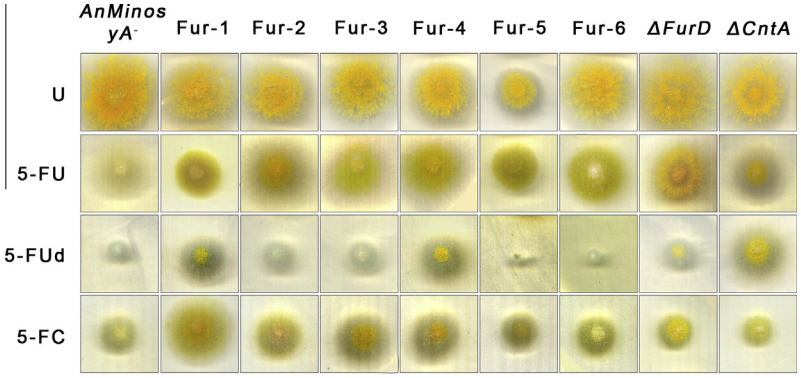

Fig. 6. 5-FU mutants obtained through *AnMinos* transposition. Growth phenotypes on 5-FU, 5-FC and 5-FUd of the original AnMinos-yA⁻ strain and 6 mutants (Fur1–6) directly selected as colonies resistant to 5-FU. Two strains genetically lacking the major uracil/5-FU transporter FurD and the general nucleoside transporter CntA, are also shown as controls.

Table 3
Molecular characterization of 5-FU mutants obtained through AnMinos transposition.

Strain	ANID	Chromosome	Activity	Region	Insertion site
Fur-1	AN2746	VI	Uncharacterized – Similarity to MFS putative monocarboxylate transporters	Upstream[a] (−265, −264)	GTCCT/ATATT
Fur-2	AN2746	VI	As above	Upstream[a] (−801, −800)	TTCTT/ATATG
Fur-3	AN2746	VI	As above	Upstream[a] (−681, −680)	ACATT/AGAGG
Fur-4	AN7502	IV	Uncharacterized – Putative uridine kinase. Predicted role in pyrimidine metabolism	6th intron (+1023, +1024)	CTGCT/AGTCT
Fur-5	AN8071	II	***strA/fsrA***, Predicted scaffold protein, required for normal sexual development and conidiation	ORF-3rd exon (+1021, +1022)	CAAGT/ATACG
Fur-6	AN5134	V	***gltA***, Glutamate synthase NAD^+-dependent GOGAT).	ORF-3rd exon (+2497, +2498)	ACTCT/AGCAT

Integration site of *AnMinos* was defined by sequencing flanking sequences and search of these sequences, using blastn, in AspGD database (see Section 2). Relevant genes affected were identified through the AspGD database.
[a] Transcription starts at −139.

3.9. Randomness of AnMinos transposition

We also sequenced the flanking regions of five random *AnMinos* integration sites, which correspond to transposition events that do not lead to any apparent mutant phenotype. Among the random transpositions characterized (Table 4), we obtained an insertion in the first exon of the *flbC* gene (AN2421). FlbC is putative C_2H_2 zinc finger transcription factor regulating conidiophore development through light-dependent activation of *brlA* transcription (Ruger-Herreros et al., 2011). However, we did not obtain the previously described mutant phenotype, which might mean that disrupting the first exon does not affect the function of FlbC. A much larger number of random transposition events should be sequenced to define the basis of preference of *AnMinos* with respect to its integration sites.

4. Discussion

The efficiency of *Minos* transposition obtained in *A. nidulans* is sufficient for it to be used as a system to isolate mutants and identify new genes. We still, however, know very little on the factors that affect the efficiency and randomness of excision and integration of *Minos*, or any other transposable element in any system. Although genetic, physiological and stress factors seem to play a role in transposition in several systems, it is still not clear how these factors affect transposition as contradictory results are sometimes obtained (Carr et al., 2010). *A. nidulans* with its unique genetic tractability permits a thorough investigation of the mechanism of *Minos* transposition. Here, for example, we have showed that *Minos* transposition in *A. nidulans* requires the NkuA protein, an homologue of Ku70, which is a key factor for NHEJ (Nayak

Table 4
Molecular characterization of random *AnMinos* transposition sites.

Strain	ANID	Chromosome	Activity	Region	Insertion site
Ran-1	AN11855	I	Uncharacterized – Unknown function	3′UTR (+635, +636)	TAGGT/AGATT
Ran-2	AN0871	VIII	Uncharacterized – Putative GPI-anchor transamidase	Promoter (−141, −140)[a]	AGCTT/ACAAC
Ran-3	AN2421	VII	***flbC***, putative C2H2 zinc finger transcription factor	ORF-1st exon (+259, +260)	TAACT/ACTCG
Ran-4	AN1001	VIII	Uncharacterized orthologue of *A. fumigatus Afu1g12770*	Promoter? (−549, −548)[b]	GAAAA/TAGAG
Ran-5	∼3.4 kb upstream from *areA*/AN8667	III	–	Intergenic	GCAGT/ATTTT

Integration sites of *AnMinos* transpositions were defined by characterizing flanking sequences and search of these sequences, using blastn, in AspGD database (see Section 2). Relevant sites of integration are defined through the AspGD database.
[a] Transcription starts from −132.
[b] Transcription starts from −304.

et al., 2006; Chapman et al., 2012). Heterologous expression of the Ac/Ds transposon system in *S. cerevisiae* also showed the involvement of the Ku complex in transposon excision (Yu et al., 2004b). Absence of Ku80, a paralogue which forms a heterodimer with Ku70, results in 80% decrease of transposition of *sleeping beauty* in mammalian cells, while the transposase molecule physically interacts with Ku70 (Izsvák et al., 2004). The mammalian cut and paste *piggyBat* transposon expressed in *S. cerevisiae* showed a 10,000-fold decrease of transposon excision in strains deleted for a number of genes encoding NHEJ proteins, including *YKU70* (She et al., 2015). The Ku proteins are also involved in the repair of P element excision gaps in *Drosophila melanogaster* (Beall et al., 1994; Min et al., 2004). Interestingly, transposons of the *piggyBac* family (such as *piggyBat*) excise precisely and do not leave footprints, at variance with *Minos* (see below and Pavlopoulos et al., 2007), *Ac/Ds* (Yu et al., 2004b) the P element and *sleeping beauty* (Izsvák et al., 2004).

Several other questions can be addressed, such as, the role of the chromatin status at the original site of transposon integration or at the transposition target site (Wood and Helfand, 2013), the effect of RNAi silencing mechanism, and the role of the expression level of the transposase or the length of the transposed sequences. (Buchon and Vaury, 2006; Ito and Kakutani, 2014). In addition, *A. nidulans*, with its complex developmental sexual and asexual pathways can also be used to address whether the developmental stage has an effect on transposition.

While reverse genetic approaches for gene functional analysis concern the generation of knock-outs, transposon-based analysis is based on the generation of mutant phenotypes, which allows the identification of genes through their hyper-expression (*hypermorphs*) or down-regulation of expression (*hypomorphs*), rather than complete knock-out. Here, we obtained evidence that at least one of the morphological mutants obtained was due to hyper-expression of the *nsdD* gene due to *AnMinos* insertion at its 5′ regulatory sequences. *AnMinos* is at least equally efficient, with respect to the frequency of transposition, with the previously developed system of *impala*. *AnMinos* seems to transpose to all chromosomes, albeit with a 50% preference in chromosome VIII, which is the chromosome which was originally inserted. We still do not know whether *AnMinos* and *impala* have the same randomness of transposition or whether they have the same target site preference. The answer to these questions will necessitate the parallel study of the two elements in genetically isogenic backgrounds and the sequencing, through high throughput approaches, of a much higher number of transposition sites. Among the nineteen transposition events of *AnMinos* characterized in the present study, seven concerned disruptions of exons, one of an intron, six probably disrupt promoters, one concerned an integration within the transcribed 3′ sequences, and only one was in an intergenic region, distant from annotated genes. Although the number of transpositions characterized is small, this result already suggests that *AnMinos* has no preference of exons, introns, regulatory regions or non-transcribed DNA. In addition, there is also already a hint that *Minos* and *impala* might have different transposition preferences. Previous studies have shown that *impala* transposes frequently into the *wA* gene and into the ribosomal repeat (Vlauchou T, Broccard-Masson, C; Drevet C., and Scazzocchio C., unpublished results) which shows a typical growth habit whereas we did not get any of these insertions using *Minos*. Inversely, *Minos* tags *brlA* frequently, but *impala* does not.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.05.007.

References

Amutan, M., Nyyssonen, E., Stubbs, J., Diaz-Torres, M.R., Dunn-Coleman, N., 1996. Identification and cloning of a mobile transposon from *Aspergillus niger* var. *awamori*. Curr. Genet. 29 (5), 468–473.

Arcà, B., Zabalou, S., Loukeris, T.G., Savakis, C., 1997. Mobilization of a Minos transposon in *Drosophila melanogaster* chromosomes and chromatid repair by heteroduplex formation. Genetics 145 (2), 267–279.

Beall, E.L., Admon, A., Rio, D.C., 1994. A Drosophila protein homologous to the human p70 Ku autoimmune antigen interacts with the P transposable element inverted repeats. Proc. Natl. Acad. Sci. USA 91 (26), 12681–12685.

Bellen, H.J., Levis, R.W., He, Y., Carlson, J.W., Evans-Holm, M., Bae, E., et al., 2011. The Drosophila gene disruption project: progress using transposons with distinctive site specificities. Genetics 188 (3), 731–743. http://dx.doi.org/10.1534/genetics.111.126995.

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., et al., 2004. The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167 (2), 761–781. http://dx.doi.org/10.1534/genetics.104.026427.

Buchon, N., Vaury, C., 2006. RNAi: a defensive RNA-silencing against viruses and transposable elements. Heredity 96 (2), 195–202. http://dx.doi.org/10.1038/sj.hdy.6800789.

Carr, P.D., Tuckwell, D., Hey, P.M., Simon, L., d'Enfert, C., Birch, M., et al., 2010. The transposon impala is activated by low temperatures: use of a controlled transposition system to identify genes critical for viability of *Aspergillus fumigatus*. Eukaryot. Cell 9 (3), 438–448. http://dx.doi.org/10.1128/EC.00324-09.

Cecchetto, G., Amillis, S., Diallinas, G., Scazzocchio, C., Drevet, C., 2004. The AzgA purine transporter of *Aspergillus nidulans*. Characterization of a protein belonging to a new phylogenetic cluster. J. Biol. Chem. 279 (5), 3132–3141. http://dx.doi.org/10.1074/jbc.M308826200.

Chang, Y.C., Timberlake, W.E., 1993. Identification of Aspergillus brlA response elements (BREs) by genetic selection in yeast. Genetics 133 (1), 29–38.

Chapman, J.R., Taylor, M.R., Boulton, S.J., 2012. Playing the end game: DNA double-strand break repair pathway choice. Mol. Cell 47 (4), 497–510. http://dx.doi.org/10.1016/j.molcel.2012.07.029.

Chun, K.T., Edenberg, H.J., Kelley, M.R., Goebl, M.G., 1997. Rapid amplification of uncharacterized transposon-tagged DNA sequences from genomic DNA. Yeast (Chichester, England) 13 (3), 233–240. http://dx.doi.org/10.1002/(SICI)1097-0061(19970315)13:3<233::AID-YEA88>3.0.CO;2-E.

Daboussi, M.-J., Capy, P., 2003. Transposable elements in filamentous fungi. Annu. Rev. Microbiol. 57, 275–299. http://dx.doi.org/10.1146/annurev.micro.57.030502.091029.

Daboussi, M.J., 1997. Fungal transposable elements and genome evolution. Genetica 100 (1–3), 253–260.

Daboussi, M.J., Langin, T., 1994. Transposable elements in the fungal plant pathogen *Fusarium oxysporum*. Genetica 93 (1–3), 49–59. http://dx.doi.org/10.1007/BF01435239.

Daboussi, M.J., Langin, T., Brygoo, Y., 1992. Fot1, a new family of fungal transposable elements. Mol. Gen. Genet.: MGG 232 (1), 12–16.

Daboussi, M.J., Langin, T., Deschamps, F., Brygoo, Y., Scazzocchio, C., Burger, G., 1991. Heterologous expression of the *Aspergillus nidulans* regulatory gene nirA in *Fusarium oxysporum*. Gene 109 (1), 155–160.

de Queiroz, M.V., Daboussi, M.J., 2003. Impala, a transposon from *Fusarium oxysporum*, is active in the genome of *Penicillium griseoroseum*. FEMS Microbiol. Lett. 218 (2), 317–321.

Deschamps, F., Langin, T., Maurer, P., Gerlinger, C., Felenbok, B., Daboussi, M.J., 1999. Specific expression of the Fusarium transposon Fot1 and effects on target gene transcription. Mol. Microbiol. 31 (5), 1373–1383.

Dufresne, M., Daboussi, M.-J., 2010. Development of impala-based transposon systems for gene tagging in filamentous fungi. Methods Mol. Biol. (Clifton, N.J.) 638, 41–54, doi: 10.1007/978-1-60761-611-5_4.

Firon, A., Villalba, F., Beffa, R., D'Enfert, C., 2003. Identification of essential genes in the human fungal pathogen *Aspergillus fumigatus* by transposon mutagenesis. Eukaryot. Cell 2 (2), 247–255.

Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R., Savakis, C., 1994. Mobile Minos elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. Proc. Natl. Acad. Sci. USA 91 (11), 4746–4750.

Franz, G., Savakis, C., 1991. Minos, a new transposable element from *Drosophila hydei*, is a member of the Tc1-like family of transposons. Nucl. Acids Res. 19 (23), 6646-6646.

Galanopoulou, K., Scazzocchio, C., Galinou, M.E., Liu, W., Borbolis, F., Karachaliou, M., et al., 2014. Purine utilization proteins in the Eurotiales: cellular compartmentalization, phylogenetic conservation and divergence. Fungal Genet. Biol. 69, 96–108. http://dx.doi.org/10.1016/j.fgb.2014.06.005.

Glayzer, D.C., Roberts, I.N., Archer, D.B., Oliver, R.P., 1995. The isolation of Ant1, a transposable element from *Aspergillus niger*. Mol. Gen. Genet.: MGG 249 (4), 432–438.

Hamari, Z., Amillis, S., Drevet, C., Apostolaki, A., Vágvölgyi, C., Diallinas, G., Scazzocchio, C., 2009. Convergent evolution and orphan genes in the Fur4p-like family and characterization of a general nucleoside transporter in *Aspergillus nidulans*. Mol. Microbiol. 73 (1), 43–57. http://dx.doi.org/10.1111/j.1365-2958.2009.06738.x.

Han, K.H., Han, K.Y., Yu, J.H., Chae, K.S., Jahng, K.Y., Han, D.M., 2001. The nsdD gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. Mol. Microbiol. 41 (2), 299–309.

Hey, P., Robson, G., Birch, M., Bromley, M., 2008. Characterisation of Aft1 a Fot1/Pogo type transposon of *Aspergillus fumigatus*. Fungal Genet. Biol.: FG & B 45 (2), 117–126. http://dx.doi.org/10.1016/j.fgb.2007.10.009.

Ito, H., Kakutani, T., 2014. Control of transposable elements in *Arabidopsis thaliana*. Chromosome Res.: Int. J. Mol. Supramol. Evolut. Aspects Chromosome Biol. 22 (2), 217–223. http://dx.doi.org/10.1007/s10577-014-9417-9.

Ivics, Z., Izsvák, Z., 2010. The expanding universe of transposon technologies for gene and cell engineering. Mobile DNA 1 (1). http://dx.doi.org/10.1186/1759-8753-1-25, 25–25.

Izsvák, Z., Stüwe, E.E., Fiedler, D., Katzer, A., Jeggo, P.A., Ivics, Z., 2004. Healing the wounds inflicted by sleeping beauty transposition by double-strand break repair in mammalian somatic cells. Mol. Cell 13 (2), 279–290. http://dx.doi.org/10.1016/S1097-2765(03)00524-0.

Joly-Lopez, Z., Bureau, T.E., 2014. Diversity and evolution of transposable elements in Arabidopsis. Chromosome Res.: Int. J. Mol. Supramol. Evolut. Aspects Chromosome Biol. 22 (2), 203–216. http://dx.doi.org/10.1007/s10577-014-9418-8.

Kempken, F., Kück, U., 1998. Transposons in filamentous fungi – facts and perspectives. BioEssays: News Rev. Mol. Cell. Develop. Biol. 20 (8), 652–659. http://dx.doi.org/10.1002/(SICI)1521-1878(199808)20:8<652::AID-BIES8>3.0.CO;2-K.

Koukaki, M., Giannoutsou, E., Karagouni, A., Diallinas, G., 2003. A novel improved method for *Aspergillus nidulans* transformation. J. Microbiol. Methods 55 (3), 687–695.

Krypotou, E., Evangelidis, T., Bobonis, J., Pittis, A.A., Gabaldón, T., Scazzocchio, C., et al., 2015. Origin, diversification and substrate specificity in the family of NCS1/FUR transporters. Mol. Microbiol. http://dx.doi.org/10.1111/mmi.12982.

Langin, T., Capy, P., Daboussi, M.-J.e., 1995. The transposable element impala, a fungal member of the Tc1-mariner superfamily. Mgg Mol. Gen. Genet. 246 (1), 19–28. http://dx.doi.org/10.1007/BF00290129.

Lee, B.-Y., Han, S.-Y., Choi, H.G., Kim, J.H., Han, K.-H., Han, D.-M., 2005. Screening of growth- or development-related genes by using genomic library with inducible promoter in *Aspergillus nidulans*. J. Microbiol. (Seoul, Korea) 43 (6), 523–528.

Li Destri Nicosia, M.G., Brocard-Masson, C., Demais, S., Hua Van, A., Daboussi, M.J., Scazzocchio, C., 2001. Heterologous transposition in *Aspergillus nidulans*. Mol. Microbiol. 39 (5), 1330–1344.

Loukeris, T.G., Arcà, B., Livadaras, I., Dialektaki, G., Savakis, C., 1995. Introduction of the transposable element Minos into the germ line of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 92 (21), 9485–9489.

Macheda, M.L., Hynes, M.J., Davis, M.A., 1999. The *Aspergillus nidulans* gltA gene encoding glutamate synthase is required for ammonium assimilation in the absence of NADP-glutamate dehydrogenase. Curr. Genet. 34 (6), 467–471.

Malavazi, I., Semighini, C.P., Kress, M.R.v.Z., Harris, S.D., Goldman, G.H., 2006. Regulation of hyphal morphogenesis and the DNA damage response by the *Aspergillus nidulans* ATM homolog AtmA. Genetics 173 (1), 99–109. http://dx.doi.org/10.1534/genetics.105.052704.

Min, B., Weinert, B.T., Rio, D.C., 2004. Interplay between Drosophila Bloom's syndrome helicase and Ku autoantigen during nonhomologous end joining repair of P element-induced DNA breaks. Proc. Natl. Acad. Sci. USA 101 (24), 8906–8911. http://dx.doi.org/10.1073/pnas.0403000101.

Miskey, C., Izsvák, Z., Kawakami, K., Ivics, Z., 2005. DNA transposons in vertebrate functional genomics. Cell. Mol. Life Sci.: CMLS 62 (6), 629–641. http://dx.doi.org/10.1007/s00018-004-4232-7.

Muñoz-López, M., García-Pérez, J.L., 2010. DNA transposons: nature and applications in genomics. Curr. Genom. 11 (2), 115–128. http://dx.doi.org/10.2174/138920210790886871.

Nayak, T., Szewczyk, E., Oakley, C.E., Osmani, A., Ukil, L., Murray, S.L., et al., 2006. A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics 172 (3), 1557–1566. http://dx.doi.org/10.1534/genetics.105.052563.

Nyyssönen, E., Amutan, M., Enfield, L., Stubbs, J., Dunn-Coleman, N.S., 1996. The transposable element Tan1 of *Aspergillus niger* var. awamori, a new member of the Fot1 family. Mol. Gen. Genet.: MGG 253 (1–2), 50–56.

Ogasawara, H., Obata, H., Hata, Y., Takahashi, S., Gomi, K., 2009. Crawler, a novel Tc1/mariner-type transposable element in *Aspergillus oryzae* transposes under stress conditions. Fungal Genet. Biol. 46 (6–7), 441–449. http://dx.doi.org/10.1016/j.fgb.2009.02.007.

Pantazopoulou, A., Diallinas, G., 2007. Fungal nucleobase transporters. FEMS Microbiol. Rev. 31 (6), 657–675. http://dx.doi.org/10.1111/j.1574-6976.2007.00083.x.

Pavlopoulos, A., Berghammer, A.J., Averof, M., Klingler, M., 2004. Efficient transformation of the beetle *Tribolium castaneum* using the Minos transposable element: quantitative and qualitative analysis of genomic integration events. Genetics 167 (2), 737–746. http://dx.doi.org/10.1534/genetics.103.023085.

Pavlopoulos, A., Oehler, S., Kapetanaki, M.G., Savakis, C., 2007. The DNA transposon Minos as a tool for transgenesis and functional genomic analysis in vertebrates and invertebrates. Genome Biol. 8 (Suppl. 1), S2. http://dx.doi.org/10.1186/gb-2007-8-s1-s2.

Plasterk, R.H., Izsvák, Z., Ivics, Z., 1999. Resident aliens: the Tc1/mariner superfamily of transposable elements. Trends Genet.: TIG 15 (8), 326–332.

Ruger-Herreros, C., Rodríguez-Romero, J., Fernández-Barranco, R., Olmedo, M., Fischer, R., Corrochano, L.M., Canovas, D., 2011. Regulation of conidiation by light in *Aspergillus nidulans*. Genetics 188 (4), 809–822. http://dx.doi.org/10.1534/genetics.111.130096.

Ryder, E., Russell, S., 2003. Transposable elements as tools for genomics and genetics in Drosophila. Briefings Funct. Genom. Proteom. 2 (1), 57–71.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Sasakura, Y., Awazu, S., Chiba, S., Kano, S., Satoh, N., 2003. Application of Minos, one of the Tc1/mariner superfamily transposable elements, to ascidian embryos as a tool for insertional mutagenesis. Gene 308, 11–20. http://dx.doi.org/10.1016/s0378-1119(03)00426-8.

She, W., Cambouris, C.B., Craig, N.L., 2015. Different DNA repair pathways are required following excision and integration of the DNA cut & paste transposon piggyBat in *Saccharomyces cerevisiae*.

Villalba, F., Lebrun, M.H., Hua-Van, A., Daboussi, M.J., Grosjean-Cournoyer, M.C., 2001. Transposon impala, a novel tool for gene tagging in the rice blast fungus *Magnaporthe grisea*. Mol. Plant-microbe Interact.: MPMI 14 (3), 308–315. http://dx.doi.org/10.1094/MPMI.2001.14.3.308.

Wang, C.-L., Shim, W.-B., Shaw, B.D., 2010. *Aspergillus nidulans* striatin (StrA) mediates sexual development and localizes to the endoplasmic reticulum. Fungal Genet. Biol.: FG & B 47 (10), 789–799. http://dx.doi.org/10.1016/j.fgb.2010.06.007.

Wood, J.G., Helfand, S.L., 2013. Chromatin structure and transposable elements in organismal aging. Front. Genet. 4. http://dx.doi.org/10.3389/fgene.2013.00274, 274-274.

Yu, J.-H., Hamari, Z., Han, K.-H., Seo, J.-A., Reyes-Domínguez, Y., Scazzocchio, C., 2004a. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol.: FG & B 41 (11), 973–981. http://dx.doi.org/10.1016/j.fgb.2004.08.001.

Yu, J., Marshall, K., Yamaguchi, M., Haber, J.E., Weil, C.F., 2004b. Microhomology-dependent end joining and repair of transposon-induced DNA hairpins by host factors in *Saccharomyces cerevisiae*. Mol. Cell Biol. 24 (3), 1351–1364.

Zagoraiou, L., Drabek, D., Alexaki, S., Guy, J.A., Klinakis, A.G., Langeveld, A., et al., 2001. In vivo transposition of Minos, a Drosophila mobile element, in mammalian tissues. Proc. Natl. Acad. Sci. USA 98 (20), 11474–11478. http://dx.doi.org/10.1073/pnas.201392398.