Chapter 4

Development of *Impala*-Based Transposon Systems for Gene Tagging in Filamentous Fungi

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Abstract

Genome sequences of many filamentous fungi are now available and additional genomes are currently being sequenced. One of the next strategic goals is to generate collections of tagged genes in order to establish a link between the several thousands of predicted genes and their function. Transposable elements have been invaluable for the identification and isolation of genes of interest as insertion of a transposon both disrupts and tags a gene. In an effort to exploit active transposons identified in the genome of *Fusarium oxysporum* as insertional mutagens, a binary system including the tagging element, MITE, and the transposase of a *Tc1* element has been established as an efficient tool for gene-tagging in *Fusarium graminearum*. In this chapter, we provide an overview of the techniques used and highlight some of the critical steps for the application of this tool to other fungal species.

Key words: *Fusarium*, *Tc1/mariner* elements, MITEs, Transposon-tagging

1. Introduction

With the availability of complete genome sequences of many filamentous fungi, the next challenging phase of research is to link sequence information to gene function. One of the best ways to ascertain function is to disrupt genes in a random and genome wide fashion. Transposon insertional mutagenesis has been used for large-scale analysis of the genome in a wide variety of organisms including microorganisms, flies, worms, mice, and plants (1, 2). A range of tagging strategies by using endogenous transposons when available or transposons from heterologous species have been developed. The identification of active transposons from the phytopathogenic fungus *Fusarium oxysporum* by trapping them in the *niaD* target gene was the first step in the development of tagging strategies (3). Among them, *impala*, a *Tc1* member, was demonstrated to transpose

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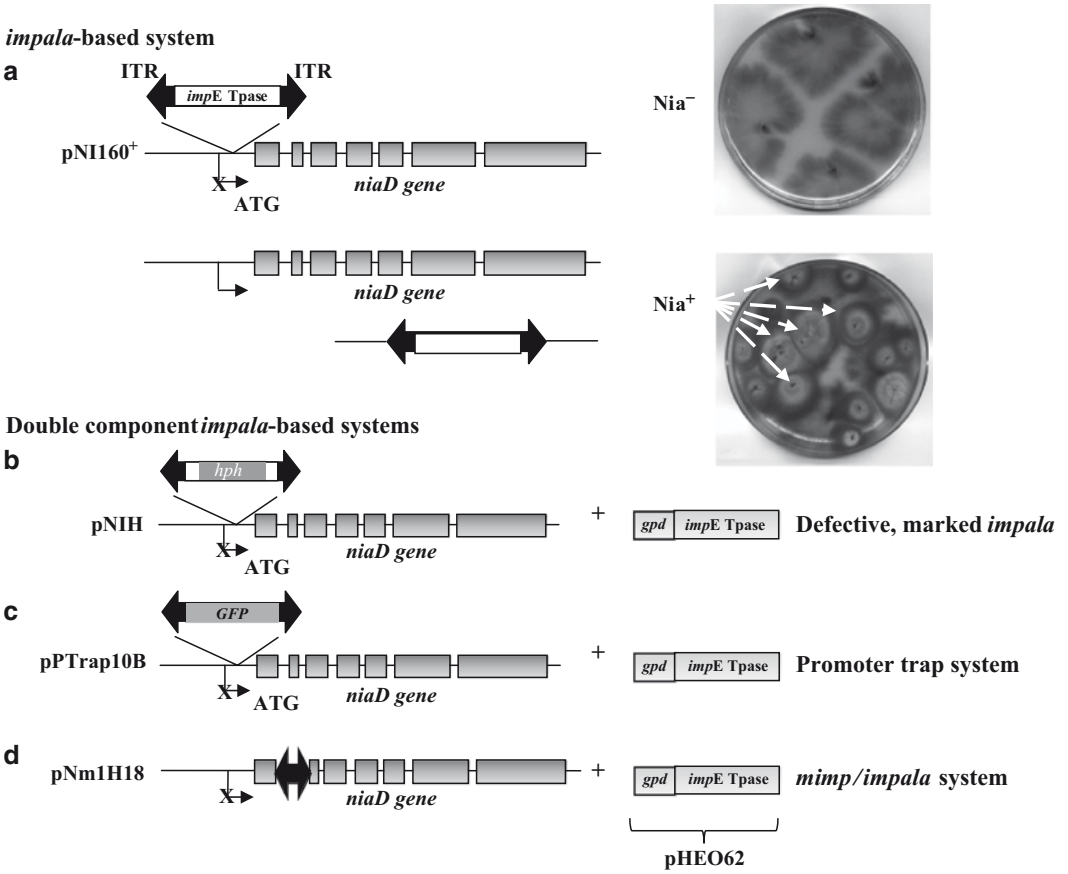

Fig. 1. Structure of the constructs and principle of the phenotypic assay for excision used in the different *impala*-based transposon-tagging systems. Plasmids pNI160$^+$, pNIH, pPTrap10B and pNm1H18 have the same skeleton consisting in a plasmid carrying the wild-type *niaD* gene of *Aspergillus nidulans* (exons are represented as open *grey boxes*, non-coding regions as a *black line*, the arrow below the line corresponds to the transcription inititation site). Large *black* opposite arrows indicate the terminal inverted repeats (TIRs) of the different transposons. (**a**) Plasmid pNI160$^+$ carries the natural transposon *impala160*, inserted in the 5′ untranslated region of *niaD*. When present, it prevents transcription of the gene leading to a *Nia*$^-$ phenotype (see upper Petri plate). Upon excision of *impala160*, *niaD* transcription is restored leading to a *Nia*$^+$ phenotype (see lower Petri plate). The principle of this phenotypic assay for excision is the same for all the transposons described in this figure. (**b**) Plasmid pNIH carries at the same position of the *niaD* gene a modified *impala* transposon in which an internal 1-kbp containing most of the *impala* coding sequence has been replaced by the bacterial gene *hph* (hygromycin B resistance) driven by the *A. nidulans trpC* promoter (9). (**c**) The vector pPTrap10B containing the *imp160::gfp* element was constructed by introducing an 862-bp *Sma*I fragment containing the coding region of the *egfp* gene into the *Sma*I site of a transposase-deleted *impala160* version containing 61 bp and 31 bp of the 5′ and 3′ border sequences, respectively. The construct was then used to replace the *impala* element previously cloned into the promoter region of the *A. nidulans niaD* gene in the pNI160$^+$ plasmid (11). (**d**) Plasmid pNm1H18 was constructed by introduction of a *mimp1* element into the first intron of the *niaD* gene (12). When defective, the transposons have to be mobilized in trans by the *impala* transposase brought by the pHEO62 construct carrying both the open reading frame encoding the *impalaE* transposase cloned between the *gpdA* promoter and the *trpC* terminator of *A. nidulans* (10) and an expression cassette conferring resistance to hygromycin B (for more detail, see ref. (12).

through a phenotypic assay for excision (Fig. 1a) based on the restoration of the activity of the nitrate reductase after *impala* excision (4). Similar to other *Tc1/mariner* elements (5), *impala* has already been demonstrated to transpose in several phylogenetically distant species (3). In some of them *impala* was successfully used to tag genes of interest (6, 7). However, *impala* reinsertion frequency, in the range of 50–75%, hampers its use as a high throughput gene-tagging system (8, 9). To overcome these difficulties, different two-component systems including the *impala* transposase and versions of the tagging element have been developed. First, *impala* has been engineered by insertion of the *hph* gene conferring resistance to the drug hygromycin B (9). This defective element is transactivated by the *impala* transposase under the control of its own promoter (9) or fused to the constitutive *Aspergillus nidulans gpdA* promoter (10) on a separate vector. This selectable marker facilitates the recovery of strains with a reinserted element on condition that only one copy of this construct has been integrated (Fig. 4.1b). Second, we establish a promoter-trap system that utilizes a promoterless *GFP* gene within the transposon so that this reporter gene should be expressed only when the excised transposon reinserts near or into an active gene (11) (Fig. 4.1c). The difficulties to detect GFP expression (weak expression, variable among replicates) among a collection of 1,000 strains tested limit the use of such a strategy and new trapping vectors have to be developed. Third, we demonstrated the ability of the *impala* transposase to transactivate *mimp1,* which shows features of MITEs (12) and developed a novel double component system based on these two partners (Fig. 1d). We assessed the *mimp1* mutagenic potential by generating *mimp*-transposed strains in *F. graminearum* isolate Fg820. We mapped the insertion sites of the tagging element in a collection of transposed strains by comparison with a published genome sequence and screened for mutant phenotypes (13). The high rate of excision of some strains, the distribution of the reinserted element throughout the genome, and the high proportion of insertions within or close to genes make this system very promising. The tools and technologies based on the *mimp1/impala* double component system we developed are presented here in order to provide a valuable resource to accelerate research on gene function in filamentous fungi.

2. Materials

2.1. Isolation of Nitrate-Reductase Deficient Strains

1. Petri plates.
2. Minimal medium agar (MM): 2% (w/v) glucose, 7.5 mM KH_2PO_4, 2 mM $MgSO_4$, 6.5 mM KCl, 6 µM $FeSO_4$ (1 mL from a 1% stock solution prepared extemporaneously). Add

the appropriate nitrogen source (5 mM urea, 23 mM sodium nitrate, 10 mM sodium nitrite or 0.7 mM hypoxanthine), adjust to pH 5.8, and solidify with 1.5% purified agar (Bacteriological agar No 1, Oxoïd).

2.2. Recovery of Cotransformants Giving Rise to Excision Events

1. Potato Dextrose Agar (PDA): We use "home-made" PDA medium: peal 200 g potatoes and let them boil in 1 L tap water for 1 h. Filtrate over gaze and cotton, add 20 g glucose, 1 g yeast extract and 1 g casein hydrolysates. Solidify with 1.5% agar and autoclave at 120°C for 15 min. A commercial product may also be used (e.g., from Difco).
2. Petri plates.
3. Cellophane disks (Focus Packaging).
4. $MgSO_4$: 1.2 M $MgSO_4$, adjust to pH 5.8 with H_3PO_4, filter sterilize on a 0.45 µm unit. Store at 4°C.
5. MS, pH 6.3: 0.6 M sorbitol, 10 mM MOPS, adjust pH to 6.3 with 0.5 N sodium hydroxide, autoclave for 15 min. Store at 4°C.
6. TMS: 1 M sorbitol, 10 mM MOPS, adjust to pH 6.3 with 0.5 N sodium hydroxide and autoclave for 15 min. Store at 4°C.
7. TMSC: 1 M sorbitol, 10 mM MOPS, 40 mM $CaCl_2$, adjust pH 6.3 with 0.5 N sodium hydroxide and autoclave for 15 min. Store at 4°C.
8. Tris–EDTA-$CaCl_2$: 10 mM Tris, pH 7.5, 1 mM EDTA, 40 mM $CaCl_2$. Store at 4°C.
9. PEG solution: 60% (w/v) PEG 4000 in 0.6 M MS. Store at RT for no more than 1 month.
10. Glucanex® (Novo Nordisk, Denmark).
11. PDAS: made as for the PDA medium except that 20% sucrose is added.
12. Hygromycin B (Fermentas).
13. MMS Top agar: MM with 20% (w/v) sucrose, 23 mM sodium nitrate, and 0.4% (w/v) purified agar.
14. Disposable 50 mL and 5 mL sterile tubes.
15. Nylon N disks (89 mm diameter, GE Healthcare).
16. Denaturation solution: 0.5 M NaOH, 5× SSC.
17. Neutralization solution: 0.5 M Tris–HCl, pH 7.5, 10× SSC. For optimal treatments, prepare the denaturation solution extemporaneously. Can be kept for weeks at room temperature (RT).
18. CL-1000 UV crosslinker® (UVP).

2.3. Optimization of the System for Large Scale Mutagenesis

1. PDA plates.
2. Cellophane disks (Focus Packaging).
3. Lysis solution: 1% sarkosyl, 100 mM Tris–HCl, pH 9.0, 10 mM EDTA.
4. Fastprep® Cell Disrupter (QBiogene).
5. Phenol solution equilibrated at pH 7.0.
6. RNase: Dissolve in water at 100 mg/mL.
7. All standard components that are needed to set up PCR reactions.

2.4. Generation of a Collection of Revertants

1. Petri plates (89 mm).
2. 96-well microtiter plates.
3. MM agar supplemented with 23 mM sodium nitrate.

3. Methods

As described above, transposition events are selected using a phenotypic assay for excision based on the restoration of the expression of the *A. nidulans niaD* gene upon excision of the mobilizable *mimp1* element (Fig. 1a, d). The successful application of our transposon-tagging system relies first on the recovery of mutants impaired in the nitrate-reductase structural gene (*nia*$^-$ mutants), without the use of a mutagen, through positively screening for resistance to chlorate (14). This selection system appears to be possible for many fungal species (15). The second step consists of introduction of the two constructs into a *nia*$^-$ mutant strain: one construct (plasmid pHEO62) contains the source of *impala* transposase together with the *hph* selectable marker conferring resistance to hygromycin B, and the second carries the tagging element inserted into the first intron of the *A. nidulans niaD* gene (plasmid pNm1H18, Fig. 1d). Cotransformants, that is transformed strains carrying both constructs, giving rise to excision events will then be selected. The most critical step will consist of the selection of the optimal cotransformants for the generation of a collection of revertants. These strains will have to fulfill a number of criteria: (1) show high rates of excision and reinsertion of the mobilizable element *mimp1*, (2) carry a single copy of this element to ensure an easy and reliable molecular analysis, and (3) give rise to reinsertion events distributed randomly throughout the recipient genome. The following part describes all these different steps.

3.1. Isolation of nia⁻ Strains

3.1.1. Isolation of Chlorate-Resistant Strains

1. Plate conidia on minimal medium (MM) with 5 mM urea and 200 mM chlorate at 10^3 conidia per plate. Prepare at least three platees (see Note 1) and incubate at 26°C.
2. Chlorate resistant colonies appear after 1 or 2 weeks as fast growing colonies (see Note 1). Pick each of colony onto fresh MM urea (5 mM) chlorate (200 mM) plates both to individualize them and to verify their resistance to chlorate.

3.1.2. Identification of nia⁻ Mutants Among Chlorate-Resistant Strains

1. Single-spore each individual chlorate-resistant mutant.
2. Transfer plugs of each of the purified chlorate-resistant mutants to MM amended with different nitrogen sources: 23 mM nitrate, 10 mM nitrite, or 0.7 mM hypoxanthine (see Note 1) and grow at 26°C for 5–7 days to determine which gene of the nitrate-reductase activity is affected. *nia*⁻ mutants will exhibit sparse mycelium on MM containing nitrate as sole nitrogen source but will develop normal aerial mycelium on the two other media.
3. Stable *nia*⁻ mutants can be screened by plating conidia (10^6 per plate, at least five plates) on minimal medium (MM) containing nitrate (23 mM) as sole nitrogen source and incubating at 26°C for 8–10 days. Reversion of the *nia* mutation can be monitored through the appearance of aerial colonies. A reversion frequency of selected *nia*⁻ mutants less than 10^{-6} viable spores is acceptable for the following steps.

3.2. Recovery of Cotransformants Giving Rise to Excision Events

3.2.1. Protoplast Transformation of a Stable nia⁻ Mutant with the Two Plasmids

1. Preparation of fungal protoplasts:
   - Prepare six PDA Petri plates with a cellophane disk, and plate 10^6 spores of the *nia*⁻ recipient strain on each of them. Incubate 17 h at 26°C.
   - Scrape the mycelium from the surface of the plates, and resuspend it in 10 mL of 1.2 M $MgSO_4$. Add Glucanex® (20 mg/mL) and incubate 2 h at 26°C with agitation at 150 rpm.
   - Purify the protoplasts by adding gently and slowly 10 mL MS on top of the digestion solution (see Note 2) and centrifugating 10 min at 750 × *g*.
   - Collect the protoplasts from the interface using a Pasteur pipette (see Note 3).
   - Add 5 mL TMS and centrifuge 5 min at 750 × *g*.
   - Resuspend the pellet of protoplasts in 200 µL TMSC. Protoplasts are ready to be counted using a 1:100 dilution in TMSC and a Thoma's cell (see Note 4).
2. Transformation of fungal protoplasts:

- Pour Petri plates of PDAS + hygromycin B at a final concentration of 50 µg/L, dry them well (see Note 5), and place at 37°C until use.
- Adjust protoplasts density to 10^7 protoplasts/100 µL (in TMSC) and incubate on ice for at least 20 min.
- Prepare a solution of the plasmids to be transformed: 5 µg of each plasmid in a total volume of 60 µL of Tris–EDTA–$CaCl_2$, pH7.5, for each transformation reaction.
- Preheat a water bath under the flow hood at 42°C, and preheat 5 mL tubes for MMS Top agar. Melt MMS Top Agar in a microwave, and dispatch 3 mL per tube (plan four tubes per transformation). Equilibrate at 42°C until use.
- Mix gently DNA (60 µL) and protoplasts (100 µL) and incubate 20 min on ice.
- Remove from the ice and add very gently, drop by drop, 160 µL of PEG solution (using a blue tip, P1000). Mix by "gentle vortexing" (half the maximal shaking), and incubate 15 min at RT.
- Add 1 mL of TMSC, mix gently by inverting the tube then by "gentle vortexing", and centrifuge for 5 min (not more than 5,500 g, in a bench top centrifuge).
- Discard the supernatant without drying the protoplasts pellet, and resuspend in 200 µL of TMSC, using a blue tip.
- Add 50 µL of the transformation reaction per tube of MMS Top Agar. Vortex quickly to mix well, and pour the content of each tube onto a PDAS + hygromycin B plate (see Note 6). Incubate at 26°C. Potential transformants usually appear after 3–5 days as fast growing hygromycin-resistant colonies.
- Transfer a plug from each hygromycin-resistant colony to a fresh PDA + hygromycin B plate. Use the recipient strain as a negative control. Purify each hygromycin-resistant transformant by single-spore isolation.

3.2.2. Identification of the Cotransformants

1. Apply Nylon N disks (GE Healthcare) onto PDA plates (one per plate).
2. Transfer a small plug (1 mm^2) of each hygromycin-resistant transformant as well as of the *nia*$^-$ recipient strain (usually, up to 15 strains can be placed on a single 89 mm diameter plate). Incubate at 26°C for 24–48 h until colonies reach 2 cm diameter.
3. Remove the Nylon N membranes, and treat them by immersion into the denaturation solution for 30 min at room temperature under shaking (150 rpm). Remove the solution and replace

it with the same volume of the neutralization solution. Incubate under the same conditions. Repeat these two treatments once again.

4. Dry the Nylon N disks on Whattman 3 MM paper. Remove any piece of agar.
5. Fix the membranes under UV using a crosslinker (3 min at 1,200 µJ/mm/s).
6. Perform molecular hybridization under standard conditions using a probe specific for the *niaD*::*mimp1* construct (for example, a *niaD*-specific probe). The proportion of cotransformants, that is strains giving a positive hybridization signal, is usually in the range of 50–75% in *Fusarium* strains. (See Note 7).

3.2.3. Phenotypic Assay for Excision

1. Grow each cotransformant onto a PDA plate for 8–10 days at 26°C.
2. Transfer four plugs (4–6 mm^2) from each strain to a plate of MM containing nitrate as sole nitrogen source and incubate at 26°C.
3. Check the plates weekly for the appearance of colonies exhibiting aerial growth ([*Nia*$^+$]), corresponding to potential excision events, above a mat of sparse mycelium ([*Nia*$^-$]), over a period of 5–6 weeks. (See Note 8).
4. Transfer a plug of each aerial colony to a fresh MM nitrate plate to verify its [*Nia*$^+$] phenotype.

3.3. Optimization of the System for Large Scale Mutagenesis

This is the most critical step. The identification of the most adequate cotransformant(s) is essential to guarantee the quality of the resulting collection of revertants that is mutant strains resulting from true transposition events of the mobilizable element. Such strains must fulfill several criteria. First, in order to ensure the feasibility of obtaining a large collection of revertants, the original cotransformant(s) must exhibit a high rate of excision. This parameter can be estimated through the phenotypic assay described above (see Subheading 3.2.3), in which, if possible, we try to select cotransformants giving rise to at least 15–20 aerial [*Nia*$^+$] colonies per plate. Second, among these cotransformants, it is essential to determine which ones carry a single copy of the tagging element. This is one of the most important points as it ensures that most mutations will be tagged (only one transposon moves into the genome) and greatly facilitates molecular analyses. Last, it is important to estimate both quantitatively and qualitatively the reinsertion step. Quantification of reinsertion events will allow estimation of the proportion of collection that is efficient. Molecular analysis of sequences flanking the element in a sample of revertants (usually 24) will give some indication about

the randomness of reinsertion events and prevent any strong bias in the collection (see Note 9). The following paragraph describes the procedure for the second and third steps.

3.3.1. Identification of Cotransformants Carrying a Single Copy of the Tagging Element

1. Select two or three revertants from each cotransformant giving rise to a good number of aerial colonies (15–20 per plate) in the phenotypic assay described above (see Subheading 3.2.3), and purify them by single-spore isolation.
2. Spread conidia of each strain (approximately 10^5 or 10^6) on a sterile cellophane disk applied on a PDA plate, and incubate 16–24 h at 26°C.
3. Recover the mycelium by scraping the cellophane disk, place the mycelium in a 2 mL Eppendorf tube, and freeze in liquid nitrogen. Store at −80°C until use.
4. Add 0.6 mL lysis solution and a few glass beads to each tube. Grind in a Fastprep twice for 30 s at 3,500 g, let settle down a few minutes, and then spin shortly.
5. Extract successively with 500 µL phenol, 500 µL phenol: chloroform (1/1 v/v), and finally with 500 µL chloroform.
6. Add 0.05 volume 3 M NaAc (usually 25 µL), 0.6 volume isopropanol (usually 320 µL), invert the tubes five times, and centrifuge 15 min at 13,000 g at RT.
7. Remove supernatant and wash the pellet with 70% ethanol (approx. 200 µL), vortex and centrifuge again 5 min at RT and at 13,000 g.
8. Vacuum dry 5 min (optional) and resuspend in 100 µL sterile water + RNase A at a final concentration of 1 mg/mL. Incubate 1–2 h at 37°C. Genomic DNA is ready for use, either for PCR (0.5 or 1 µL per PCR reaction) or for digestion by restriction endonucleases for Southern blot analysis (usually 60–80 µL per digest depending on the efficiency of the extraction). Tubes can be stored either at 4°C or at −20°C until use.
9. Perform a PCR test using 50 ng of genomic DNA and primers *niaD*144 (5′-GTTCATGCCGTGGTCGCTGC-3′) and *niaD*754r (5′-AGTTGGGAATGTCCTCGTCG-3′), which are specific for the *A. nidulans niaD* gene and surrounding the insertion site of the tagging element within the construct. PCR reactions should be performed under the following conditions: 4 min at 94°C, then 30 cycles of 1 min at 94°C, 1 min at 59°C, and 2 min at 72°C. A schematic representation of typical results is presented in Fig. 2a. A 717-bp PCR product corresponding to a *niaD* locus still carrying the tagging element is expected for cotransformants (Fig. 2a, COTR1 and COTR2). In revertants, two situations might be encountered. A single

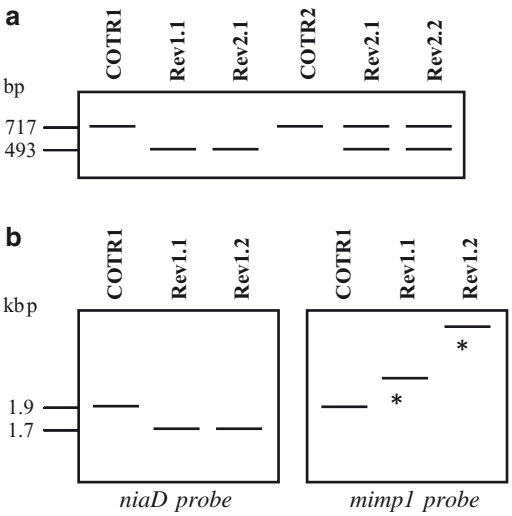

Fig. 2. Schematic representation of typical results of the molecular analysis of different sets of strains each corresponding to a cotransformant and the derived revertants. (**a**) PCR using the *niaD*144/*niaD*754r primer pair: numbers correspond to sizes in base pairs expected for a *niaD* donor site either empty (493 bp) or still carrying *mimp1* (717 bp). (**b**) Southern blot analysis of cotransformant 1 (COTR1) and two corresponding revertants (Rev1.1 and Rev1.2). Genomic DNA was digested with *Xba*I and membranes were successively hybridized with a *niaD* probe (*left*), and a *mimp1* probe (*right*). The stars indicate the reinsertion of excised copies.

493 bp PCR product corresponding to the size of the wild-type *niaD* fragment plus the typical excision footprints left by the element (Fig. 2a, Rev1.1 and Rev1.2) indicates excision of tagging element from the *niaD* gene in these revertants and that the cotransformant (COTR1) they derive from is supposed to contain a single copy of the tagging element. Besides this 493 bp fragment, in other revertants (Fig. 2a, Rev2.1 and Rev2.2), an additional PCR product that corresponds to a *niaD* locus still carrying the tagging element (717 bp) can be observed. These revertants also result from an excision event but at least one additional copy of the *mimp1*-interrupted *niaD* construct was present in the original cotransformant COTR2. Strains corresponding to the latter case should be excluded from further analysis.

10. To further characterize the strains at the molecular level, Southern blot experiments are highly recommended. Genomic DNA of each cotransformant (carrying a single copy of the tagging element) and of 2–3 corresponding revertants is digested using the *Xba*I restriction enzyme, separated electrophoretically on 0.7% agarose gels and transferred onto nylon membranes. Molecular hybridizations are then performed using successively two probes on each membrane: a probe matching the *niaD* gene and overlapping the initial

insertion site of the tagging element and a probe specific for the tagging element itself. As illustrated in Fig. 2b (*left panel*), using the *niaD* probe, cotransformants are characterized by the presence of a single 1.9 kb *Xba*I hybridization band while revertants show a single 1.7 kb *Xba*I signal. This first molecular hybridization thus confirms the results obtained by PCR (see previous step). Using the *mimp1* element as a probe, reinsertion is observed as hybridizing bands of different sizes (Fig. 2b, *right panel*). The proportion of revertants for which such a hybridizing signal is detected corresponds to the reinsertion frequency.

3.3.2. Verification of the Randomness of Reinsertion Events

1. On a sample of purified revertants (24), perform thermal asymmetric interlaced (TAIL)-PCR reactions to recover the sequences flanking the tagging element (see Note 10). In the primary PCR (TAIL1-PCR), the arbitrary degenerate (AD) primer AD2, 5′-AG(A/T)GNAG(A/T)ANCA(A/T)AGA-3′, and the specific primer m1Div53F, 5′-GCCTCTTTGAGCCACG-CTAC-3′, are used with a reduced-stringency and a high-stringency annealing temperatures of 44°C and 66°C, respectively. The TAIL1-PCR program is the following: 92°C 2 min, 95°C 1 min, five cycles consisting of 94°C 30 s, 66°C 1 min and 72°C then 2 min 30 s, 94°C 30 s, 30°C 3 min and a ramping to 72°C over 2 min, 72°C 2 min 30 s and finally 15 supercycles consisting of 94°C 30 s, 66°C 1 min, 72°C 2 min 30 s, 94°C 30 s, 66°C 1 min, 72°C 2 min 30 s, 94°C 30 s, 44°C 1 min, 72°C 2 min 30 s. The secondary PCR (TAIL2-PCR) is set up with 2 µL of a 1/100 dilution of the primary PCR, using the same AD2 primer and Div149 (5′-GCAGGCTAAACTCCAAAT-AGGC-3′) as the second specific primer. The TAIL2-PCR program is as follows: 12 supercycles consisting of 94°C 30 s, 66°C 1 min, 72°.
2. C 2 min 30 s, 94°C 30 s, 66°C 1 min, 72°C 2 min 30 s, 94°C 30 s, 44°C 1 min, 72°C 2 min 30 s followed by an elongation step at 72°C for 5 min.
3. Sequence the resulting PCR products and search for homology with the genome sequence of the species you are working on. If reinsertion occurs randomly, matches at different genomic loci should be obtained.

3.4. Generation of a Collection of Revertants

After selection of strains exhibiting a high rate of excision and verification that reinsertion is not biased, saturation mutagenesis of *Fusarium* genes can be performed. To cover the genome thousands of transposed *mimp*-strains have to be recovered. Two strategies can be used depending on the rate of excision. Revertants can be picked on plates (see Subheading 3.2.3.) or they can be

obtained on microtiter plates ensuring that they result from independent excision events.

1. For the microtiter plate method, inoculate single wells of a 96-well plate containing nitrate minimal agar medium (200 µL per well) with a single germinating spore per well.
2. Collect revertants as they appeared, through the appearance of dense mycelium (Fig. 1a).
3. Purify by single spore isolation and store either on microtiter plate with PDA at −80°C or in eppendorf tubes (containing 500 µL PDA) at 4°C.

4. Notes

1. In *Fusarium* strains, we observed an influence of the nitrogen source in the minimal medium containing chlorate on the occurrence of *nia*− mutants among other nitrate non-utilizing mutants. For other fungal species, urea might have to be replaced by other nitrogen sources such as glutamine, asparagine, uric acid or proline. Because some fungi may exhibit a low sensitivity to chlorate, concentration might be increased up to 500 mM chlorate. Finally, the detection of chlorate-resistant as well as of *nia*− mutants is not always obvious. In these cases, mutants are isolated and identified using morphological differences as compared with the wild-type strain, such as the formation of sectors or colonies with more aerial mycelium or faster growth on the selection media.
2. Alternatively, tubes containing 10 mL MS each can be prepared. The digestion solution can then be slowly poured at the bottom of the tube as it is denser than the MS itself.
3. We usually bend the Pasteur pipette in a flame. Alternatively a 1 mL tip can be used.
4. When resuspended in an osmotic solution such as TMSC, protoplasts can be kept on ice for at least 2 h.
5. Proper drying of the plates under a flow hood prevents condensation and will ensure a correct adhesion of the Top agar layer on the solid PDAS bottom plate.
6. Hygromycin B concentration should be adjusted, depending on the sensitivity of the recipient strain. For *F. graminearum* strains, we usually use hygromycin B at a final concentration of 50 µg/mL. For more sensitive strains, adding the antibiotic directly into the bottom plates might be too vigorous. Another selection method consists in pouring the transformed protoplasts into MMS Top agar on PDAS plates and incubate

the plates without any selection pressure for 16–24 h. This step will allow protoplast regeneration. Only after this incubation time, a second MMS Top agar containing the antibiotic at the adequate concentration is then poured onto the first one, allowing the selection of transformants.

7. In order to increase the proportion of cotransformants carrying a single-copy of the tagging element, the amount of plasmid DNA used in the transformation experiment (particularly the one carrying the tagging element) might be lowered to 2 µg.
8. Two criteria, necessary but not sufficient, are used to estimate the quality of excision events. First, aerial colonies should appear in the first 3 weeks; later events might be enriched in spontaneous mutations corresponding to the reversion of the endogenous *nia* mutation. Second, appearance of colonies should be progressive along the incubation period, which indicates that events are independent.
9. In the case of integration of both constructs at the same genomic locus, we observed two categories of bias: (1) aberrant transposition events resulting from excision of the tagging element together with genomic sequences (16) or (2) transposition of the tagging element at proximal sites, mainly in the *A. nidulans niaD* promoter sequences.
10. Alternatively, flanking sequences can be recovered using inverse PCR (12).

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