Fungal Genetics and Biology 45 (2008) 1552–1561

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Transposon-tagging identifies novel pathogenicity genes in *Fusarium graminearum*

Marie Dufresne [a,*], Theo van der Lee [b], Sarrah Ben M'Barek [a,b], Xiude Xu [b,c], Xu Zhang [b,d], Taiguo Liu [a,e], Cees Waalwijk [a], Wenwei Zhang [b,e], Gert H.J. Kema [b], Marie-Josée Daboussi [a]

[a] Univ. Paris-Sud 11, CNRS, UMR8621, Institut de Génétique et Microbiologie, 91405 Orsay, France
[b] Plant Research International B.V., P.O. Box 16, 6500 AA, Wageningen, The Netherlands
[c] Plant Protection Institute, Liaoning Academy of Agricultural Sciences, Shenyang 110161, China
[d] Institute of Genetics, Jiangsu Academy of Agricultural Sciences, Zhongling Street 50, Nanjing, Jiangsu 210014, China
[e] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agriculture Sciences, Beijing 100094, China

ARTICLE INFO

Article history:
Received 16 April 2008
Accepted 3 September 2008
Available online 23 September 2008

Keywords:
Transposon mutagenesis
Fusarium graminearum
Functional screening
Pathogenicity
Sexual development

ABSTRACT

With the increase of sequenced fungal genomes, high-throughput methods for functional analyses of genes are needed. We assessed the potential of a new transposon mutagenesis tool deploying a *Fusarium oxysporum* miniature inverted-repeat transposable element *mimp1*, mobilized by the transposase of *impala*, a *Tc1*-like transposon, to obtain knock-out mutants in *Fusarium graminearum*. We localized 91 *mimp1* insertions which showed good distribution over the entire genome. The main exception was a major hotspot on chromosome 2 where independent insertions occurred at exactly the same nucleotide position. Furthermore insertions in promoter regions were over-represented. Screening 331 mutants for sexual development, radial growth and pathogenicity on wheat resulted in 19 mutants (5.7%) with altered phenotypes. Complementation with the original gene restored the wild-type phenotype in two selected mutants demonstrating the high tagging efficiency. This is the first report of a MITE transposon tagging system as an efficient mutagenesis tool in *F. graminearum*.

1. Introduction

In the last few years the number of sequenced fungal genomes has increased tremendously (Soanes et al., 2007). A feature shared by all these organisms is the large number of genes for which no function is known and that cannot even be postulated due to lack of similarity with functionally characterized genes. Therefore, one of the main challenges in the post-genomic era is to establish a link between the several thousands of genes predicted through sequencing of these genomes and biological functions. In haploid filamentous fungi, several strategies have been developed since the late 1990s. These include targeted disruption or gene replacement as well as random integration of circular or linear plasmids or T-DNA in fungal genomes (Weld et al., 2006). Using random disruption strategies, several mutants with altered pathogenicity or distorted development were obtained in a variety of fungal species. However, the number of tagged mutants among all mutants displaying an altered phenotype in these experiments was low (Sweigard et al., 1998; Balhadère et al., 1999; Seong et al., 2006; Blaise et al., 2007). This poor tagging efficiency hampers the large-scale functional screening of mutants. These untagged mutants may arise from large deletions and/or chromosomal rearrangements that are known to occur during transformation with heterologous DNA (Sweigard et al., 1998; Mullins et al., 2001; Choi et al., 2007). Imperfect repair after enzyme treatment during the REMI (restriction enzyme-mediated integration) procedure (Sweigard et al., 1998; Balhadère et al., 1999) may lead to mutants that are not tagged. Other bottlenecks in random disruption are caused by the lack of transformation efficiency of protoplasts that varies strongly among fungi and difficulties in recovering sequences flanking the DNA insertion site due to complex integration patterns as well as the occurrence of multiple insertions (Blaise et al., 2007; Choi et al., 2007). The use of transposons as tools for insertional mutagenesis offers several advantages. Transposon mutagenesis does not need efficient protoplast regeneration and DNA transposons often insert as a single copy at the new location without major genomic rearrangements, which facilitates the recovery of genomic regions flanking the new insertion site (Spradling et al., 1999; Maes et al., 1999). Among the DNA transposons isolated from the phytopathogenic fungus *Fusarium oxysporum*, *impala*, a *Tc1*-like element has already been demonstrated to transpose in several phylogenetically distant species (Daboussi and Capy, 2003). In some of them, *impala*-based systems were successfully used to tag genes of interest: a novel pathogenicity gene in *Magnaporthe grisea* (Villalba et al., 2001) and several genes essential for fungal growth in *Aspergillus fumigatus* (Firon et al., 2003). However, *impala* reinsertion frequency was shown to be low (50–75%) hampering its use as a high-throughput gene-tagging system (Migheli et al., 2000, Hua-Van et al., 2001).

* Corresponding author. Fax: +33 1 69 15 70 06.
E-mail address: marie.dufresne@igmors.u-psud.fr (M. Dufresne).

1087-1845/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2008.09.004

More recently, we demonstrated the ability of the *impala* transposase (under the control of a constitutive promoter) to transactivate *mimp1* which shows features of MITEs (Hua-Van et al., 2000). Mobilisation of *mimp1* has been demonstrated in *F. oxysporum* as well as in *Fusarium graminearum*, where it reinserted at a very high frequency (from 83% to 91% depending on the set of revertants tested), preferentially close to genes (Dufresne et al., 2007). Hence, the efficiency of tagging genes is enhanced and associated phenotypic changes are detected in functional screens.

In the present study, we applied this mutagenesis strategy in *F. graminearum* Schwabe (teleomorph *Gibberella zeae*), a ubiquitous fungal pathogen of cereal crops, including wheat, barley and maize. It is the prevalent causal agent of Fusarium Head Blight (FHB) in regions with a temperate climate. FHB is of particular concern because it causes significant losses of yield and seed quality (McMullen et al., 1997). In addition, infection is often associated with the production of mycotoxins like deoxynivalenol, nivalenol and zearalenone that are harmful for human and animal health, rendering the produce unfit for consumption (Bennet and Klish, 2003).

Despite a substantial progress in the field on genomics, and the recent sequencing of the genome of *F. graminearum* (Cuomo et al., 2007) only few pathogenicity determinants are known in *F. graminearum* and major questions regarding which genes are involved in the infection process remain unsolved. In this study, we generated and analyzed a collection of mutants obtained through transposition of the MITE element *mimp1*. The mutagenic potential of *mimp1* has been estimated by screening the collection for radial growth on a set of media, development of perithecia, and pathogenicity on wheat heads. The tools and technologies described in this study will provide a valuable resource to accelerate research on gene function, a major challenge in the post-genomic era.

2. Materials and methods

2.1. Fungal strains and culture conditions

Fusarium graminearum strain *Fg*820 and derivatives were maintained on PDA plates. To obtain spores either for single-spore purification or for mycelium production on cellophane disks, strains were inoculated on liquid mung bean medium and incubated with moderate shaking (120–150 rpm) at 25–28 °C for 2 to 5 days. The mung bean medium was prepared by boiling 40 g of mung beans in one liter of distilled water until the first peels became detached. The filtrate was subsequently autoclaved for 20 min at 120 °C. To test for the stability of *mimp1* reinsertions, spores from three revertants, 112, 154 and 237, were obtained in liquid mung bean medium and subsequently plated onto PDA plates covered with cellophane disks (10^6 spores per plate). Plates were then incubated at 26 °C for 16 h until mycelium recovery. Alternatively, to test for the stability of *mimp1* insertions under stress conditions, plates were UV-treated (50 mJ mm^{-2} s^{-1}) then incubated at 26 °C for 24 h. Three replicates were done for each treatment and each strain. Mycelia were then recovered, frozen into liquid nitrogen and kept at −80 °C until genomic DNA extraction.

2.2. Plasmids

Plasmid pHEO62 contains an ORF encoding the *impala* transposase (Hua-Van et al., 1998), cloned between the gpdA promoter and the trpC terminator of *Aspergillus nidulans*. It also carries a hygromycin resistance gene (hph) from *Escherichia coli* (Carroll et al., 1994). pNm1H18, also referred to as the *niaD*::*mimp1* construct, carries a *mimp1* element located in the first intron of the *A. nidulans niaD* gene (Dufresne et al., 2007). Vectors for functional complementation of revertants 112 and 237 were constructed according to the following strategy. First, a wild-type genomic fragment carrying the entire ORF (FGSG_10057, for mutant 112 and FGSG_01974 for mutant 237) and large 5′ and 3′ regions was amplified using genomic DNA of the *F. graminearum* strain PH-1 (NRRL31084) and primer pairs 112COMPF/112COMPR and 237COMPF/237COMPR, respectively, with the Expand High Fidelity Taq polymerase (Roche Diagnostics, Meylan, France), according to the instructions of the manufacturer. Sizes of the whole fragment, of 5′- and 3′-regions as well as primer sequences are indicated for each amplified genomic region in Supplementary Table 1. The PCR product was subsequently cloned into pGEM-T vector (Promega, Charbonnières-les-Bains, France). The resulting plasmid was linearized with SpeI and ligated with a 2.1-kb XbaI restriction fragment from plasmid pSm334 carrying a cassette conferring geneticin resistance (Marek et al., 1989). The resulting constructs used for functional complementation of revertants 112 and 237 were named pCOMP112 and pCOMP237, respectively.

2.3. Transformation experiments and revertant selection

The transformation procedure was as described before (Hua-Van et al., 2001). When geneticin was used as a selection marker, it was directly incorporated into the bottom plates at a final concentration of 100 mg/l.

To obtain revertants, plugs of each original transformant (or "donor line") were placed on plates of nitrate minimal agar medium as described before (Dufresne et al., 2007). Revertants were detected as patches of aerial mycelium with a wild-type phenotype on a background of sparse mycelium corresponding to a *niaD* mutant. The high excision frequency observed for transformant 6/11 forced us to develop a methodology to ensure that revertants resulted from independent excision events. Revertants were retrieved from single wells of a 96-well plate containing nitrate minimal agar medium, previously inoculated by a single germinating spore per well.

2.4. DNA preparation and Southern blot analysis

DNA extractions from *F. graminearum* strains were conducted as previously described (Dufresne et al., 2007). For Southern blot analyses, ten micrograms of genomic DNA were digested with the appropriate restriction enzyme, separated by electrophoresis on 0.7% agarose gels and transferred on nylon membranes, using a vacuum blotter. DNA templates were ^{32}P-labeled using the rediprime™II kit (Amersham Biosciences). Hybridizations were conducted under standard conditions (Sambrook et al., 1989).

2.5. Polymerase chain reaction and primer sequences

Excision of *mimp1* was checked by PCR using 50 ng of genomic DNA from single-spore cultures of putative revertants and using primers *niaD*144 and *niaD*754r as described before (Dufresne et al., 2007). The sizes of the expected PCR products are 717 bp strains carrying the *niaD* gene disrupted by the *mimp1* element while for the wild-type *niaD* gene a 485 bp PCR fragment is expected. The *mimp1* and *niaD* fragments used as probes in molecular hybridization experiments were obtained by PCR as described previously (Dufresne et al., 2007).

Re-insertions of *mimp1* in purified revertants were assessed using *mimp1*-specific primers: SPE*mimp1*-5′ (5′-CAATAAGTTTGAATACCGGGCGTG-3′) and SPE*mimp1*-3′ (5′-GTTTGAATACCTTTTGATTTG-3′), both overlapping TIRs (italics) and internal sequences (underlined). A full-length *mimp1* element results in a PCR product of 197 bp (Hua-Van et al., 2000). PCR conditions were the following: a denaturation step of 5 min at 95 °C followed by 35 cycles of 1 min 94 °C, 1 min 53 °C, 30 s 72 °C and a last elongation step of 10 min at 72 °C.

Potential excision of *mimp1* from reinsertion sites was assessed in revertants 112, 154 and 237 using primers surrounding the target TA dinucleotide in these strains (Supplementary Table 1). PCR conditions were as follows: a denaturation step of 5 min at 95 °C followed by 45 cycles of 1 min 94 °C, 1 min 60 °C, 1 min 72 °C and a final elongation step of 10 min at 72 °C. The expected sizes of the amplicons with or without the *mimp1* element are indicated in Supplementary Table 1.

PCR analysis of geneticin-resistant transformants obtained from functional complementation experiments was done with primers annealing on both sides of the *mimp1* TA insertion site in the corresponding revertant. Amplicons with different fragment sizes allowed us to distinguish between the mutant allele, carrying *mimp1*, and the wild-type allele (Supplementary Table 1). In all cases, PCR conditions were as follows: a denaturation step of 5 min at 95 °C followed by 35 cycles of 1 min 94 °C, 1 min 60 °C, 1 min 72 °C and a final elongation step of 10 min at 72 °C.

2.6. Amplification of mimp1 flanking sequences

mimp1 flanking sequences were recovered using a modified TAIL-PCR or by an inverse PCR strategy using the HaeIII restriction enzyme, both described previously (Dufresne et al., 2007).

2.7. Cloning of PCR products and DNA sequencing

PCR products were directly cloned into the pGEM-T vector (Promega, Charbonnières-les-Bains, France) using 3 μl of either rough or purified PCR products, following the manufacturer's instructions. Sequencing of PCR products either directly or cloned into the pGEM-T vector, was performed by Genome Express (Meylan, France) using an ABI Big Dye Terminator kit (Perkin Elmer) and the appropriate primer(s).

2.8. Computational analyses

Searches for matches of nucleotide sequences in the Fusarium Comparative Database (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) used the BLASTN algorithm (Altschul et al., 1997). The occurrence of TA dinucleotides in the *F. graminearum* chromosomal sequences was determined using the compseq algorithm in the Emboss package (http://emboss.bioinformatics.nl/). A set of 500 TA dinucleotides were selected randomly in the genome as a reference sample. Their location relative to predicted ORFs in the *F. graminearum* genome sequence was automatically determined using an Excel based tool (available upon request). For the determination of the consensus sequence of *mimp1* insertions, 5 bp upstream and downstream of the TA target site were analyzed with the SeqLogo program (Schneider and Stephens, 1990). The "oligoanalysis" algorithm (http://rsat.scmbb.ulb.ac.be/rsat/) in the Regulatory Sequence Analysis Tools interface was used to search for enrichment of particular trinucleotides in the insertion site sequences. The potential to form secondary structures was determined using RNAfold available in the Pasteur Institute website (http://bioweb.pasteur.fr/seqanal/interfaces/rnafold-simple.html).

2.9. Perithecial development

Perithecium induction was performed by plating a 2.5% aqueous solution of Tween 60 (Sigma, St. Quentin Falladier, France) onto the surface of 7-day-old mycelia grown on carrot agar at 24 °C under continuous white and black fluorescent lighting (Klittich and Leslie, 1988; Trail et al., 2003). Plates were incubated for 4 to 5 weeks and weekly examined for perithecium formation. Mutants were compared to the reference strains *Fg*820 *nia1*, *nia5* and *nia6*.

2.10. Pathogenicity assays

Inoculation was performed by placing approximately 500 spores (5 μl of a 10^5 spores/ml suspension) into the floral cavity of the third, eight and thirteenth maturing spikelets starting from the stembase. Initial screenings were performed on cultivars Lavett grown in the field. Retesting of candidates and complemented strains were done on the cultivar Thasos grown in the greenhouse. All plants were inoculated in growth stage 61 to 65 (Zadocs Scale). Wheat heads were detached from the stem just prior to inoculation and after inoculation the wheat heads were maintained in plastic containers. The first 24 h inoculated heads were kept in the dark and subsequently they were incubated with a photoperiod of 16 light and 8 h darkness at 20 °C. After 48 to 72 h the inoculated heads were air dried for 30 min every day to limit mycelium growth on the outer surface of the glume. The virulence of the mutants was scored 6 or 7 days after inoculation by recording the visual symptoms. No visual symptoms: score 0; only the inoculated spikelet shows browning or bleaching: score 1; the inoculated spikelet as well as 1–2 adjacent spikelets show browning or bleaching: score 2; the inoculated spikelet as well as 3 or more adjacent spikelets show browning or bleaching: score 3. For each mutant the average score of nine inoculated spikelets was determined. Mutants with an average score less than 2 were classified as strongly reduced, mutants with an average score between 2 and 2.7 were classified as possibly reduced, mutants with an average score of 2.7 or more were considered similar to the wild-type strain.

2.11. Radial growth tests

Isolates were grown on five different media. These included potato dextrose agar (PDA), as a reference and water agar (WA) to mimic extreme poor nutrient conditions. Strains were also grown on agar supplemented with mung bean medium (MB) which is shown to induce sporulation. Finally, we applied nutrient stress by growing the isolates on nutrient poor medium (SNA, Nirenberg, 1976) or SNA with 0.9 M NaCl (SNA-5) to resemble an osmotic stress. Spores were obtained from cultures grown in MB (5 days at 25 °C 180 rpm), diluted to 10^5 spores/mL and 500 spores were spotted on 20 × 20 cm plates with any of these five growth media. Plates were incubated at 15 and 25 °C and growth was compared by including the wild-type *Fg*820 strain on each plate. Germination and outgrowth into subsequent colonies was monitored on days 1, 2, 3, 4 and 7 by marking the colony edge and photographic recordings from above as well as the reverse side.

3. Results

3.1. Generation of a collection of revertants

Previously, we reported on the generation of transformants of *F. graminearum* (*Fg*820) carrying the *mimp1* element inserted into the A. nidulans *niaD* gene as well as the *impala* transposase (Fig. 1A, Dufresne et al., 2007). In total 33 of such transformants showed transposition of the *mimp1* element as displayed by the occurrence of Nia^+ colonies, named revertants, when grown on minimal medium agar plates.

To develop a collection of transposon mutants for further functional genetics studies, we first selected transformants carrying a single copy of the *niaD*::*mimp1* construct. This selection was performed by PCR amplification on Nia^+ colonies. As shown in Fig. 1B, the size of the PCR products indicated the presence or absence of *mimp1* at the original *niaD* site. A 493-bp PCR product corresponding to the size of the wild-type *niaD* gene (485 bp) plus the typical 8-bp excision footprints left by the element (lanes 2, 4 and

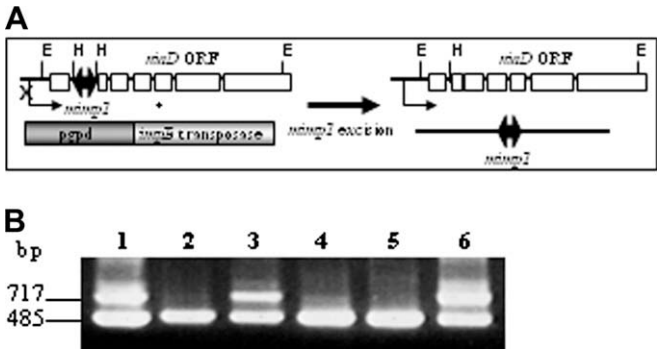

Fig. 1. (A) Scheme of the rationale of *mimp1* mobilization by the *impala* transposase. (B) PCR products obtained using primers *niaD*144 and *niaD*754r on genomic DNA of purified revertants. Each revertant is derived from a different transformant. Numbers indicated on the left correspond to sizes in base pairs expected for a *niaD* site either empty (493 bp) or still carrying *mimp1* (717 bp). Lane 1, revertant 1/3.1; lane 2, revertant 1/2.1; lane 3, 1/8.1; lane 4, 6/11.1; lane 5, 5/19.1; lane 6, 5/7.1.

5) indicates excision of *mimp1* element from the *niaD* gene in transformants and these revertants are supposed to contain a single *niaD*::*mimp1* copy. Besides this 493 bp fragment, in other revertants (Fig. 1B, lanes 1, 3 and 6 an additional fragment of 717 bp is found, that corresponds to a *niaD* locus still carrying the *mimp1* element (717 = 493 + 224). These revertants also resulted from an excision event but additional copies of the *mimp1*-interrupted *niaD* construct were present in the original transformant. The analysis of the whole set of 33 transformants that were able to revert to a Nia^+-phenotype, twelve gave rise to a single 493 bp PCR fragment and hence carried a single copy of the *niaD*::*mimp1* construct. Eight of these showed high excision frequencies (15 to 20 Nia^+ colonies per plate) and these transformants, referred to as donor lines were selected to generate a collection of revertants (Table 1).

To evaluate the *mimp1* reinsertion frequency, PCR analysis using *mimp1*-specific primers was conducted on 24 revertants (three revertant for each of the eight donor lines). In 23 of the 24 (95.8%) revertants, a *mimp1* element had re-inserted at a new location, as was detected by the presence of a 197 bp-PCR product using *mimp1*-specific primers (data not shown). This high re-insertion frequency allowed direct phenotypic analysis of the revertants without pre-selection of mutants in which the *mimp1* element reinserted.

3.2. Insertion preference of the mimp1 element

Identification of the insertion sites of the *mimp1* element in the *F. graminearum* genome was conducted by TAIL-PCR or inverse

Table 1
niaD$^+$ revertants obtained after *mimp1* excision

Initial transformant	Number of revertants
1/6[a]	43
½[a]	24
1/12[a]	16
1/22[a]	11
1/23[a]	1
1/24[a]	49
5/19[b]	1
6/4[c]	12
6/11[c]	176
6/23[c]	10

[a] Transformants obtained from the recipient strain *Fg*820 *nia1*.
[b] Transformant obtained from the recipient strain *Fg*820 *nia5*.
[c] Transformants obtained from the recipient strain *Fg*820 *nia6*.

PCR. Subsequent BLASTN analysis (Altschul et al., 1997) was used to place re-insertion sites on the *F. graminearum* genome (release 3 of the *F. graminearum* assembled and annotated genome available in the Fusarium Comparative Database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html). Four Nia^+ strains out of the 95 analysed still showed the *mimp1* copy into the *A. nidulans niaD* intron and were consequently excluded from further analysis. Fig. 2 shows the distribution of *mimp1* re-insertions over the four chromosomes of the *F. graminearum* genome. Most re-insertions were unique however, over the 91 insertion sites analyzed, 7 were hit more than once. Among a total of 66 independent genomic loci, 59 sites were inserted once, five sites were inserted twice, one site was inserted three times and one site was inserted 19 times. The most striking example of frequently hit genomic sites is the one on chromosome 2, where the *mimp1* element inserted at the same TA dinucleotide (at position 977,305) 19 times. Insertions in this hotspot were found in revertants originating from four different donor lines (Supplementary Table 2) demonstrating the independence of the transposon insertion events. A Chi-square analysis indicated that the distribution is non-uniform because of the distortion causes by chromosomes 2 and 3 ($P \leqslant 0.05$). Indeed, while insertions on chromosome 1 and 4 are as frequent as expected, fewer insertions were found on chromosome 3, while higher on chromosome 2 due to the hotspot of insertion. The distribution of TA dinucleotides, a prerequisite for *mimp1* insertion, does not explain this preference (Table 2). To determine whether *mimp1* has a preference for re-insertion at particular nucleotide sequences, we examined insertions sequences flanking the TA target sites. Apart from the TA insertion site already mentioned, no consensus sequence could be identified (Supplementary Fig. 1). Moreover, a search for a bias

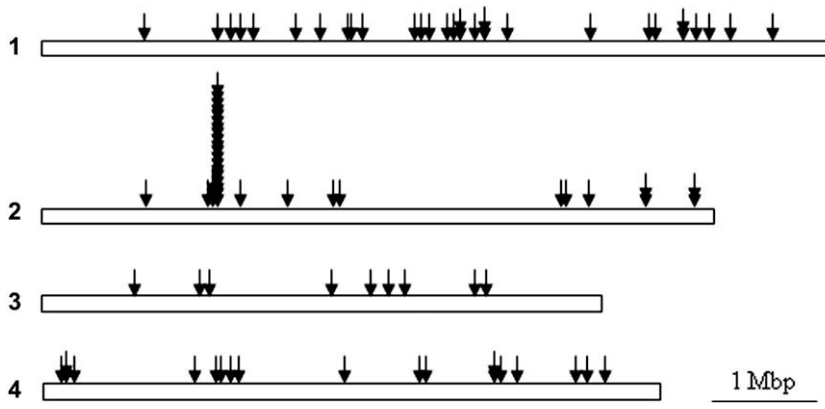

Fig. 2. Distribution of the 91 *mimp1* insertions on the four *F. graminearum* chromosomes. Genomic loci hit by *mimp1* insertions are indicated by arrows. Note that some loci were hit more than once: two doublets on chromosome 1, one doublet, one triplet and a major hotspot (19 insertions) on chromosome 2, and two doublets on chromosome 4.

Table 2
Observed and expected numbers of *mimp1* insertions on the four *F. graminearum* chromosomes

	Chromosome				Total
	1	2	3	4	
TA targets	543,481	431,023	360,659	372,165	1,707,328
Observed insertions	30	32[a]	9[a]	20	91
Expected insertions	29	23	19	20	91

The calculations were made assuming all TA dinucleotides are equally accessible.
[a] χ^2-test, $P \leqslant 0.05$

of particular trinucleotides did not reveal any preference (data not shown). Testing the potential of sequences at *mimp1* insertions to form stable secondary structures did not give significant results either.

Of the 66 characterized insertions in the *F. graminearum* genome, half (33/66) were found to be within or close to (500 bp upstream or 500 bp downstream) predicted genes (Table 3). When comparing with a reference sample consisting in 500 randomly selected TA dinucleotides, significant differences were observed (Table 3). As determined using a Chi-square analysis ($P \leqslant 0.05$), *mimp1* target sites were found much less frequently into ORFs whereas they were over-represented in close or more distal 5′-regions of predicted genes (Table 3).

3.3. Stability of the mimp1 element at reinsertion loci

In the *mimp1*/*impala* double component system, the source of *impala* transposase is constitutively expressed through the control of the promoter region of the *A. nidulans* GPDA gene. In order to test for the stability of the *mimp1* element in revertants, once reinserted at new genomic loci, we developed a PCR method allowing the detection of excision events. Revertants 112, 154 and 237 were selected for these experiments and grown either under standard in vitro conditions on PDA or UV-treated (see Section 2), a stressful condition already described to induce transposition of some eukaryotic transposable elements (Daboussi and Capy, 2003; Bouvet et al., 2008). Only PCR products corresponding for the reinsertion sites carrying the *mimp1* element could be detected for the three strains, in the two growth conditions tested (data not shown). These results demonstrated that, at least under these conditions and for the three strains tested, *mimp1* reinsertions are stable and do not give rise to re-excision events.

3.4. Screening for mutants with alterations in the development of perithecia

In order to estimate the mutagenic potential of the *mimp1*/*impala* double component system, we first assessed 331 revertants for their capacity to produce perithecia. *F. graminearum* is a homothallic fungus and sexual reproduction can be performed routinely under laboratory conditions (Klittich and Leslie, 1988; Trail et al., 2003). A primary screening identified 22 mutants that were disabled in perithecium production as compared with reference strains *Fg*820 *nia1*, *nia5* and *nia6* (Fig. 3A). In a secondary screen performed on these 22 potential mutant strains, 11 were shown to retain their mutant phenotypes (Table 4). These strains fell into three categories: three mutants were unable to produce any perithecia (Fig. 3B), four produced few perithecia of reduced size (Fig. 3C) and four produced perithecial initials (Fig. 3D) but these rarely emerged from the medium even after a prolonged incubation time (4–5 weeks).

3.5. Screening for pathogenicity on wheat heads

We screened the same set of 331 revertants for differences in virulence in 6 batches and all batches showed successful infection of wheat heads. Six or seven days after inoculation, the wild-type strain *Fg*820 and most of the mutants showed infection of the inoculated spikelet as well as one or two adjacent spikelets above and beneath the inoculated spikelet. However, 26 mutants showed a reduced virulence phenotype as judged on the spread of the infection from the inoculated spikelet. Inoculation with these mutants only showed visual symptoms on the inoculated spikelet and even this inoculated spikelet did not show the characteristic blight observed upon inoculations with the wild-type strain (Supplementary Fig. 2). Twenty-five candidate mutants were retested and eleven (44%) showed again clear reduced symptoms (only the inoculated spikelet showed visual symptoms or even the inoculated spikelet did not show the characteristic blight or browning, see Table 4), eight (32%) showed spread to other spikelets but its speed seemed reduced and six (24%) showed symptoms similar to the phenotype of wild-type isolate *Fg*820.

3.6. Screening for radial growth

We applied a range of growth conditions to expose the mutants to a wide variety of stresses which are likely to induce different sets of genes. Among the more than 300 *mimp1*-transposon insertion mutants tested, revertant 112 was the only one which showed substantial growth reduction (Fig. 4B, and Table 4). This retardation was evident on all media tested and both at 15 °C as well as at 25 °C.

3.7. Molecular analysis of the identified mutants

All 19 identified mutant strains but one were shown to correspond to a "cut-and' paste" event of the *mimp1* element (excision followed by reinsertion). The mutant strain 39 was shown not to carry *mimp1* anymore, although resulting from excision of the element from the *niaD* donor site (data not shown). *mimp1* flanking

Table 3
Observed numbers of *mimp1* insertions towards predicted ORFs as compared with a set of TA dinucleotides randomly selected in the *F. graminearum* genome sequence

	5′ side			ORF	3′ side		
	>1000	500–1000	0–500		0–500	500–1000	>1000
mimp1 insertions[a]	13.5[c]	16.7[c]	28.8[c]	15.2[c]	6.1[c]	4.5	15.2[c]
Random TAs[b]	6.2	6.6	18.2	46.6	14.2	5	3.2

The calculations were made assuming all TA dinucleotides are equally accessible.
To determine the distance of *mimp1* reinsertion sites or random TAs to genes, the more recent annotated version of the *F. graminearum* genome available in the *Fusarium* Comparative database (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) was used. Location is either in an ORF, in 5′- or 3′-regions, indicated as 5′ or 3′ with the distance relative to the start codon or stop codon, respectively, in bp.
[a] Percentages determined from original values on a set of 66 *mimp1* reinsertion sites and of 500 randomly selected TAs for the reference sample.
[b] Percentages determined from original values on a set of 66 mimp1 reinsertion sites and of 500 randomly selected TAs for the reference sample.
[c] Values significantly different from the reference sample as determined using a χ^2-test ($P \leqslant 0.05$).

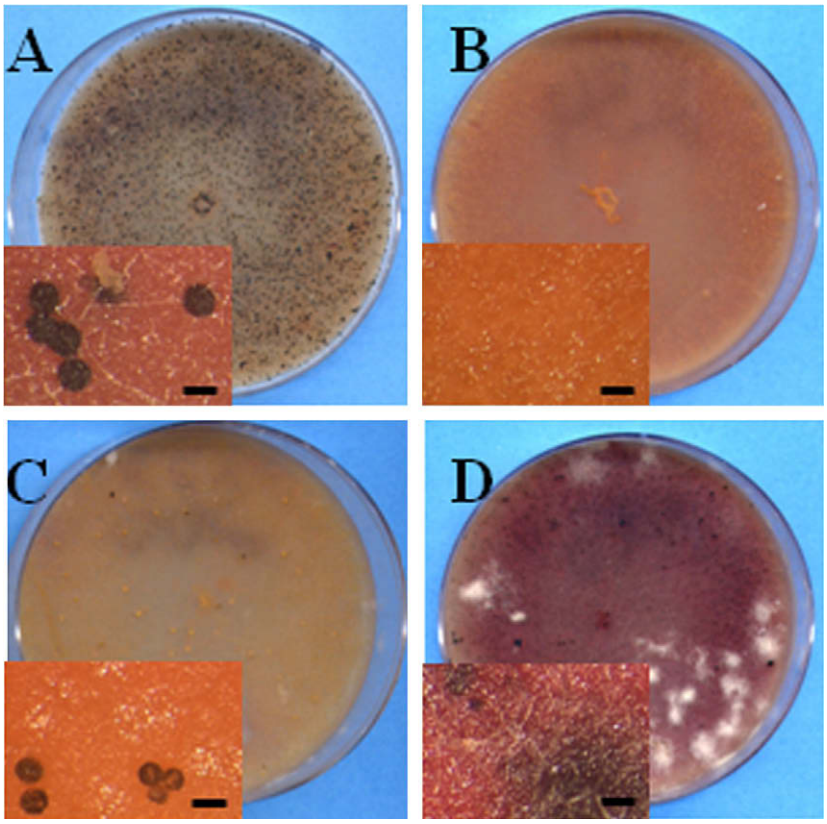

Fig. 3. Functional complementation experiments conducted on revertant 112. (A) Southern blot analysis of a set of potential functional complemented revertants obtained from revertant 112. Genomic DNA was digested by the MscI restriction enzyme, electrophoretically separated on an agarose gel (0.5× TAE, 0.7% agarose), transferred onto a Nylon membrane and probed using the radiolabeled 443-bp PCR product obtained using the 112F/112R primers pair (Supplementary Table 1). All the complemented strains exhibit the expected 1.5-kbp hybridization signal corresponding to the wild-type allele brought by transformation. Three out of the four complemented revertants (112C5, 112C6 and 112C8) analyzed also show a second hybridizing band (1.7 kbp), also observed for revertant 112, corresponding to the mutant allele. Strain 112C4 only carries the wild-type allele, indicating that it results from a replacement event. (B) Compared in vitro growth of the wild-type strain *Fg*820, revertant 112 and a transformant (112C8) resulting from the functional complementation experiment. Colonies correspond to an 8-day growth onto PDA plates. (C) Restoration of pathogenicity on detached wheat leaves following functional complementation. Detached wheat heads of cultivar Thasos were spot inoculated at anthesis. Next to the first inoculated spikelet (indicated by the arrows) every fifth spikelet is inoculated. In the mutant line 112 only the inoculated spikelets show the characteristic symptoms of bleaching while in the complemented line 112C8 infection spreads similar to the wild-type (left panels: whole spikes, right panels: cross sections).

Table 4
Phenotypes and genes affected in *mimp1*mutant strains

Revertant	Perithecia development	Radial growth	Pathogenicity[a]	Closest predicted ORF[b]	Predicted function[c]
5	WT	WT	PathII	FGSG_00416 (5′-670)	CHP (Major Facilitator Superfamily)
38	Perithecium initials in agar	WT	WT	FGSG_04510 (ORF)	CHP (Common central domain of tyrosinase)
39	No perithecia	WT	PathI	No *mimp1* reinsertion	
41	Few perithecia developed	WT	WT	FGSG_09759 (3′-3102)	CHP (Cation efflux family)
71	WT	WT	PathII	FGSG_02549 (5′-144)	CHP (Phosphoglycerate mutase family)
86	Perithecium initials in agar	WT	PathII	n.d.	n.d.
112	WT	Drastic reduction	PathII	FGSG_10057 (ORF)	CHP
118	WT	WT	PathI	n.d.	n.d.
147	Few perithecia developed	WT	WT	FGSG_08737 (3′-390)	CHP
154	WT	WT	PathII	FGSG_12753 (3′-1025)	Predicted protein
184	WT	WT	PathI	FGSG_02077 (5′-203)	HP (CFEM domain)
191	Perithecium initials in agar	WT	WT	FGSG_04610 (5′-313)	HP similar to maltose permease (Major Facilitator Superfamily)
198	Perithecium initials in agar	WT	WT	FGSG_09905 (5′-127)	Predicted protein
226	Few perithecia developed	WT	WT	n.d.	n.d.
234	No perithecia	WT	WT	FGSG_07062 (5′-865)	CHP (EXS family)
237	No perithecia	WT	PathII	FGSG_01974 (5′-73)	HP similar to HET-C2 (Glycolipid transfer protein)
266	Few perithecia developed	WT	WT	n.d.	n.d.
316	WT	WT	PathII	FGSG_12019 (5′-1729)	HP
317	WT	WT	PathII	n.d.	n.d.

WT, wild-type phenotype, determined as compared with the reference strain Fg820 and its *nia*-derivatives.

[a] Pathogenicity phenotypes defined as follows: PathI, average score between 0 and 1; PathII, average score between 1 and 2; WT, average score above 2 (see Section 2).

[b] The BlastN algorithm (Altschul et al., 1997) was used to search for significant matches in the *Fusarium* Comparative database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html). The closest predicted gene to the *mimp1* insertion site is indicated. *mimp1* location towards this gene is indicated in brackets, either in an ORF (ORF), in 5′- or 3′-regions (5′ or 3′, respectively) with the distance relative to the start codon or stop codon, respectively, in bp.

[c] Putative function of the predicted proteins as indicated in the *Fusarium* Comparative database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html). Specific protein domains are indicated in brackets. CHP: conserved hypothetical protein; HP: hypothetical protein.

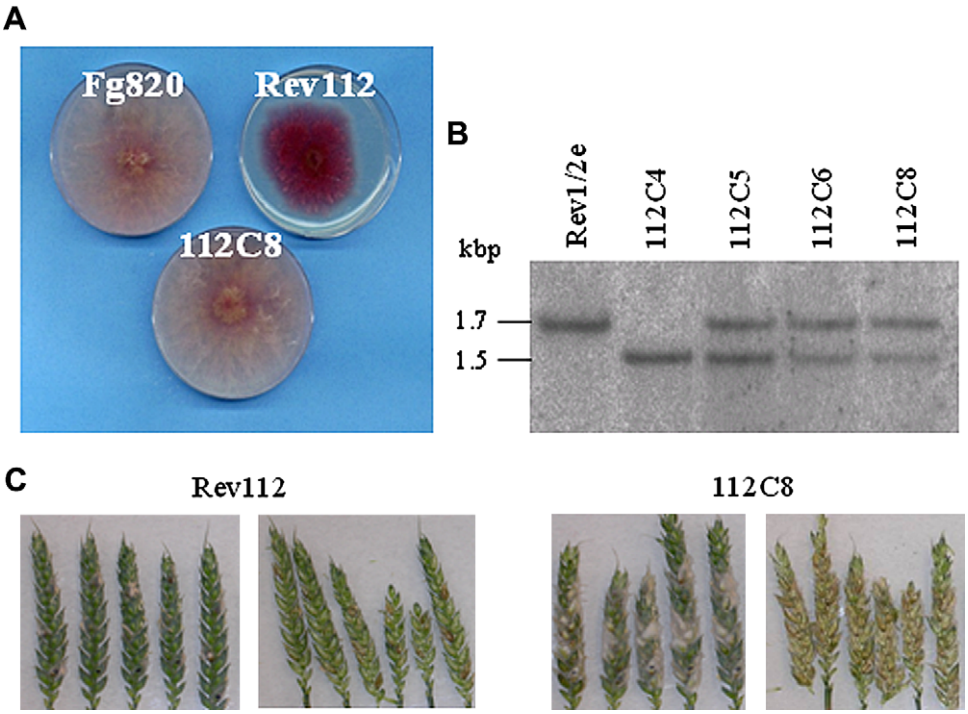

Fig. 4. Functional complementation experiments conducted on revertant 112. (A) Southern blot analysis of a set of potential functional complemented revertants obtained from revertant 112. Genomic DNA was digested by the MscI restriction enzyme, electrophoretically separated on an agarose gel (0.5X TAE, 0.7% agarose), transferred onto a Nylon membrane and probed using the radiolabeled 443-bp PCR product obtained using the 112F/112R primers pair. All the complemented strains exhibit the expected 1.5–kbp hybridization signal corresponding to the wild-type allele brought by transformation. Three out of the four complemented revertants (112C5, 112C6 and 112C8) analyzed also show a second hybridizing band (1.7 kbp), also observed for revertant 112, corresponding to the mutant allele. Strain 112C4 only carries the wild-type allele, indicating that it results from a replacement event. (B) Compared in vitro growth of the wild-type strain Fg820, revertant 112 and a transformant (112C8) resulting from the functional complementation experiment. Colonies correspond to an 8-day growth onto PDA plates. (C) Restoration 1 of pathogenicity on detached wheat leaves following functional complementation. Detached wheat heads of cultivar Thasos were spot inoculated at anthesis. Next to the first inoculated spikelet (indicated by the arrows) every fifth spikelet is inoculated. In the mutant line 112 only the inoculated spikelets show the characteristic symptoms of bleaching while in the complemented line 112C8 infection spreads similar to the wild type (left panels: whole spikes, right panels: cross sections).

sequences were recovered in 13 out of the 19 mutant strains. 61.6% (8/13) were found to be within or at less than 500 bp from predicted genes (Table 4): two were shown to result from *mimp1* reinsertion into a predicted ORF and six carried the element at less than 500 bp from a predicted ORF (five in the 5′ and one in the 3′-regions, respectively). Most of the genes potentially tagged by *mimp1* correspond to conserved hypothetical proteins encountered in other ascomycete species (see Supplementary Table 2).

3.8. Functional complementation

Two mutants, revertants 112 and 237, were chosen for their interesting phenotypic and/or molecular characteristics (Table 4) to assess the relationship between re-insertion of *mimp1*and their phenotypes. Revertant 112 exhibited drastic reduction in radial growth on PDA medium. At 15 °C the average increase in diameter 24 and 48 h was 0.42 cm compared to 1.0 cm for the wild-type and complemented strains ($P < 0.05$ based on a *T*-test). At 25 °C the average increase in diameter between 24 and 48 h of revertant 112 was 0.63 cm while the average the wild-type and complemented strains was 1.6 cm ($P < 0.05$ based on a *T*-test). Revertant 112 was only slightly altered in conidiospore production on mungbean medium (data not shown). Assays on wheat heads have shown that it was unable to spread beyond the initial infected spikelet (Fig. 4C). Molecular analysis of revertant 112 revealed that *mimp1* had inserted into an ORF encoding a putative transcription factor (FGSG_10057, Table 4), which shows characteristics typical for the GAL4_like fungal $Zn(II)_2Cys_6$ family of transcription factors (Supplementary Fig. 3). Revertant 237 showed a wild-type phenotype in vitro under standard conditions but did not develop any perithecia (Fig. 5B). This mutant was also unable to spread to adjacent spikelets after infection of the initial spikelet (Fig. 5C). Re-insertion of *mimp1* in this mutant occurred in the putative promoter region of an ortholog of the *Podospora anserina* hetc2 gene, (FGSG_01974, see Table 4 and Supplementary Fig. 4), a gene involved in vegetative incompatibility. Functional complementation experiments were conducted by transforming each of these revertants with a construct carrying both a large genomic region including the wild-type gene and flanking regions as well as a geneticin resistance cassette. Twenty-six and thirty-eight geneticin-resistant transformants were obtained for revertants 112 and 237, respectively. Among these, a set of 12 transformants of each revertant were further analyzed. Introduction of the wild-type allele into either revertant led to the restoration of mutant phenotypes, demonstrating that the *mimp1* insertions were responsible for the altered phenotypes in both revertants. All analyzed geneticin transformants of revertant 112 carried an additional intact copy of FGSG_10057 including flanking sequences, but transformant 112C4 in which the *mimp1* disrupted FGSG_10057 gene was replaced by an intact wild-type copy of the gene (Fig. 4A). In all cases growth rate on PDA plates and virulence towards wheat spikes were restored to wild-type (Figs. 4B and 4C). Similarly, functional complementation of revertant 237 was conducted. All five transformants analyzed were found to result from the replacement of the mutant allele by the intact wild-type copy of the gene (Fig. 5A) and restored both the development of perithecia (Fig. 5B) and wild-type virulence on wheat spikes (Fig. 5C).

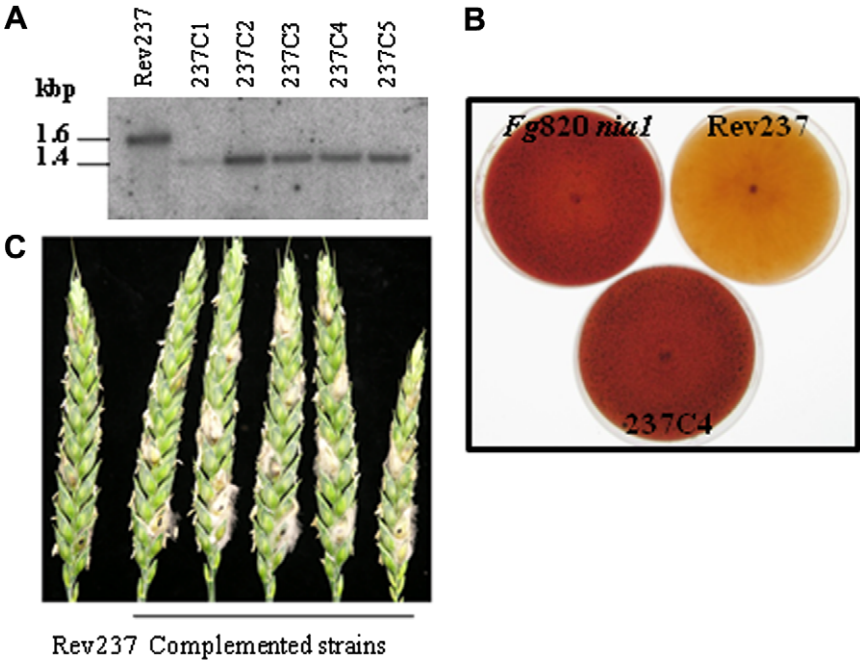

Fig. 5. Functional complementation experiments conducted on revertant 237. (A) Southern blot analysis of a set of potential functional complemented revertants obtained from revertant 112. Genomic DNA was digested by the HindIII restriction enzyme, electrophoretically separated on an agarose gel (0.5X TAE, 0.7% agarose), transferred onto a Nylon membrane and probed using the radiolabeled 443-bp PCR product obtained using the 237F/237R primers pair (see Supplementary Table 1). All the complemented strains exhibit a single 1.4–kbp hybridization signal corresponding to the wild-type allele brought by transformation, indicating that they result from a replacement event. (B) Restoration of perithecial development following functional complementation. Perithecium induction was performed by plating a 2.5% aqueous solution of Tween 60 onto the surface of 7-day-old mycelia grown on carrot agar at 24°C under continuous white and black fluorescent lighting (see Materials and Methods). Pictures were taken 10 days after perithecium induction. (C) Restoration of pathogenicity on detached wheat heads following functional complementation. Detached wheat heads of cultivar Thasos were spot inoculated at anthesis. Next to the first inoculated spikelet (indicated by a black dot) every fifth spikelet is inoculated. In the mutant line 237 only the inoculated spikelets show the characteristic symptoms of bleaching while in the five complemented lines infection spreads similar to the wild type.

4. Discussion

This study is the first report on the use of a MITE as an insertional mutagen for large-scale generation of mutants. Although mobilization was shown for some MITEs (Jiang et al., 2003; Kikuchi et al., 2003; Nakasaki et al., 2003; Macas et al., 2005; Menzel et al., 2006; Han and Korban, 2007), in few cases the source of transposase responsible for their transposition is known. The functional relationship between a MITE and a full-length DNA transposon has only been proven in *Arabidopsis thaliana* for the plant MITE *mPing* and two members of the *PIF/Harbinger* superfamily, Ping and Pong (Yang et al., 2007) and in *Fusarium* for *mimp1* and the related autonomous *Tc1*-like element *impala* (Dufresne et al., 2007). So far only *mimp1* was developed as a tagging tool.

In this paper, we demonstrated that the *mimp1*/*impala* system exhibits very interesting transposition characteristics. In *F. graminearum* transformants that carry a single copy of *mimp1*, the MITE was shown to excise as well as reinsert at high frequencies. These characteristics are of particular interest since nearly all revertants have the *mimp1* inserted into a new location. Consequently this system allows the rapid generation of large numbers of revertants without the laborious pre-selection using molecular analyses for mutants typical for other tagging systems. We also demonstrated that, in spite of the constitutive source of transposase, the reinserted MITE copies were stable under the in vitro conditions tested which included a strong UV treatment that is known to induce transposition (Daboussi and Capy, 2003; Bouvet et al., 2008) and therefore could represent a critical test. Although instability might be observed under specific conditions, we considered that the observed stability was suitable for more detailed studies of genes tagged by a *mimp1* insertion.

A critical aspect in transposon-mediated global mutagenesis is the ability of the transposon to hit most of the genome with a reasonable frequency. To assay this point and evaluate insertional preferences of *mimp1*, we performed molecular analysis of *mimp1* flanking sequences in 95 randomly chosen revertants. Among these, 91 were shown to correspond to transposition events and used to locate *mimp1* re-insertion sites. *mimp1* was shown to insert into all four *F. graminearum* chromosomes without any preference towards a particular nucleotide sequence adjacent to the TA dinucleotide target site. A similar behaviour has already been reported for Minos of *Drosophila melanogaster*, another member of the *Tc1*/mariner superfamily, for which insertions depend weakly, if at all, on sequences apart from the TA insertion site (Metaxakis et al., 2005). However, our analysis also revealed the existence of seven genomic loci hit more than once by the transposon: five doublets, one triplet and a hotspot of insertion located on chromosome 2. In six out of these seven cases, such re-insertions loci were identified in revertants originating from different donor lines, ensuring the independence of the re-insertion events and indicating that the preferential insertion into these loci is not likely to be caused by the original location of the element. Insertional bias of transposons has already been described. The extensive use of the *P* element as a tagging tool in *D. melanogaster* has enlightened a high insertion preference for specific euchromatic regions (Spradling et al., 1999). Insertional mutagenesis conducted in *Caenorhabditis elegans* using of the Mos element showed the existence of a major hotspot at the rDNA locus (Granger et al., 2004). However, while in these studies, hotspots were identified as windows of several kilobase pairs, in our case, the insertion site is the same at the nucleotide level for each genomic site hit more than once. Despite a detailed molecular analysis of theses genomic regions, in particular of the locus hit 19 times, no peculiar characteristics could be found. Addi-

tional experiments are needed to better understand this bias and characterize these particular genomic regions.

The location of of *mimp1* insertions towards *F. graminearum* predicted genes was examined. Our analyses revealed that 50% of the insertions were located in the close vicinity (at less than 500 bp away) of predicted ORFs. In addition, insertions were found more frequently in upstream regions as compared to ORFs and 3′-UTRs which appeared depleted of *mimp1* insertions. Given that the median size of a *F. graminearum* intergenic region is 1179 bp, the high number of insertions of *mimp1* in 5′ close (< 500 bp) or more distal (500 to 1000 bp) regions is biased, as this category is on average over twice the size of the 500 bp windows. Such a distribution suggests that *mimp1* preferentially inserts far from genes which contrasts with what has already been described for other MITEs (Bureau and Wessler, 1994a, 1994b; Santiago et al., 2002). Nevertheless, as shown by our results, this mutagenic tool allowed the recovery of mutants in *F. graminearum* given the high gene density of its genome (Cuomo et al., 2007).

mimp1 mutagenic potential was further evaluated on a collection of 331 revertants using three phenotypic screens: perithecial development, radial growth and virulence on wheat spikes. These screens led to the identification of 19 putative mutants, representing 5.7% of the collection. The mutagenic rate of the *mimp1*/*impala* system is much higher than that obtained with REMI or Agrobacterium tumefaciens-mediated transformation (ATMT) Sweigard et al., 1998; Balhadère et al., 1999; Mullins et al., 2001; Seong et al., 2006; Blaise et al., 2007). With other insertional mutagens the proportion of mutants in various filamentous fungi ranged between 0.2% and 2%. The higher mutagenic rate of the *mimp1* transposon may result from the combination of three different phenotypic screens in our study. It is likely to be also correlated with the high gene density of the *F. graminearum* genome (Cuomo et al., 2007). Among the 19 identified mutants, only one strain exhibits a clear difference in radial growth. In addition, we noticed some morphological differences that were harder to quantify (data not shown). We anticipated that among the >300 revertants tested more mutants would show some kind of aberration in growth characteristics as compared to the wild-type strain *Fg*820. Although we expect different sets of genes to be expressed and required to efficiently exploit the different nutritional conditions, the level of differentiation in mycelium is limited. Possibly the genes required for a more general process such as mycelium growth are more likely to be redundant than genes involved in specific processes such as particular stages in the infection process or perithecial development. In the screens for virulence and perithecial development we found more mutants that showed aberrations from the wild-type phenotype. Interestingly, mutants exhibiting altered phenotypes in more than one of these characteristics could be identified. While eight mutants were only altered in virulence, and seven only in the development of perithecia, one strain exhibited both a drastic growth reduction and a virulence defect. The three remaining mutants were characterized by both virulence and sexual development defects (see Table 4).

Molecular analysis of the mutants has shown that all but one strain correspond to a true "cut-and-paste" event. In one mutant strain, although excised from the donor site, the *mimp1* element was not found in the genome. This result might be explained by an absence of *mimp1* reinsertion but raises the question of the origin of the mutation. Alternatively, although experiments conducted on three independent mutant strains have shown that *mimp1* insertions are stable, the transposon may have first reinserted into a genomic locus, leading to the mutation and this event has been rapidly followed by a secondary excision of the element. Given the footprints left upon excision, the mutation might have been maintained without the presence of *mimp1*. Sequences flanking *mimp1* insertions have been recovered for 13 mutant strains. Their analysis showed that 61.6% carried the element at less than 500 bp from a predicted ORF. This percentage is slightly higher than the one determined on the random sample of revertants, which was expected as phenotypic screens might select for tagged mutations. Most of the genes potentially tagged in this set of mutants corresponded to conserved hypothetical proteins having orthologs in other ascomycetes, in particular in the rice blast pathogen *M. grisea*. In the present work, functional complementation demonstrated the relationship between *mimp1* re-insertion and the phenotypic alterations in two revertant strains. Revertant 112 was shown to be altered in wheat spike colonization and exhibits also a drastic radial growth. The gene tagged in this mutant encodes a transcription factor characterized by a $Zn(II)_2Cys_6$ DNA-binding domain. Interestingly, the *M. grisea* ortholog (MGG_09263) had previously been tagged using T-DNA (Jeon et al., 2007). The corresponding mutant was shown to be altered both in appressorium formation and pathogenicity on rice seedlings but not importantly in growth rate (Jeon et al., 2007), therefore showing partial functional conservation with the *F. graminearum* ortholog. The second strain, revertant 237 is a virulence mutant unable to spread upon the first inoculated spikelet and is also impaired in the development of perithecia. In this mutant, *mimp1* reinserted in the putative promoter region of a gene similar to the *P. anserina* het c2 gene, predicted to encode a glycolipid transfer protein. Notably, another strain (revertant 193, see Supplementary Table 2) exhibiting a wild-type phenotype in all three screens was shown to carry a *mimp1* insertion into the 5′-region of the same predicted gene but located further from the start codon (378 bp versus 73 bp in revertants 193 and 237, respectively), giving some indication about the minimal promoter region required for functionality of this gene. In *P. anserina*, the het c2 gene has been shown to be involved nonallelic vegetative incompatibility and, interestingly, in ascospore production (Saupe et al., 1994). Considering its similarity to glycolipid transfer proteins, these authors suggested a possible involvement of the HET-C2 protein in cell-wall biosynthesis (Saupe et al., 1994). The *F. graminearum* mutant 237 also exhibited an alteration in sexual development although more drastic than in *P. anserina* as no perithecia are produced. How such a protein function might also alter fungal growth in planta remains unclear and will need further investigation. Further evaluation of the *mimp1* tagging efficiency is in progress through functional complementation of additional mutant strains.

Altogether, these results demonstrate that a MITE-based transposon-tagging strategy is an efficient tool for genome-wide insertional screens in *F. graminearum*. The *mimp1*-based transposon-tagging strategy allowed the rapid generation of large collections of mutants ready for phenotypic screens. We expect that the range of heterologous species in which *mimp1* retains its transposition characteristics is as wide as the one determined for its master element *impala* (*Magnaporthe* sp., *Penicillium* sp., *Aspergillus*, *Colletotrichum* sp., for a review see Daboussi and Capy, 2003), therefore this transposon-tagging system has potential for functional genomics in filamentous ascomycete fungi.

Acknowledgments

Part of this work was supported by funding from CNRS and from KNAW-MOST (04-PSA-BD-05, 2004CB720407). We are grateful to Daniel Gautheret and Eric Espagne for their help with bioinformatics tools and to Frances Trail for precious advices on perithecial development.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.09.004.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Balhadère, P.V., Foster, A.J., Talbot, N.J., 1999. Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. Mol. Plant-Microbe Interact. 12, 129–142.

Bennet, J.W., Klish, M., 2003. Mycotox. Clin. Microb. Rev. 16, 497–516.

Blaise, F., Rémy, E., Meyer, M., Zhou, L., Narcy, J.-P., Roux, J., Balesdent, M.-H., Rouxel, T., 2007. A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool of pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal Genet. Biol. 44, 123–138.

Bouvet, G.F., Jacobi, V., Plourde, K.V., Bernier, L., 2008. Stress-induced mobility of OPHIO1 and OPHIO2, DNA transposons of the Dutch elm disease fungi. Fungal Genet. Biol. 45, 565–578.

Bureau, T.E., Wessler, S.R., 1994a. Mobile inverted-repeat elements of the *Tourist* family are associated with the genes of many cereal grasses. Proc. Natl. Acad. Sci. USA 91, 1411–1415.

Bureau, T.E., Wessler, S.R., 1994b. Stowaway: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. Plant Cell 6, 907–916.

Carroll, A.M.C., Sweigard, J.A., Valent, B., 1994. Improved vectors for selecting resistance to hygromycin. Fungal Genet. Newsl. 41, 22.

Choi, J., Park, J., Jeon, J., Chi, M.-H., Goh, J., et al., 2007. Genome-wide analysis of T-DNA integration into the chromosomes of *Magnaporthe oryzae*. Mol. Microb. 66, 371–382.

Cuomo, C.A., Güldener, U., Xu, J.-R., Trail, F., Turgeon, G., Di Pietro, A., Walton, J.D., et al., 2007. The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. Science 317, 1400–1402.

Daboussi, M., Capy, P., 2003. Transposable elements in filamentous fungi. Annu. Rev. Microbiol. 57, 275–299.

Dufresne, M., Hua-Van, A., Abdel Wahab, H., Ben M'Barek, S., Vasnier, C., et al., 2007. Transposition of a fungal MITE through the action of a *Tc1*-like transposase. Genetics 175, 441–452.

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, 247–255.

Gouet, P., Courcelle, E., Stuart, D.I., Metoz, F., 1999. ESPript: multiple sequence alignments in PostScript. Bioinformatics 15, 305–308.

Granger, L., Martin, E., Ségalat, L., 2004. Mos as a tool for genome-wide insertional mutagenesis in *Caenorhabditis elegans*: results of a pilot study. Nucleic Acids Res. 32, e117.

Han, Y., Korban, S.S., 2007. Spring: a novel family of miniature inverted-repeat transposable elements is associated with genes in apple. Genomics 90, 195–200.

Hua-Van, A., Héricourt, F., Capy, P., Daboussi, M.J., Langin, T., 1998. Three highly divergent subfamilies of the *impala* transposable element coexist in the genome of the fungus *Fusarium oxysporum*. Mol. Gen. Genet. 259, 354–362.

Hua-Van, A., Davière, J.M., Langin, T., Daboussi, M.J., 2000. Genome organization in *Fusarium oxysporum*: clusters of class II transposons. Curr. Genet. 37, 339–347.

Hua-Van, A., Pamphile, J.A., Langin, T., Daboussi, M.J., 2001. Transposition of autonomous and engineered *impala* transposons in *Fusarium oxysporum* and a related species. Mol. Gen. Genet. 264, 724–731.

Jeon, J., Park, S.Y., Chi, M.H., Choi, J., Park, J., Rho, H.S., Kim, S., et al., 2007. Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. Nat. Genet. 39, 561–565.

Jiang, N., Bao, Z., Zhang, X., Hirochika, H., Eddy, S.R., McCouch, S.R., Wessler, S.R., 2003. An active DNA transposon family in rice. Nature 421, 163–167.

Kikuchi, K., Terauchi, K., Wada, M., Hirano, H.Y., 2003. The plant MITE mPing is mobilized in anther culture. Nature 421, 167–170.

Klittich, C.J.R., Leslie, J.F., 1988. Nitrate reductase mutants of *Fusarium moniliforme* (*Gibberella fujikoroi*). Genetics 118, 417–423.

Macas, J., Koblizkova, A., Neumann, P., 2005. Characterization of Stowaway MITEs in pea (*Pisum sativum* L.) and identification of their potential master elements. Genome 48, 831–839.

Maes, T., De Keukeleire, P., Gerats, T., 1999. Plant tagnology. Trends Plant Sci. 4, 90–96.

Marek, E.T., Schardl, C.L., Smith, D.A., 1989. Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal homology. Curr. Genet. 15, 421–428.

McMullen, M., Jones, R., Gallenberg, D., 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis. 81, 1340–1348.

Menzel, G., Dechyeva, D., Keller, H., Lange, C., Himmelbauer, H., Schmidt, T., 2006. Mobilization and evolutionary history of miniature inverted-repeat transposable elements (MITEs) in *Beta vulgaris* L. Chromosome Res. 14, 831–844.

Metaxakis, A., Oehler, S., Klinakis, A., Savakis, C., 2005. Minos as a genetic and genomic tool in *Drosophila melanogaster*. Genetics 171, 571–581.

Migheli, Q., Steinberg, C., Davière, J.M., Olivain, C., Gerlinger, C., et al., 2000. Recovery of mutants impaired in pathogenicity after transposition of *Impala* in *Fusarium oxysporum* f. sp. *melonis*. Phytopathology 90, 1279–1284.

Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M., Kang, S., 2001. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91, 173–180.

Nakasaki, T., Okumoto, Y., Horibata, A., Yamahira, S., Teraishi, M., Nishida, H., Inoue, H., Tanisaka, T., 2003. Mobilization of a transposon in the rice genome. Nature 421, 170–172.

Nirenberg, H.I., 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. Mitt. Biol. Bundesanst. Land Forstwitsch. Berlin-Dahlem 169, 1–117.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, New York.

Santiago, N., Herraiz, C., Goni, J.R., Messeguer, X., Casacuberta, J.M., 2002. Genome-wide analysis of the *Emigrant* family of MITEs of *Arabidopsis thaliana*. Mol. Biol. Evol. 19, 2285–2293.

Saupe, S., Descamps, C., Turcq, B., Bégueret, J., 1994. Inactivation of the *Podospora anserina* vegetative incompatibility locus het-c, whose product resembles a glycolipid transfer protein, drastically impairs ascospore production. Proc. Natl. Acad. Sci. USA 91, 5927–5931.

Schneider, T.D., Stephens, R.M., 1990. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. 18, 6097–6100.

Seong, K., Hou, Z., Tracy, M., Kistler, H.C., Xu, J.-R., 2006. Random insertional mutagenesis identifies genes associated with virulence in the wheat scab fungus *Fusarium graminearum*. Phytopathology 95, 744–750.

Soanes, D.M., Richards, T.A., Talbot, N.J., 2007. Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity? Plant Cell 19, 3318–3326.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., et al., 1999. The Berkeley Drosophila Genome Project gene disruption project: single P-element insertions mutating 25% of vital Drosophila genes. Genetics 153, 135–177.

Sweigard, J.A., Carrol, A.M., Farrall, L., Chumley, F.G., Valent, B., 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. Mol. Plant-Microbe Interact. 11, 404–412.

Trail, F., Xu, J.-R., San Miguel, P., Halgren, R.G., Kistler, H.C., 2003. Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). Fungal Genet. Biol. 38, 187–197.

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. 14, 308–315.

Weld, R.J., Plummer, K.M., Carpenter, M.A., Ridgway, H.J., 2006. Approaches for functional genomics in filamentous fungi. Cell Res. 16, 31–44.

Yang, G., Zhang, F., Hancock, C.N., Wessler, S.R., 2007. Transposition of the rice miniature inverted repeat transposable element *mPing* in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 104, 10962–10967.