Transposon *impala*, a Novel Tool for Gene Tagging in the Rice Blast Fungus *Magnaporthe grisea*

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impala, a *Tc1-mariner* transposable element from *Fusarium oxysporum*, was introduced into the rice blast fungus *Magnaporthe grisea* to develop transposon-based insertional mutagenesis. A construct (pNIL160) containing an autonomous *impala* copy inserted in the promoter of *niaD* encoding *Aspergillus nidulans* nitrate reductase was introduced by transformation into a *M. grisea* nitrate reductase-deficient mutant. *impala* excision was monitored by restoration of prototrophy for nitrate. Southern analysis of *niaD*⁺ revertants revealed that *impala* was able to excise and reinsert at new loci in *M. grisea*. As observed for its host *Fusarium oxysporum*, *impala* inserted at a TA site left a typical excision footprint of 5 bp. We have shown that a defective *impala* copy was inactive in *M. grisea*, yet it can be activated by a functional *impala* transposase. A transformant carrying a single copy of pNIL160 was used to generate a collection of 350 revertants. Mutants either altered for their mycelial growth (Rev2) or nonpathogenic (Rev77) were obtained. Complementation of Rev77 with a 3-kb genomic fragment from a wild-type locus was successful, demonstrating the tagging of a pathogenicity gene by *impala*. This gene, called *ORP1*, is essential for penetration of host leaves by *M. grisea* and has no sequence homology to known genes.

Transposable elements (TEs) are DNA segments that are ubiquitous in prokaryotic and eukaryotic organisms and have the ability to move and replicate within genomes (Kidwell and Lisch 1997). TEs are classified into two classes in accordance with their structure and mode of transposition (Finnegan 1992). Class I TEs are retroelements that transpose by reverse transcription of an RNA intermediate. Class II TEs transpose directly from DNA to DNA by a “cut-and-paste” mechanism. These elements are characterized by small inverted repeats (10 to 200 bp) that border an internal transposase-encoding open reading frame (ORF) that is necessary for their transposition. Fungal TEs were first identified in the yeast *Saccharomyces cerevisiae* (Boeke 1989), and were recently detected in

filamentous fungi (Daboussi et al. 1992). Transposons representing all major classes of eukaryotic TEs were identified in fungi either through the characterization of repeated genomic sequences or their trapping in target genes (Daboussi 1996; Kempken 1999). Autonomous copies were identified for a few fungal Class II TEs such as *Fot1* (*Fusarium oxysporum* transposon) and *impala* from *Fusarium oxysporum* (Hua-Van et al. *in press*; Migheli et al. 1999) or Restless from *Tolypocladium inflatum* (Kempken and Kück 1996). Transposition of autonomous fungal Class I TEs also was demonstrated for Tad from *Neurospora crassa* (Cambareri et al. 1994) and for MAGGY from *Magnaporthe grisea* (Nakayashi et al. 1999). Tagging genes of interest with fungal TEs is not well documented, apart from their initial discovery as an insertion in a known gene and the use of Restless in its host species to identify a nitrogen pathway-specific regulator gene (Kempken and Kück 2000).

Several class I transposons have been identified in *M. grisea* (Farman et al. 1996; Kachroo et al. 1994). *Pot2* and *Pot3* (*Pyricularia oryzae* transposon) ORFs are related to *Fot1* (Daboussi et al. 1992), but their transposition was not established clearly (Shull and Hamer 1994) until the recent characterization of a *Pot3* insertion in the avirulence gene AVR-Pita (S. Kang, *personal communication*). Because *Pot2* and *Pot3* are present in high copy number in the *M. grisea* genome, the identification of their insertion sites is a difficult task. To circumvent these drawbacks, we used *impala* from the distant fungal species *F. oxysporum* (Langin et al. 1995) for which an autonomous copy was identified (*imp160*) (Hua-Van et al. *in press*). *impala* was isolated from *F. oxysporum* as a spontaneous insertional mutation upstream of the start codon of the *Aspergillus nidulans niaD* gene. This 1,280-bp DNA transposon has 37-bp inverted terminal repeat sequences (ITRs) and a 339-bp ORF, with strong homology to transposases from the *Tc1-mariner* superfamily (Hua-Van et al. 1998). As for this family of TEs, *impala* inserts into a TA site that is duplicated during insertion and leaves a typical set of footprints after its excision (Hua-Van et al. 1998; Langin et al. 1995). Transformation of a *F. oxysporum* strain devoid of *impala* with a plasmid carrying this element inserted in the *niaD* promoter (*niaD::imp160* from pNI160) showed that *imp160* excises from the *niaD* promoter, restoring a functional nitrate reductase gene, and inserts at a new locus (Hua-Van et al. *in press*). *imp160* also transposes autonomously in the related

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fungus species *Fusarium moniliforme* (Hua-Van et al. *in press*).

Here, we provide evidence for transposition of *impala* in *M. grisea* on the basis of a phenotypic assay for its excision from the *niaD* gene promoter and molecular analysis of these revertants by Southern blot hybridization and DNA sequence analyses. In addition, we demonstrate that *impala* could be used to inactivate and tag a pathogenicity gene.

RESULTS

Transposition of *impala* in *Magnaporthe grisea* monitored by a phenotypic assay.

Plasmid pNI160 contains a copy of *impala* (*imp160*) inserted in a *A. nidulans niaD* promoter shortened to 0.3 kb (p0.3). The p0.3 promoter is sufficient for the expression of *niaD* in *F. oxysporum*, leading to prototrophic revertants after *impala* excision (Hua-Van et al. *in press*; Migheli et al. 1999). In *M. grisea*, the *niaD* gene from *A. nidulans* carried by the plasmid pAN301 is able to complement a *NIAI*⁻ mutant (Daboussi et al. 1989). In pAN301, the *niaD* gene is expressed under the control of a 1.3-kb promoter (p1.3) corresponding to the full intergenic region between the start codon of the *niaD* gene and the start codon of the divergently transcribed *niiaA* gene encoding nitrite reductase (Punt et al. 1995). To assay the effect of these different promoters on *niaD* expression in *M. grisea*, we transformed the *NIAI*⁻ mutant G11.174 with a plasmid (p11Δ*NdeI*) carrying *niaD* under the control of the p0.3 promoter (Migheli et al. 1999). Transformation of this mutant, used as a control, was achieved with the plasmid pAN301Δ*NdeI* carrying *niaD* under the control of the p1.3 promoter. Plasmids were introduced into *M. grisea* by transformation with plasmid pCB1179 conferring hygromycin resistance (Sweigard et al. 1997). Transformants were selected on nitrate minimal medium containing hygromycin. Transformants displaying a *niaD*⁺ phenotype were obtained only with the plasmid carrying the p1.3 promoter (pAN301Δ*NdeI*),

showing that the p0.3 *niaD* promoter (p11Δ*NdeI* and pNI160) was not capable of driving the expression of *niaD* in *M. grisea*. Therefore, we constructed a plasmid (pNIL160) carrying *imp160* inserted at the same location as in pNI160 in which the p0.3 promoter was replaced by p1.3 promoter. pNIL160 was introduced into *NIAI*⁻ mutant G11.174 by transformation with plasmid pCB1179. To increase transformation efficiency, experiments were performed with REMI. Transformants (80%) were detected among hygromycin-resistant colonies by polymerase chain reaction (PCR) with *impala* transposase-specific primers. Twelve random transformants carrying pNIL160 were plated on minimal medium. After 3 weeks, all transformants gave rise to wild-type revertants, suggesting that *imp160* excised in *M. grisea* (Table 1). Emergence time and number of revertants per plate varied, depending on transformant. According to these criteria, transformants were classified into four classes. Class I and II transformants gave rise to early revertants (10 to 14 days after plating), whereas class III and IV gave rise to late revertants (14 to 18 days after plating). The number of revertants per plate was independent of inoculum concentration for class I and III transformants, whereas the number of revertants was proportional to inoculum for class II and IV transformants.

Molecular analysis of *niaD*⁺ revertants.

Transformants CTRF-6 and CTRF-8 carrying one copy of pNIL160 and their revertants were analyzed by Southern hybridization with *niaD* or *impala* as probes (Fig. 1A and B). In revertants, we did not detect the 4.0-kb *EcoRI* fragment carrying *niaD::imp160* from pNIL160 (Fig. 1A) that was present in the initial transformants CTRF-6 and CTRF-8 (Fig. 1A and B, lanes 1 and 8). When probed with *niaD*, these revertants displayed a 2.7-kb *EcoRI* fragment hybridizing only to *niaD* (Fig. 1B) and corresponding to the size of the *niaD* *EcoRI* fragment after *impala* excision. These patterns suggest that *impala* excised from the *niaD* promoter in all of the revertants analyzed. A fragment of variable size hybridizing only to the

Table 1. Selection of *niaD*⁺ revertants from *Magnaporthe grisea* transformants carrying pNIL160^a

Plating	10 ⁶ Spores			10 ⁵ Spores		
	14 days ^b	18 days	Total	14 days	18 days	Total
Class I ^b						
Trf-13	60 ^c	11	71	07	25	32
Trf-24	40	13	53	04	03	07
Trf-30	19	Not determined	Not determined	03	11	14
Trf-36	36	Not determined	Not determined	05	22	27
Class II						
Trf-08	15	68	83	04	19	23
Trf-15	05	60	65	01	19	20
Trf-26	08	Not determined	Not determined	03	07	10
Class III						
Trf-16	0	25	25	0	03	03
Trf-17	01	28	29	0	02	02
Trf-21	0	41	41	0	04	04
Class IV						
Trf-05	0	02	02	0	0	0
Trf-06	0	05	05	0	03	03

^a The G11.174 nitrate reductase-deficient mutant was stable when plating a high inoculum level of spores or protoplasts onto minimal medium.

^b Transformants were classified according to their pattern of reversion. Class I = early revertants (14 days), number not proportional to inoculum. Class II = early revertants (14 days), number not proportional to inoculum. Class III = late revertants (18 days), number proportional to inoculum. Class IV = late revertants (18 days), number not proportional to inoculum.

^c Number of revertants per plate (data from a single experiment). Transformants were inoculated on minimal medium (10⁵ to 10⁶ spores per plate) and incubated for 3 weeks at 26°C. Revertants were counted at 14 and 18 days after plating. Total = number of revertants obtained per plate.

impala probe also was detected in these revertants, a result which could correspond to the insertion of *impala* at a new locus. These revertants were analyzed by PCR with primers flanking the *imp160* insertion in the *niaD* promoter. Amplification of a 217-bp product confirmed that *impala* had excised from the *niaD* promoter. The sequence of these PCR products showed a typical footprint of five nucleotides, as described in *F. oxysporum* (Langin et al. 1995). These footprints corresponded to the duplication of the TA target site and three nucleotides from either the 5' (CTGTA for three revertants) or 3' end (CAGTA for six revertants) of *impala* ITRs. These results demonstrate that *impala* transposes in *M. grisea* by a cut-and-paste mechanism, as it does in *F. oxysporum*. Southern blot

analysis of 45 revertants isolated from eight different transformants showed that *impala* was reinserted into *M. grisea* genome at different rates (30 to 100%), depending on the transformants (Table 2). Overall, *impala* was inserted at a new location in *M. grisea* genome at a high rate (35 over 45: 77%). The stability of *impala* at these new loci was assessed with the use of six revertants from the class II CTRF-8 and three revertants from the class IV CTRF-6. Southern blot analysis of these six revertants after their subculturing on rice agar or liquid medium for 1 week revealed that their *impala* integration patterns remained stable (data not shown).

impala may transpose autonomously in *Magnaporthe grisea*.

Transposons with significant homology to *impala* have been identified in the *M. grisea* genome. As *impala*, *Pot2*, and *Pot3* have short inverted repeats, an ORF with significant homology to *Tc1-mariner* transposases and inserts at a dinucleotide TA site (Farman et al. 1996; Kachroo et al. 1994). To assess the influence of these TEs on *impala* transposition in *M. grisea*, we used a defective *impala* element where most of the transposase was replaced by the *hph* gene (Hua-Van et al. *in press*). This plasmid (pNIH) was modified with the *niaD* p1.3 promoter instead of the p0.3 promoter to produce the pNIHL160 that was introduced by transformation into the *M. grisea* *NIA1*⁻ mutant G11.174. Among the six hygromycin-resistant transformants tested, we did not detect any prototrophic revertant on minimal medium. This result indicates that the defective *imp160::hph* element from pNIHL160 cannot be mobilized by *M. grisea* endogenous transposable elements. The *NIA1*⁻ mutant strain was transformed by pNIHL160 and a second plasmid (pEO6) carrying the ORF encoding *impala* transposase under the control of the *A. nidulans* *gpdA* promoter (Hua-Van 1998). Transformants were detected by PCR among hygromycin-resistant colonies with *impala* transposase-specific primers. Transformants CTRF-DC1 and CTRF-DC9 gave rise to 11 and 23 *niaD*⁺ revertants per 10⁶ spores plated, respectively. Southern analysis of three revertants from CTRF-DC9 confirmed the excision and reinsertion of modified *impala* from pNIHL160 (Fig. 2). Presence of a 4.5-kb *EcoRI* fragment in these revertants showed that at least two copies of pNIHL160 carrying the defective element were inte-

Fig. 1. Genomic hybridization patterns of *Magnaporthe grisea* transformants carrying the plasmid pNIHL160 and their *niaD*⁺ revertants. Genomic DNA (4 µg) was digested with *EcoRI*, submitted to agarose gel electrophoresis, and transferred onto a nylon membrane. Membrane was hybridized with **A**, radiolabeled 560-bp *impala* polymerase chain reaction product or **B**, radiolabeled *niaD* 2.7-kb *EcoRI* fragment. Lane 1, CTRF-8; lanes 2 to 7, revertants from CTRF-8; lane 8, CTRF-6; lanes 9 to 11, revertants from CTRF-6.

Table 2. Insertion of *impala* in *Magnaporthe grisea* *niaD*⁺ revertants

Transformant analyzed	Number of revertants carrying <i>impala</i> ^a	Percent
Class I		
Trf-13	3/5	60
Trf-30	2/6	33
Trf-36	5/5	100
Class II		
Trf-08	6/6	100
Trf-15	5/6	83
Trf-26	4/5	80
Class III		
Trf-21	4/6	67
Class IV		
Trf-06	6/6	100

^a Number of revertants with *impala* inserted at a new locus/number of revertants analyzed. All revertants with *impala* corresponded to independent excision events, except two revertants from CTRF-13 with identical *impala* insertion patterns.

grated into their genome. These data demonstrate that a defective *impala* can be activated in *M. grisea* by an *impala* transposase in *trans*, but not by endogenous transposons.

Screening for insertional mutants with *impala*.

With the exception of its trapping in the promoter of *niaD* (Langin et al. 1995), there is not yet demonstration that *impala* can insert into genes in *F. oxysporum*. To test the ability of *impala* to insert into *M. grisea* genes, we screened a collection of 350 *niaD*⁺ revertants obtained from CTRF-6 (five revertants per 10⁶ spores plated, 100% of *impala* reinsertion) for mutants with growth or pathogenicity defects. From this screen, we identified one revertant (Rev2) with morphological defects (reduced radial mycelial growth, reduced aerial hyphae, and deep-brown pigmentation). Flanking regions to the *impala* insertion site in Rev2 were rescued by inverse-PCR and sequenced. This analysis showed that *impala* was inserted in Rev2 at a TA dinucleotide located in an ORF (Rev2-ORF) located 23 bp downstream of its putative start codon. Database searches with T-BLAST-X (Altschul et al. 1990) revealed significant homologies (*E* value: 2.10⁻³⁰) between Rev2-ORF and the *S. cerevisiae* DNA mismatch repair gene *MLH1* (Fig. 3). The protein deduced from Rev2-ORF had 63% identity to Mlh1p.

One nonpathogenic revertant (Rev77) was identified that did not cause lesions on leaves of rice and barley. This mutant differentiated melanized appressoria but was unable to penetrate into rice leaves, as shown by the absence of infectious hyphae in peeled barley epidermis 2 days after inoculation (data not shown). Flanking regions to *impala* insertion in Rev77 were rescued by inverse-PCR. Two cosmid clones (20J4 and 2C16) hybridizing to Rev77 inverse-PCR probe

were isolated from a *M. grisea* wild-type strain genomic library (Dioh et al. 2000). Sequencing of junction fragments revealed that *impala* was inserted at a TA dinucleotide site located in an ORF of 1,719 (Rev77-ORF) at 1,154 bp from its putative start codon. A 3.0-kb *EcoRI*-*NotI* fragment from cosmid (2C16) that starts 1.3 kb upstream of the putative start codon from Rev77-ORF and terminates at the end of Rev77-ORF (Fig. 4) was cloned in the plasmid pCB1531 conferring Basta resistance (Sweigard et al. 1997). This plasmid (pCB13) was used to transform Rev77 protoplasts. Among the 10 transformants obtained carrying pCB13, nine were pathogenic on barley (Fig. 5). Because the 3.0-kb subclone we used was able to complement the Rev77 pathogenicity defect, we can conclude that Rev77 mutant phenotype is caused by the insertion of *impala* into Rev77-ORF, which could therefore correspond to a gene without introns that encodes for a putative protein of 573 amino acids. Databases searches (BLAST, Psi-BLAST, and Fasta) at the nucleotide or protein levels did not reveal significant homologies between Rev77-ORF and known sequences (GenBank and EMBL). Therefore, Rev77-ORF defined a new orphan pathogenicity gene, called *ORP1*, for orphan gene required for pathogenicity (GenBank accession number AF315717).

DISCUSSION

Transposon tagging was developed in *M. grisea* with the *Tc1-mariner* transposon *impala* from *F. oxysporum* (Langin et al. 1995). The *impala* element *imp160* was shown to be autonomous in *F. oxysporum* and *F. moniliforme* (Hua-Van et al. *in press*) and in *A. nidulans* (Li Destri Nicosia et al. *in press*). The results we describe demonstrate that *imp160* is able to transpose in another distant fungal species, *M. grisea*. We showed that *imp160* excises from *niaD* promoter of pNIL160 plasmid integrated in the *M. grisea* genome and that it reinserts at new genomic loci. *impala* transposition was shown to be autonomous in *M. grisea* as a defective *impala* element, was active only when complemented in *trans* by a functional *impala* transposase. Duplication of a TA target site during integration in the *M. grisea* genome and the presence of excision footprints similar to those obtained in *F. oxysporum* suggest that *impala* transposes in *M. grisea* by the same mechanism as in *F. oxysporum*. This result strongly suggests that *impala* transposes by a conserved mechanism in fungi that does not require host-specific factors, as described for most *Tc1-mariner* transposable elements (Plasterk et al. 1999).

During the monitoring of *impala* transposition in *M. grisea*, we observed that the frequency of revertants obtained (2 to 83 per 10⁶ spores plated) and the rate of *impala* insertion (33 to 100%) varied according to transformants. These results suggested that *impala* transposition could be influenced by the genomic location of the initial pNIL160 donor site. A more detailed analysis of this variability was performed on a set of transformants (Tables 1 and 2). A first category of transformants from class I (CTRF-13, 24, 30, and 36) gave rise to early revertants (10 to 14 days after plating) in numbers proportional to the amount of spores plated, as observed for mixtures of wild-type and mutant spores (data not shown). Revertants from class I transformants are likely to be present in the inoculum before plating. They could result either from

Fig. 2. Genomic hybridization patterns of *Magnaporthe grisea* *niaD*⁺ revertants obtained from transformants carrying the plasmid pNIHL160. Genomic DNA (4 µg) of *niaD*⁺ revertants (lanes 1 to 3) from CTRF-DC9 (lane 4) was digested with *EcoRI*, submitted to gel electrophoresis, transferred onto a nylon membrane, and probed with a radiolabeled 870-bp *hph* polymerase chain reaction product or a *niaD* 2.7-kb *EcoRI* fragment.

one or a few independent excision events that occurred during subculturing and could have multiplied prior to plating. Southern blot analysis of six random revertants from CTRF-13 revealed that two revertants (30%) had the same *impala* integration pattern (data not shown), demonstrating that these two insertions originated from the same excision event prior to plating. In class II (CTRF-8, 15, 26) and IV (CTRF-5, 6, 22, and 23) transformants, revertants appeared with a frequency independent of the number of spores plated. All the 23 revertants from class II and IV carried *impala* reinsertions at different locations in their genome, showing that they are the result of independent excision events that occurred after plating. The delay we observed for revertant emergence between class II and IV transformants could result from a position effect specific of the genomic region where pNIL160 was integrated.

In order to lower the risk of picking a revertant resulting from the same *impala* excision event, we selected a class IV transformant (CTRF-6). We then generated a collection of 350 revertants from CTRF-6 and identified two mutants. The first mutant (Rev2) has several morphological defects (reduced mycelial growth, reduced aerial hyphae, and brown pigmentation). We showed that this mutant has an insertion of *impala* in a gene with significant homology to the yeast *MLH1* gene (63% identity at protein level) that is involved in DNA mismatch repair. Yeast *MLH1* mutants exhibit an elevated spontaneous mutation rate during vegetative growth (Prolla et al. 1994), a lower frequency of crossing over, and an increase in non-Mendelian segregations (Hunter and Borts 1997). There is no obvious correlation, however, between the Mlh1- yeast

mutant phenotype and the morphological alterations we observed in Rev2. This discrepancy led us to hypothesize that the Rev2 phenotype results from a spontaneous mutation at a locus other than *MHL1*. This mutation could have occurred as a result of a Mlh1p impairment that might increase the spontaneous mutation rate in *M. grisea*, as observed for yeast. We identified a second mutant, Rev77, that was not pathogenic on rice and barley leaves. Rev77 differentiated an appressorium defective for penetration of host leaves. Characterization of genomic sequences flanking *impala* insertion in Rev77 revealed that *imp160* was inserted in a large ORF (1,719 bp), 1,154 bp after its putative start codon. A wild-type genomic fragment of 3.0 kb containing the Rev77-ORF (1.7 kb) and its putative promoter (1.3 kb) was able to complement Rev77, demonstrating that insertion of *impala* in Rev77-ORF was responsible for Rev77 mutant phenotype. The Rev77-ORF defines a new pathogenicity gene, called *ORP1*, with no significant homology to known genes. Additional experiments such as the determination of its expression during the infection process or biological and biochemical characterization of mutant appressoria are required to better understand the role of *ORP1* in penetration.

These results clearly demonstrate that *impala* could be used to generate tagged pathogenicity mutants in *M. grisea*. Although we obtained only one pathogenicity mutant, the mutant frequency estimated by our sample (1/350: 0.3%) is similar to the mutant frequency (0.5%) obtained with REMI in *M. grisea* (Balhadère et al. 1999; Sweigard et al. 1998). Compared to REMI, transposon mutagenesis is simpler to set up when starting from an available transformant with a transposon in a

Fig. 3. Alignment between the protein deduced from Rev2-open reading frame and mismatch repair proteins. Identical amino acids between at least three proteins are in bold. Asterisks mark identical amino acids between Rev2p (AF320085) and Mlh1p. Mlh1p (U07187) and Pms1p (M29688) were isolated from *Saccharomyces cerevisiae*, MutL (A33588) from *Salmonella typhimurium*, and Hexb (A33589) from *Streptococcus pneumoniae*.

construct allowing the monitoring of its excision. It does not require repeated transformations of wild type, and a large number of revertants (> 5000) can be produced in a relatively short time (3 weeks). A drawback of REMI mutagenesis is the existence of a variable proportion of untagged pathogenicity mutants (40 to 60%) that could result from spontaneous mutations either generated during protoplast transformation or caused by REMI-induced DNA repair. We can expect that transposon mutagenesis will not generate untagged mutants because there is no transformation involved. Additional studies with larger collections of revertants resulting from *impala* excision are required to validate this hypothesis.

Other transposable elements, in addition to *impala*, could be used for insertional mutagenesis. Recently the *Gypsy*-like retrotransposon *MAGGY* from *M. grisea* was shown to transpose autonomously in strains from either its host species or a distant fungal species *Colletotrichum lindemuthianum* (Nakayashi et al. 1999), but there is no report yet of its insertion in a gene of interest. The control of retrotransposition is a likely drawback when using this element for insertional mutagenesis. *MAGGY* was shown to be amplified in high copy number after its introduction by transformation in strains lacking this element (Nakayashi et al. 1999), preventing the identification of tagged genes without intensive genetic studies. We did not observe such amplification of *impala* after its introduction into the *M. grisea* genome. This could be the result of its mechanism of transposition with lower chances of amplification than retroelements and its probable low transposition rate.

Overall, we have shown that *impala* transposes autonomously in *M. grisea* and could be used to obtain a tagged pathogenicity mutant. This transposable element will be very useful for insertional mutagenesis in fungi because it does not seem to have severe host restriction and is easily modified without losing its transposition properties.

MATERIALS AND METHODS

Biological material.

The pathogenic *MAI*⁻ mutant G11.174 (Daboussi et al. 1989) was obtained from isolate Guy11 (Notteghem et al. 1992). G11.174 was stored on dried filter paper disks (Silué et al. 1992). Conditions for culture and composition of rice–agar medium for *M. grisea* maintenance and sporulation were as described in Silué et al. 1998. Fungal strains were purified by monospore isolation after an overnight germination at 26°C on water–2% agar.

Rice cultivar Sariceltik and barley cultivar Express are highly susceptible to *M. grisea*. Rice seedlings were cultivated at 25°C during the day and 20°C at night for 20 days at 70% relative humidity. Barley seedlings were cultivated at 20°C during the day and 15°C at night for 10 days at 70% relative humidity. Rice plants were fertilized with ammonium sulfate (5 g m⁻²) once a week and 1 to 2 days before inoculation.

Plasmids.

pNI160 (Hua-Van et al. *in press*) carry *imp160* inserted into a 0.3-kb promoter from the *A. nidulans niaD* gene. pNI160 was obtained by adding a 1.0-kb *EcoRI*–*Bam*HI fragment from the *niaD* promoter (Malardier et al. 1989) at the *Bam*HI site of the 0.3-kb *niaD* promoter (p0.3) from pNI160, resulting

in a 1.3-kb promoter (p1.3). This plasmid was used in transformation experiments with pCB1179 (Sweigard et al. 1997) carrying the *hph* gene conferring hygromycin resistance. pNIHL160 was obtained by adding a 1.0-kb *EcoRI*–*Bam*HI fragment from the *niaD* promoter at the *Bam*HI site of the 0.3-kb *niaD* promoter (p0.3) from pNIH (Hua-Van et al. *in press*), resulting in a 1.3-kb promoter (p1.3). pEO6 (Hua-Van 1998), carries the ORF encoding *impala* transposase from *imp160* under the control of the *gpdA* promoter from *A. nidulans* (Punt et al. 1990). pGEM-T Easy vector (Promega, Charbonnières, France) was used for cloning PCR products.

Fungal transformation, selection of revertants, and phenotypic analysis.

Transformation of *M. grisea* was performed as described in Sweigard et al. 1998. For REMI experiments, four units of *Nde*I restriction enzyme per ml were added to 1 µg of plasmid DNA in transformation buffer and incubated with protoplasts prior to the addition of polyethylene glycol. Transformants

Fig. 4. Schematic representation of *impala* insertion in Rev77-open reading frame (ORF). The 3.0-kb *NotI*–*EcoRI* genomic fragment from Rev77-ORF locus complemented the Rev77 mutant phenotype. *impala* was inserted at 1,154-bp from putative ATG of the 1,719-bp Rev77-ORF.

Fig. 5. Pathogenicity of Rev77. Fragments of barley leaves were inoculated with spores suspension (1×10^5 spores per ml) from **A**, wild-type G11.174 strain; **B**, mutant Rev77; and **C**, Rev77 complemented by 3-kb *EcoRI*–*NotI* genomic fragment from wild type. Symptoms were recorded 7 days after inoculation.

were selected on Tanaka complete medium (Daboussi et al. 1989) supplemented with 20% sucrose and 240 µg of hygromycin per ml (Sigma-Aldrich, Saint Quentin Fallavier, France) or 30 to 40 µg of Basta per ml and transferred to Tanaka complete medium supplemented with 120 µg of hygromycin per ml or 30 to 40 µg of Basta per ml and purified by monospore isolation. Purified transformants were cultivated for 14 days on rice agar for sporulation. Spores from transformants were plated on minimal medium gelified with 8 g of agarose per liter at 1×10^5 to 1×10^6 spores per 12×12 cm petri dishes and incubated at 26°C for 3 weeks. Minimal medium used was Tanaka-glucose with vitamins (10^{-3} g of medium with thiamin per liter and 5.10^{-6} g of medium with biotin per liter), without yeast extract (Daboussi et al. 1989).

Detached leaf assays were performed with 3-cm fragments from the mid part of the last-developed rice or barley leaves, placed on water–1% agar plus 2.10^{-3} g of kinetin per liter in 4×4 multicompartiment, square petri dishes. Detached leaves were moistened and inoculated by spreading spore suspensions with cotton tips. Spore suspensions of each transformant were prepared by scraping a 10-day-old mycelial rice–agar culture in 10 ml of sterile water and adjusting to 1×10^5 spores per ml. Symptoms were recorded 4 to 7 days after incubation at 23°C. Four-leaf fragments of rice and barley cultivars were inoculated with spores of each transformant. Assays on whole plants were carried out by spraying 24-day-old rice seedlings with spore suspensions adjusted to 3×10^4 spores per ml in water–0.1% gelatin. Symptoms were recorded 7 days after inoculation.

Molecular analysis of transformants and revertants.

Genomic *M. grisea* DNA for Southern blot analysis was extracted from digested mycelium according to Sweigard et al. (1990). Large-scale DNA preparation for inverse-PCR experiments were performed as described in Daboussi et al. (1989). Southern blots were performed according to Sambrook et al. (1989). Nucleic acids were transferred to positive nylon membranes (Appligene-oncor, Illkirch, France) with a vacuum blotter (Appligene-oncor). Radio-actives probes were synthesized with the Megaprime DNA labeling system (Amersham, Les Ulis, France) and $\alpha^{32}\text{P}$ labeled dCTP (Amersham). PCR reactions were carried out with a PTC-100 thermocycler (MJ-Research, Waltham, MA, U.S.A.), using Taq polymerase from Appligene and sodium salt nucleotides from Boehringer Mannheim (Meylan, France). Primers SPE5 (AGAACACA-ACCCTGCCACGG) and SPE3 (TCCGGGCGGTATGC-ACAGAG) specific of *impala* were used to detect transformants. Primers C1 (CGCTGCGAATTCTTCAGT) and *niaX* (CTAGACTTAGAACCTCGG) corresponding to sequences on both sides of *imp160* in the *niaD* promoter were used to characterize *impala* footprints. Primers were used at the concentration of 0.5 µM with 100 ng of DNA, 2.5 µl of $10\times$ buffer, 0.1 mM deoxynucleoside triphosphate, one unit of Taq polymerase, and sterilized water up to 25 µl. The program was one cycle of 5 min at 95°C and 30 cycles of 40 s at 95°C, 40 s at 60°C, and 1 min 30 s at 72°C, followed by one cycle of 10 min at 72°C. Amplification products were separated by gel electrophoresis in TAE with 1% agarose at 3 volts per cm with the 1-kb ladder as molecular weights marker (GIBCO BRL, Gaithersburg, MD, U.S.A.). For inverse-PCR, 3 µg of genomic DNA was digested by 10 units of *Bam*HI or *Hind*III.

After one phenol–chloroform extraction, DNA was precipitated by 1/2 volume of 7.5 M ammonium acetate, three vol of 100% ethanol and recovered in 40 µl of water. Ligation was performed during 10 min at room temperature with 8 µl of recovered DNA and the rapid DNA ligation kit from Boehringer Mannheim according to the supplier's instructions. DNA was precipitated as described above, recovered in 10 µl of water and used for inverse-PCR with primers *ImpE5* (GGCATTGAAAACGCGGTCCC) and *ImpE3* (CAGCA-GCAAAACAGCTGCCC), as described for PCR, except that the reaction volume was 50 µl and elongation time at 72°C was 2 min 30 s.

Molecular cloning

of pathogenicity gene inactivated in Rev77.

The inverse-PCR product corresponding to junction fragments of *impala* with the genome of Rev77 was used to screen a *M. grisea* cosmid library from wild-type strain 96/0/76 (Dioh et al. 2000) blotted on nylon membranes. Two cosmids (20J4 and 2C16) with the same restriction pattern were identified. A 3.0-kb *Eco*RI–*Not*I fragment from cosmid 2C16 that hybridizes to the inverse-PCR probe corresponding to the *impala* junction fragments was extracted from agarose gel with the GFX band purification kit (Amersham) and cloned in pCB1531 (Sweigard et al. 1997) carrying the *bar* gene for Basta resistance to give plasmid pCBSN77. Complementation assays were performed by transformation of Rev77 protoplasts with pCBSN77 to give Basta resistance. Sequencing was performed by Genome Express S.A. (Grenoble, France) and sequences were analyzed with BLAST software (Altschul et al. 1990).

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LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410.
- Balhadère, P. V., Foster, A. J., and 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.
- Boeke, J. D. 1989. Transposable elements in *Saccharomyces cerevisiae*. Pages 335–374 in: *Mobile DNA*. D. E. Berg and M. M. Howe, eds. American Society for Microbiology Press, Washington, DC.
- Cambareri, E. B., Helber, J., and Kinsey, J. A. 1994. *Tad1-1*, an active LINE-Like element from *Neurospora crassa*. *Mol. Gen. Genet.* 242:658–665.
- Daboussi, M. J. 1996. Fungal transposable elements: Generators of diversity and genetic tool. *J. Genet.* 75:325–339.
- Daboussi, M. J., Djeballi, A., Gerlinger, C., Blaiseau, P. L., Bouvier, I., Cassan, M., Lebrun, M.-H., Parisot, D., and Brygoo, Y. 1989. Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans*. *Curr. Genet.* 15:453–456.
- Daboussi, M. J., Langin, T., and Brygoo, Y. 1992. *Fot1*, a new family of fungal transposable elements. *Mol. Gen. Genet.* 232:12–16.
- Dioh, W., Tharreau, D., Nottoghem, J. L., Orbach, M., and Lebrun, M.-H. 2000. Mapping of avirulence genes in the rice blast fungus, *Magnaporthe grisea*, with RFLP and RAPD markers. *Mol. Plant-Microbe Interact.*

- Interact. 13:217-227.
- Farman, M. L., Taura, S., and Leong, S. A. 1996. The *Magnaporthe grisea* DNA fingerprinting probe MGR586 contains the 3' end of an inverted repeat transposon. *Mol. Gen. Genet.* 251:675-681.
- Finnegan, D. J. 1989. Eukaryotic transposable elements and genome evolution. *Trends Genet.* 5:103-107.
- Hua-Van, A. 1998. Caractérisation de la famille d'éléments transposables *Impala* et développement d'un outil de mutagenèse insertionnelle chez le champignon phytopathogène *Fusarium oxysporum*. Ph.D. thesis. Université Paris-6, France.
- Hua-Van, A., Héricourt, F., Capy, P., Daboussi, M. J., and 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., Pamphile, J. A., Langin, T., and Daboussi, M. J. Transposition in *Fusarium oxysporum* and related species using autonomous and engineered *impala* transposons. *Mol. Gen. Genet. In press.*
- Hunter, N., and Borts R. H. 1997. Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis. *Genes Dev.* 11:1573-1582.
- Kachroo, P., Leong, S. A., and Chattoo, B. B. 1994. *Pot2*, an inverted repeat transposon from the rice blast fungus *Magnaporthe grisea*. *Mol. Gen. Genet.* 245:339-348.
- Kempken, F. 1999. Fungal transposons: From mobile elements towards molecular tools. *Appl. Microbiol. Biotechnol.* 52:756-760.
- Kempken, F., and Kück, U. 1996. *Restless*, an active Ac-like transposon from the fungus *Tolytlocadium inflatum*: Structure, expression and alternative RNA splicing. *Mol. Cell Biol.* 16:6563-6572.
- Kempken, F., and Kück U. 2000. Tagging of a nitrogen pathway-specific regulator gene in *Tolytlocadium inflatum* by the transposon *Restless*. *Mol. Gen. Genet.* 263:302-308.
- Kidwell, M. G., and Lisch, D. 1997. Transposable elements as sources of variation in animals and plants. *Proc. Natl. Acad. Sci. USA* 94:7704-7711.
- Langin, T., Capy, P., and Daboussi M. J. 1995. The transposable element *Impala*, a fungal member of the *Tc1-mariner* superfamily. *Mol. Gen. Genet.* 246:19-28.
- Li Destri Nicosia, M. G., Masson, C., Demais, S., Hua-Van, A., Daboussi, M.-J., and Scazzocchio, C.. Heterologous transposition in *Aspergillus nidulans*. *Mol. Microbiol. In press.*
- Malardier, L., Daboussi, M. J., Julien, J., Roussel, F., Scazzocchio, C., and Brygoo, Y. 1989. Cloning of the nitrate reductase gene (*niaD*) of *Aspergillus nidulans* and its use for transformation of *Fusarium oxysporum*. *Gene* 78:147-156.
- Migheli, Q., Lauge, R., Daviere, J. M., Gerlinger, C., Kaper, F., Langin, T., and Daboussi, M. J. 1999. Transposition of the autonomous *Fot1* element in the filamentous fungus *Fusarium oxysporum*. *Genetics* 151:1005-1013.
- Nakayashiki, H., Kiyotomi, K., Tosa, Y. and Mayama, S. 1999. Transposition of the retrotransposon *MAGGY* in heterologous species of filamentous fungi. *Genetics* 153:693-703.
- Notteghem, J. L., and Silué, D. 1992. Distribution of the mating type alleles in *Magnaporthe grisea* populations pathogenic on rice. *Phytopathology* 82:421-424.
- Plasterk, R. H. A., Isvak, Z., and Ivics, Z. 1999. Resident aliens, the *Tc1*/mariner superfamily of transposable elements. *Trends Genet.* 15:326-332.
- Prolla, T. A., Christie, D. M., and Liskay, R. M. 1994. Dual requirement in yeast DNA Mismatch repair for *MLH1* and *PMS1*, two homologs of the bacterial *mutL* gene. *Mol. Cell Biol.* 14:407-415.
- Punt, P. J., Dingemanse, M. A., Kuyvenhoven, A., Soede, R. D., Pouwels, P. H., and Van den Hondel, C. A. 1990. Functional elements in the promoter region of *Aspergillus nidulans* *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* 93:101-109.
- Punt, P. J., Strauss, J., Smit, R., Kinghorn, J. R., Van den Hondel, C. A., and Scazzocchio, C. 1995. The intergenic region between the divergently transcribed *niaA* and *niaD* genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. *Mol. Cell Biol.* 15:5688-5699.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Shull, V., and Hamer J. E. 1994. Genomic structure and variability in *Pyricularia oryzae*. Pages 65-86 in: *Rice Blast Disease*. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International, Wallingford, U.K.
- Silué, D., Notteghem, J. L., and Tharreau, D. 1992. Evidence for a gene-for-gene relationship in the *Oryza sativa*-*Magnaporthe grisea* pathosystem. *Phytopathology* 82:577-582.
- Silué, D., Tharreau, D., Talbot, N. J., Clergeot, P.-H., Notteghem, J. L., and Lebrun, M.-H. 1998. Identification and characterization of *apf* in a non-pathogenic mutant of the rice blast fungus *Magnaporthe grisea* which is unable to differentiate appressoria. *Physiol. Mol. Plant Pathol.* 53:239-251.
- Sweigard, J. A., Orbach, M. J., Valent, B., and Chumley, F. G. 1990. A miniprep procedure for isolating genomic DNA from *Magnaporthe grisea*. *Fungal Genet. Newsl.* 37:4.
- Sweigard, J. A., Chumley, F. G., Carroll, A. M., Farrall, L., and Valent, B. 1997. A series of vectors for fungal transformation. *Fungal Genet. Newsl.* 44:52-53.
- Sweigard, J. A., Carroll, A. M., Farrall, L., Chumley, F. G., and Valent, B. 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant-Microbe Interact.* 11:404-412.