Recovery of Mutants Impaired in Pathogenicity After Transposition of *Impala* in *Fusarium oxysporum* f. sp. *melonis*

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Accepted for publication 21 July 2000.

ABSTRACT

Migheli, Q., Steinberg, C., Davière, J.-M., Olivain, C., Gerlinger, C., Gautheron, N., Alabouvette, C., and Daboussi, M.-J. 2000. Recovery of mutants impaired in pathogenicity after transposition of *Impala* in *Fusarium oxysporum* f. sp. *melonis*. Phytopathology 90:1279-1284.

The ability of transposon *impala* to inactivate genes involved in pathogenicity was tested in *Fusarium oxysporum* f. sp. *melonis*. Somatic excision of an *impala* copy inserted in the nitrate reductase-encoding *niaD* gene was positively selected through a phenotypic assay based on the restoration of nitrate reductase activity. Independent excision events were analyzed molecularly and shown to carry reinserted *impala* in more than 70% of the cases. Mapping of reinserted *impala* elements on large *Not*I-restriction fragments showed that *impala* transposes randomly. By screening 746 revertants on plants, a high proportion (3.5%) of mutants impaired in their pathogenic potential was recovered. According to the kinetics of wilt symptom development, the strains that were impaired in pathogenicity were clustered in three classes: class 1 grouped two strains that never induced Fusarium wilt symptoms on the host plant; class 2 and class 3 grouped 15 and 9 revertants which caused symptoms more than 50 and 30 days after inoculation, respectively. The first results demonstrate the efficiency of transposition in generating mutants affected in pathogenicity, which are usually difficult to obtain by classical mutagenesis, and open the possibility to clone the altered genes with *impala* as a tag.

Additional keywords: melon, transposon tagging.

Fusarium oxysporum Schlechtend.:Fr. is a common soilborne pathogen with a worldwide distribution, which can cause disease on a wide range of economically important crops (46). It exists in many specialized forms, grouped into formae speciales and physiological races depending on their pathogenicity towards particular plant species or cultivars (3). *F. oxysporum* also includes nonpathogenic strains that play an important role in the natural suppression of pathogenic strains (1,2). At present, little is known on the evolutionary biology of this fungus and only limited information is available on pathogenicity determinants (22). A detailed knowledge of such determinants would be invaluable in developing our understanding of the interaction between *F. oxysporum* and host plants. Generation of mutants impaired in pathogenicity and the characterization of their defects represent a powerful strategy for identification of pathogenicity genes. Attempts to obtain such mutants have been made in the past by chemical or UV treatment (8; M.-J. Daboussi, *unpublished data*). Strains selected in such a way often present low stability and their analysis can be complicated by the presence of multiple site-mutation events (8).

Recent developments in the molecular biology of this fungus (16,29,32) offer new perspectives for cloning genes involved in different steps of the pathogenic process with an insertional mutagenesis approach. This method consists in the generation of mutants after insertion of a DNA fragment into a gene of interest. The mutated copy of the gene can be isolated by the inserted DNA as a tag (4,35,52). Insertional mutagenesis has a significant advantage over conventional cloning techniques, i.e., it is possible to clone a gene without prior knowledge of the gene product. This is of great value in the molecular dissection of the phytopathogenic process because of the lack of information on the pattern of expression of genes involved in the plant–pathogen interaction (50). The so-called "black box" approach proved valuable in the understanding of bacterial pathogenesis (23,40) and could now be applied in fungal pathogens either by plasmid-mediated transformation or transposon tagging. Development of DNA-mediated transformation techniques in fungi (19), recently coupled with the restriction enzyme-mediated integration of plasmids (45), proved effective in identifying pathogenicity factors in plant pathogenic fungi such as *Cochliobolus heterostrophus* (34), *Ustilago maydis* (7), *Magnaporthe grisea* (5), and *Colletotrichum lindemuthianum* (17). However, this approach may suffer from problems associated with a low frequency of simple integration events and by nonrandom insertion of transforming DNA (9).

Another efficient tagging system is represented by the use of transposons, which are segments of DNA capable of moving from one location to another in the genome. These elements have been successfully used as mutagens in plants, bacteria, and insects (20,30,33,41,51). Recently, their potential application to filamentous fungi has been stressed (9,10,28). The identification of different families of active transposons in *F. oxysporum* (11) makes such a strategy, which was never used in other filamentous fungi, already appropriate for this pathogen. The aim of this work was to test the efficiency of the transposable element *impala* (25,26,31) for generating random mutations in *F. oxysporum*.

Here we report on the generation of pathogenicity mutants in *F. oxysporum* f. sp. *melonis*, causal agent of vascular wilt on melon, by transposition of the transposon *impala*. This element is a member of the *Tc1-mariner* superfamily. It is typically 1.3 kb long, with near perfect 37-bp inverted terminal repeats (ITRs) and con-

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Publication no. P-2000-0821-01R

tains a single open reading frame (ORF) potentially encoding a 340-amino acid transposase (26). Like all *Tc1-mariner* elements, it transposes by an excision-reinsertion mechanism and specifically inserts into a TA dinucleotide which is duplicated upon insertion (11). The first known *impala* member, *imp160*, was identified as an insertion (mutation *niaD160*) in the *niaD* gene of *Aspergillus nidulans* that had been introduced in strain FOM24 of *F. oxysporum*. The *imp160* transposed copy is able to excise from *niaD*, leading to the restoration of nitrate reductase activity, and frequently reinserts into a new genomic position (10). Based on this evidence, an experimental design was set up to test the ability of *impala* transposition to inactivate genes involved in pathogenicity.

MATERIALS AND METHODS

Fungal strains. A *F. oxysporum* f. sp. *melonis* transformant, TR7, containing a single copy of the *niaD* (nitrate reductase) gene from *A. nidulans*, and derived from the pathogenic isolate FOM24 (FPFS culture collection, INRA Dijon, France), was subject to chlorate resistance selection (11). Two mutants, coded *niaD*::*imp160* and *niaD*::*imp161*, were generated upon insertion of an *impala* copy in the 5′ leader of the *niaD* gene (31). It is not clear whether these mutants correspond to independent insertion events, because both insertions are at the same site and in the same orientation. However, these mutants can be distinguished by the number of *impala* copies (discussed below), and thus were both considered in the course of this study. A nonpathogenic isolate of *F. oxysporum*, FO47 (1), was used in pathogenicity assays as a control.

All strains were grown to sporulation on potato dextrose agar and stored as plugs under mineral oil at 12°C.

Phenotypic excision assays. Monoconidial cultures of mutants *niaD*::*imp160* and *niaD*::*imp161* (eight from *niaD*::*imp160* and seven from *niaD*::*imp161*) were grown for 48 h in potato dextrose broth (13) at 26°C on a rotary shaker (150 rpm). Conidial suspensions were obtained by filtering through a 100 µm of nylon filter, washed twice with sterile-distilled water and plated (10^2 to 10^3 spores ml^{-1}) onto a solid mineral medium containing nitrate as the sole nitrogen source (MM-nitrate) (13). To avoid background mutations, multiple monoconidial cultures of each mutant were grown on MM-nitrate. Excision events were identified by the appearance of the wild-type phenotype after 14 to 21 days of incubation at 26°C. Each patch of aerial mycelium developed on solid MM-nitrate was considered an independent reversion event. There were 9 to 165 revertants isolated from each of the monoconidial culture. Single spore colonies were purified on MM-nitrate and stored at 12°C before inoculation on plants and Southern blot analysis.

DNA preparation and Southern blot analysis. DNA for Southern blot analysis was obtained by a miniprep extraction method (32). For Southern blot analysis, ≈10 µg of DNA were digested in the presence of 100 units of the restriction enzymes *Eco*RI or *Xba*I (Roche, Mannheim, Germany) and subjected to electrophoresis through 0.6% agarose. Southern transfer was performed by standard methods (44) onto Hybond N nylon membranes (Amersham, Little Chalfont, UK) and fixed by UV crosslinking. DNA probes corresponded to polymerase chain reaction (PCR) products. Primers used for the amplification of the *niaD* specific probe (probe A) were *niaD144* (5′-GTTCATGCCGTGGTCGCTGCG-3′) and *niaD145* (5′-CCCGGCCAAAGCCTCGAATTCG-3′). For the *impala* specific probe (probe B), a unique primer (5′-CAGTGGGGTACAATAAGTTTGAATACA-3′), deduced from the ITR sequence, was used (31). PCR was performed according to Saiki et al. (43) in a thermal cycler (Biomed 60, Biomed, Orsay, France) with 100 to 300 ng of total genomic DNA and 1 unit of *Taq* polymerase (Oncor Appligene, Gaithersburg, MD) for 30 cycles (1 min 30 s at 94°C, 1 min at 60°C, and 1 min 30 s at 72°C), followed by an additional extension step of 15 min at 72°C. The two probes were ^{32}P-labeled with a random primer extension kit (Pharmacia Upjohn, North Peapack, NJ) following manufacturer's instructions. The presence and location of the *niaD* gene and the *impala* element was assessed by probes A and B, respectively. Hybridization was performed under standard conditions (65°C and 0.2× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 sodium citrate, pH 7.0]).

Transposition pattern of *impala* copies. To determine the transposition pattern of *impala* copies in the genome of *F. oxysporum*, intact chromosomes of isolate FOM24, mutant *niaD160*, and of the 2 revertants, rev4 and rev8 were obtained from protoplasts prepared by growing and digesting mycelia as described by Langin et al. (32). The protoplasts were treated as described by Mäntylä et al. (36). Plugs were washed twice for 1 h in adequate buffer and digested overnight at 37°C with 40 units of the restriction enzyme *Not*I (Roche). Separation of digested bands was performed using the CHEF-Mapper System (Bio-Rad Laboratories, Hercules, CA). Conditions of separation were: 10°C, running buffer 0.5× Tris-borate-EDTA changed every 24 h, 1% pulsed-field certified agarose (Bio-Rad), gel, pulse time gradient from 3 to 30 s during 36 h at 6 V cm^{-1}, and then from 40 to 90 s during 20 h at 5 V cm^{-1} both with a field angle of 120°. Chromosomal DNAs from *Saccharomyces cerevisiae* (Bio-Rad) were used as molecular size reference markers.

Pathogenicity assay. A large-scale screening of 746 revertants was first conducted to select those impaired in their pathogenic potential on melon. The selected revertants were then subjected to two additional pathogenicity tests under different conditions to confirm the pathogenicity impairment. Screening for putative loss of pathogenicity and pathogenicity tests were performed on melon (*Cucumis melo*) cv. Jerac F1 (Clauses, Bretigny-sur-Orge, France). This cultivar is susceptible to isolate FOM24 (race 1-2 Y), whereas strain FO47 does not cause any disease symptom.

Suspensions of conidia of *F. oxysporum* f. sp. *melonis* isolate FOM24, transformant TR7, mutants *niaD*::*imp160* and *niaD*::*imp161*, revertants from both mutants, and the nonpathogenic *F. oxysporum* isolate FO47 were prepared for the large scale-screening as described (47). Six seedlings were inoculated to test each strain.

Starting 2 weeks after inoculation, the plants were checked twice a week for 5 weeks and those exhibiting typical yellowing of the leaves indicative of Fusarium wilt of melon were uprooted. Thirty revertants for which at least five plants were still healthy 35 days after the inoculation were retested for pathogenicity.

For the two subsequent pathogenicity tests, inocula were produced as above, but conidia were washed twice with sterile-distilled water by centrifugation (4,000 × *g* for 20 min) before inoculation. The first pathogenicity test was performed in a growth chamber under artificial light (17,000 Lux). Seedlings were cultivated in 100 ml of rockwool cubes, and the inocula were brought to 1×10^4 conidia ml^{-1} in rockwool. Growing conditions were 20°C during night hours (8 h) and 25°C during day hours (16 h). Six seedlings were inoculated to test each revertant. The test lasted 58 days after inoculation.

The second experiment was conducted in a glasshouse (February to April) with 400 ml of steamed potting mixture as plant growth substrate for each seedling. Inoculation provided 1×10^5 conidia ml^{-1} of potting mixture. Growing conditions were the same as for the large screening. Twelve seedlings were inoculated with each revertant. The test lasted 72 days after inoculation. Twenty-nine revertants were tested and one revertant was lost during subculture.

In both experiments, controls consisted of seedlings inoculated with water, strain FO47, or the wild-type isolate FOM24. Symptoms were evaluated as described above.

Statistical analysis. When inoculated in steamed potting mixture, the number of plants (12) used to assess pathogenicity impairment for each strain allowed statistical analysis with a survival data analysis method. This analysis estimated the survival function ($S(t)$), which is the probability that the failure time (typical

symptom appearance) is at least *t* for each plant, *t* being the time elapsed since the day of inoculation (27). A mean life time (MLT) was evaluated for the melon plants inoculated with a given revertant, provided that at least 1 of the 12 plants exhibited symptoms (49). MLT were compared with the Logrank test.

Isolation of revertants after inoculation. To verify that wilt symptoms were caused by the introduced Fusarium strains, isolation from H_2O_2 surface-sterilized root or stem fragments was conducted as follows: root pieces and stem pieces were dipped in a 5% H_2O_2 solution for 5 s and were respectively rinsed into three consecutive sterile-distilled H_2O dippings. The root and stem pieces were blotted dry onto sterile filter paper and cut into 3- to 4-mm fragments. These fragments were placed on Malt extract agar medium (10 g liter^{-1}) (Biokar Diagnosis, Beauvais, France) supplemented with citric acid (250 mg liter^{-1}) in petri dishes and incubated for 24 to 72 h at 25°C. The appearance of *F. oxysporum* hyphae at the fragment ends was checked every 24 h. Similarly, isolation was performed from root and stem fragments of asymptomatic plants to assess whether the inoculated *Fusarium* sp. had penetrated the root.

RESULTS

Selection of somatic excision. The mutant strains, *niaD*::*imp160* and *niaD*::*imp161*, resulting from the insertion of an *impala* copy in the *niaD* gene, were highly unstable, giving rise to a large number of nitrate-utilizing colonies (up to 100 colonies per petri dish). These colonies were easily visualized on nitrate-MM as patches of aerial mycelium (revertants) in a background of sparse mycelium (mutant) and resulted from the excision of the *impala* copy inserted in *niaD*, thus allowing the fungal strain to utilize nitrate. The excision frequency was difficult to estimate, firstly because *niaD* mutant spores germinate and develop a very sparse mycelium on MM, increasing the number of nuclei; secondly, although multiple reversion events could occur on the same microthallus, they could collectively only be scored as a single reversion.

Fate of the excised copy. The low representation of *impala* in the genome of the TR7 isolate (Fig. 1), consisting of four major hybridizing bands that correspond to genomic copies (*impA*, *impB*, *impC*, and *impE*) belonging to the E subfamily and two low-intensity bands corresponding to subfamilies F *(impF*) and D (*impD*) (26), offers the opportunity to follow the variation in copy number associated with transposition events. In the mutant strain *niaD*::*imp161*, there is one additional copy, corresponding to that inserted in the *niaD* gene, whereas in strain *niaD*::*imp160*, we observed two additional copies, one inserted in *niaD* and the other elsewhere. Sequencing of genomic copies indicate that *imp160* and *imp161* are identical to *impE* and thus assumed to derive from a duplicative transposition of *impE* (26).

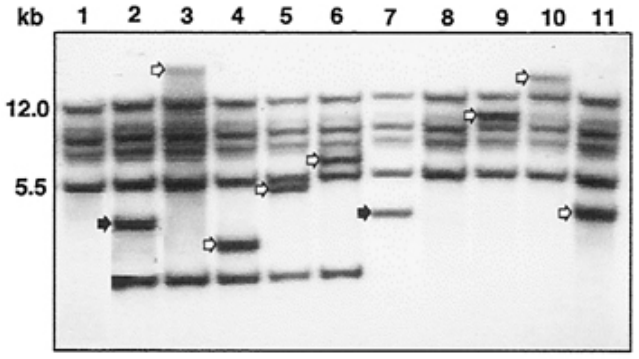

Fig. 1. Transposition of *impala* in *Fusarium oxysporum* f. sp. *melonis*. Each lane contains genomic DNA digested with *Eco*RI and probed with the cloned *imp160* copy. From left to right; lane 1: transformant TR7 showing four major hybridizing bands corresponding to copies belonging to the E subfamily and two low-intensity bands corresponding to copies belonging to the D subfamily (26); lanes 2 and 7: mutants *niaD*::*imp160* and *niaD*::*imp161*, respectively, resulting from transposition of an *impala* copy in the *niaD* gene; lanes 3 through 6: independent revertants from *niaD*::*imp160*; lanes 8 through 11: independent revertants from *niaD*::*imp161*. The position of *Eco*RI fragments bearing the *niaD*::*impala* gene is indicated by a filled arrow. The position of *Eco*RI fragments bearing the reinserted *impala* copy is indicated by an empty arrow. Molecular weight marker bands (in kilobases) are indicated in the left margin.

Southern blot analysis of 34 revertants digested with *Eco*RI and probed with a cloned *impala* copy showed that the excision of the *impala* copy from the *niaD* gene was associated in the majority of the cases (more than 70%) with its reintegration into a new genomic position (Table 1). This frequency has been confirmed with the restriction enzyme *Xba*I which has no restriction sites within *impala* (data not shown). The pattern of reinsertion of the transposed *impala* copy was different for most of the revertants analyzed, indicating that reintegration occurred at different sites in the genome and that each revertant corresponds to an independent transposition event. In addition, the pattern of *impala* insertion appeared to be very stable in culture. Indeed, no modification in the genomic position of endogenous copies as well as the new transposed copy has been observed through subculturing in a sample of 40 revertants analyzed (data not shown). The pattern of the reinserted *impala* copy was analyzed with chromosomal digests obtained with the rare-cutting enzyme *Not*I, which generates fragments ranging from 30 to 900 kb. Transposition of *impala* in the 10 revertants analyzed occurred on different *Not*I-restricted fragments (Fig. 2), suggesting that *impala* does not transpose preferentially to sites in the proximity of the donor site and is potentially able to mutagenize the whole genome.

Pathogenicity tests and characterization of pathogenicity mutants. Isolate FOM24, transformant strain TR7, and *niaD*::*imp160* and *niaD*::*imp161* mutants caused typical Fusarium wilt symptoms on the susceptible melon cv. Jerac within 22 to 28 days after inoculation, whereas antagonistic *F. oxysporum* strain FO47 did not affect melon.

A total of 746 revertants were tested on melon in order to detect those impaired in their pathogenic potential. Most of the revertants tested caused Fusarium wilt symptoms on the six inoculated plants within 28 to 35 days. In the case of 30 revertants tested, five or six plants were still healthy 35 days after the inoculation (data not shown). These revertants were selected for subsequent pathogenicity tests.

TABLE 1. Summary of *impala* reinsertion data. Southern blot analysis was carried out with *Eco*RI-digested DNAs from 34 *F. oxysporum* f. sp. *melonis* revertants derived from mutants *niaD*::*imp160* and *niaD*::*imp161* and hybridized to the *impala* probe

Revertants from *niaD*::*imp161*	Reinsertion (size in kb)[z]	Revertants from *niaD*::*imp160*	Reinsertion (size in kb)[z]
Rev3	–	Rev2	–
Rev4	–	Rev3	+ (6.6)
Rev5	+ (10.5)	Rev4	+ (3.2)
Rev6	–	Rev6	+ (2.0)
Rev7	+ (10.5)	Rev7	+ (6.1)
Rev8	+ (3.7)	Rev8	+ (4.9)
Rev9	+ (7.8)	Rev9	+ (6.0)
Rev20	+ (9.0)	Rev23	+ (2.0)
Rev24	+ (11.0)	Rev24	+ (5.2)
Rev25	+ (4.0)	Rev25	+ (5.8)
Rev26	+ (11.5)	Rev26	+ (12.0)
Rev27	+ (2.4)	Rev27	–
Rev28	–	Rev28	–
Rev29	+ (4.0)	Rev29	+ (12.0)
Rev30	+ (9.0)	Rev30	–
Rev32	–	Rev249	+ (6.0)
Rev34	+ (7.2)	…	…
Rev35	+ (12.0)	…	…

[z] – Indicates the transposed *impala* copy has not reinserted into a new genomic position upon excision from the donor site. + Indicates the transposed *impala* copy has reinserted into a new genomic position (the molecular weight of the new hybridizing *Eco*RI fragment is indicated in brackets).

The first test performed in the growth chamber confirmed that 27 of the 30 revertants selected in the large-scale screening were altered in their pathogenicity; twenty-three of the thirty did not cause any symptom on melon plants and four revertants caused wilt on one of the six plants. Three other revertants caused wilt on two or four plants: they were therefore considered as having retained their pathogenic potential toward melon and consequently discarded. However, wilting of the plants (two sets of six plants) in the presence of strain FOM24 was achieved gradually: nine plants suffered from Fusarium wilt within 26 days after inoculation, two more within 41 days, and the last plant survived until day 51.

According to the kinetics of wilt symptom development in the second pathogenicity test, the strains were clustered into three classes. This grouping was well represented by evaluating the survival function $S(t)$ of the population of melons of each class according to the Kaplan-Meier estimate (Fig. 3). Class 1 grouped two strains that never induced Fusarium wilt symptoms on the host plant (Table 2). Revertants of this class (rev127 and rev157) behaved similarly to the nonpathogenic *F. oxysporum* strain FO47. Class 2 and class 3 grouped 15 and 9 revertants that caused symptoms after more than 50 and 30 days from inoculation, respectively (Table 2; Fig. 3).

The MLT of melon plants submitted to revertants of class 1 could not be evaluated because no plant mortality occurred during the test. Although the kinetics of Fusarium wilt development were different (aggressiveness of revertants grouped in class 3 was noticeable at an earlier stage compared with revertants grouped in class 2), MLT of melons of these two classes were not significantly different ($P = 0.05$) (Table 3; Fig. 3).

DISCUSSION

As demonstrated in other pathosystems, the identification of pathogenicity mutants has the great potential for finding novel genes that encode determinants of pathogenicity, so that these steps may be targeted for the development of efficient and durable disease control (5,7,17,29). Autonomous copies of elements belonging to the *Tc1-mariner* class have been identified in *F. oxysporum* (12,25,37), and the properties of their transposition appear pertinent when designing a transposon tagging method. Therefore, we decided to test the efficacy of the *impala* element in generating pathogenicity mutants of *F. oxysporum* f. sp. *melonis*. An experimental design was set up allowing the selection of *impala* transposition and the subsequent mutational events. Selection of transposition events was followed by a phenotypic assay based on the restoration of *niaD* expression upon excision. The reinsertion of the excised copy was determined by Southern blot analysis, enabling recovery of independent transposition events which were screened for altered pathogenicity. In a first screening, the two mutants *niaD*::*imp160* and *niaD*::*imp161* and the transformed strain TR7 were as pathogenic on melon as the wild-type isolate FOM24, indicating that neither the transformation or the insertion of *impala* in *niaD* gene impaired the pathogenicity of the strains. The high proportion (3.5%) of revertants inducing no or slight symptoms of Fusarium wilt in this screening suggests that the *impala* element has a high mutagenic potential and that a large number of genes may be involved in the pathogenic potential of *F. oxysporum* f. sp. *melonis*.

Based on how fast melons inoculated with revertants exhibited typical Fusarium wilt symptoms, three classes were proposed to group revertants with similar pathogenicity. Although classes 2 and 3 had similar MLT, revertants grouped in class 2 showed a very long lag phase before causing symptoms, but once this phase elapsed, they showed a degree of virulence similar to that presented by the wild type. Conversely, revertants grouped in class 3 showed a shorter lag phase and symptoms appeared more gradually than in class 2. It is therefore obvious that revertants from classes 2 and 3, although still pathogenic toward the susceptible cultivar of melon used, are impaired in their pathogenicity, compared with the wild-type isolate FOM24, but different mechanisms are likely to be responsible for such deficiency. These mechanisms are part of the "pathogenic machinery" but are not essential to inhibit the full achievement of disease on the host plant. Saprophytic competence in the plant growth substrate, root colonization, or root penetration abilities might have been im-

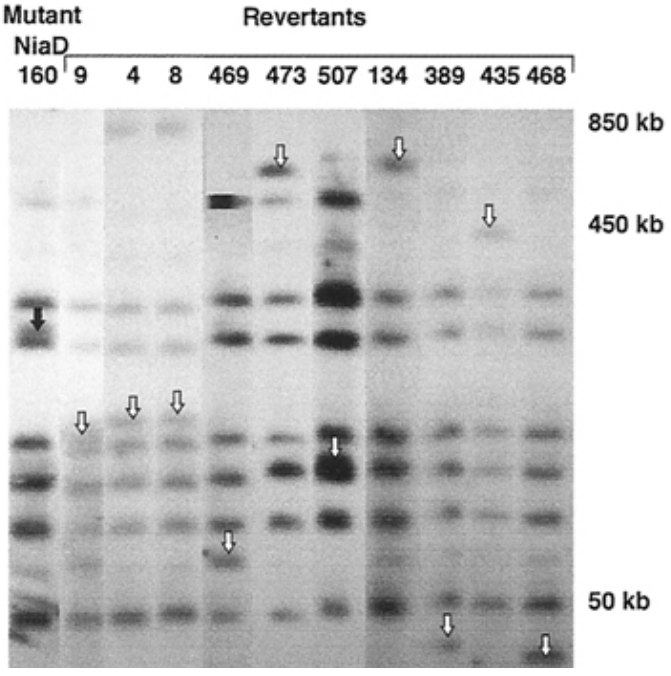

Fig. 2. Pattern of *impala* reinsertion on chromosomal DNAs from mutant *niaD*::*imp160* and 10 independent revertants digested with the rare-cutting enzyme *Not*I, separated by pulsed field gel electrophoresis, and probed with the *imp160* copy. The position of the *Not*I fragment bearing the *niaD*::*impala* gene in mutant *niaD*::*imp160* is indicated by a filled arrow. The position of *Not*I fragments bearing the reinserted *impala* copy in revertants is indicated by an empty arrow. Molecular weight marker bands (in kilobases) are indicated in the right margin.

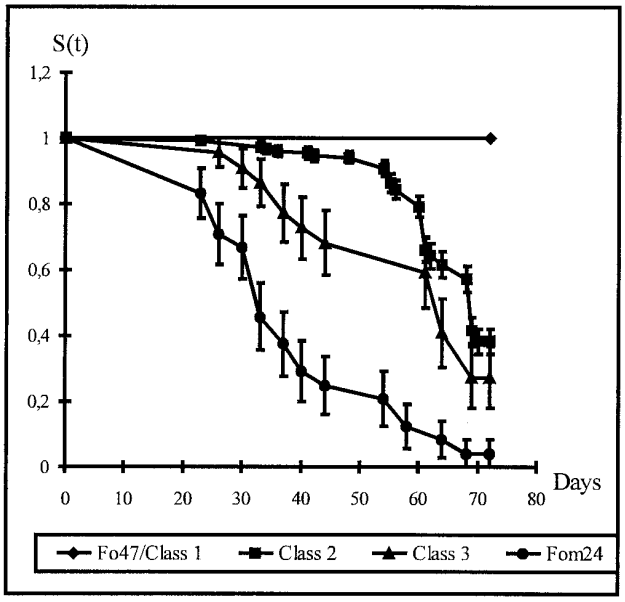

Fig. 3. Nonparametric estimation (Kaplan-Meier estimate) of the survival function of melon ($S(t)$) for each class of *Fusarium oxysporum* f. sp. *melonis* revertants. Classes were determined by how fast melons challenged with revertants exhibited typical Fusarium wilt symptoms.

paired. Although reduced in their pathogenicity, mutants of classes 2 and 3 were still capable of colonizing host vessels and growing beyond the site of penetration. Similar results were observed for nonpathogenic mutants of *Nectria haematococca* (29) and *Colletotrichum magna* (21,42).

Finally, 2 of the 746 revertants tested never induced any symptom on the susceptible host and can therefore be considered nonpathogenic mutants of strain FOM24. Rev157 was reisolated from roots but not from shoots of asymptomatic melon plants (data not shown); this revertant was still able to colonize and penetrate root tissues, but was unable to reach the vessels. This may explain why it did not cause symptoms of Fusarium wilt. Indeed, Olivain and Alabouvette (38,39) showed that both pathogenic and nonpathogenic strains of *F. oxysporum* were able to penetrate the root of a host plant but, while growth of the nonpathogenic strain was restricted to the superficial cell layers, the pathogen was able to colonize the vessels. Rev127 could also be altered in root colonization ability, as it was seldom reisolated from root tissues (data not shown). Population dynamics of revertants in the plant growth substrate, and on the roots of host plants, as well as cytological studies of root tissue colonization need to be performed to determine the precise mechanisms involved in the partial (classes 2 and 3) or total (class 1) impairment of pathogenicity in the revertants.

TABLE 2. Pathogenicity mutants selected, fate of transposed *impala* copy, and pathogenicity class of revertants from *Fusarium oxysporum* f. sp. *melonis niaD*::*imp160* and *niaD*::*imp161* tested on melon

Monoconidial culture	Revertants	Mutants	Code	Reinsertion of *impala* (kb)[y]	Class[z]
niaD::*imp160*					
C1	9	0			
C2	99	1	Rev69	+ (5.5)	2
C3	64	3	Rev384	–	2
			Rev389	+ (12.0)	2
			Rev401	NT	3
C4	38	3	Rev427	+ (12.5)	2
			Rev429	+ (5.5)	2
			Rev435	+ (10.5)	2
C5	18	5	Rev446	+ (5.0)	2
			Rev449	+ (6.8)	2
			Rev451	+ (>12.0)	3
			Rev460	+ (11.0)	3
			Rev463	+ (>12.0)	P
C6	36	6	Rev467	–	3
			Rev468	+ (6.0)	P
			Rev469	+ (10.0)	2
			Rev473	+ (8.0)	2
			Rev483	–	3
			Rev493	+ (10.0)	2
C7	9	3	Rev505	+ (10.5)	2
			Rev507	+ (>12.0)	2
			Rev510	+ (6.8)	3
niaD::*imp161*					
C11	9	0			
C12	91	1	Rev55	+ (12.0)	2
C13	37	8	Rev124	–	P
			Rev125	+ (3.8)	3
			Rev127	+ (9.4)	1
			Rev134	+ (5.0)	3
			Rev144	+ (>12.0)	3
			Rev156	+ (>12.0)	L
			Rev157	+ (4.0)	1
			Rev160	NT	2
C14	142	8	NT	NT	NT
C15	165	4	NT	NT	NT
C16	29	0	NT	NT	NT

[y] + Indicates that the transposed *impala* copy has reinserted into a new genomic position, and the molecular weight of the new hybridizing *Eco*RI fragment is indicated in brackets. – Indicates that the transposed *impala* copy has not reinserted into a new genomic position upon excision from the donor site. NT = not tested.

[z] Pathogenicity class on melon in second test. P = revertant that retained pathogenicity toward melon. L = lost during subculturing.

The revertants affected in pathogenicity have been analyzed by Southern blot analysis in order to determine if the excised *impala* copy has reinserted in most of these mutants. The results indicate that in the majority (26 of 30), *impala* has reintegrated into a new genomic position. Among the revertants in which reinsertion has not occurred, two induced reduced symptoms on melon and two, selected in the same experiment, were not pathogenic. This result may be explained by the ability of the *impala* element to leave a footprint upon excision, generally 5 bp (31), thus causing a switch in the ORF.

Although most of the mutants present a reintegration, the fact that some of them have lost the transposed copy addresses several questions about the origin of the mutant phenotype: is the transposed copy stable through mitotic division under laboratory conditions or during the interaction with the host plant? Is it stable under stress conditions? Are other transposable elements present in *F. oxysporum* f. sp. *melonis* mobilized at the same rate of *impala*? Are there chromosomal rearrangements?

The distribution of *impala* copies on chromosomes of *F. oxysporum* f. sp. *melonis* separated by CHEF electrophoresis was previously determined by flanking regions of cloned *impala* copies (26) from a genomic cosmid library of isolate FOM24 (15). The *impala* copies appeared to be randomly distributed on the chromosomes; with the exception of the copies coded *impD* and *impF*, which were located on a doublet of 4.8 Mb, the other copies marked specifically a different chromosome each (14). Mapping of new transposed *impala* elements showed that *impala* is able to transpose onto different chromosomes. These data, together with the fact that different *Not*I-digests hybridized to the transposed *impala* copy in all the revertants analyzed, indicate that *impala* does not transpose preferentially in the proximity of the donor site and is potentially able to mutagenize the entire genome.

Although the new mutants obtained in the present study are not yet fully characterized, the high percentage of revertants impaired in their pathogenic potential suggests that *impala* has a great advantage over other insertional mutagenesis techniques, which were reported to have efficiencies of 0.4 to 1.4% (5,7,17, 29,48).

An additional advantage of transposon-mediated mutagenesis is the reversibility of the insertion process which can be of great importance in asexual fungi to confirm that the mutant phenotype is associated with the insertion of the transposon at a specific site (37). However, to use efficiently the active elements as gene tags, it is necessary to better stabilize the mutagenic copy after transposition. Efficient mutagenic elements are being constructed by linking the ORF of the transposons *impala* and *Fot*I to strong promoters from *A. nidulans* genes, both constitutive (*gpdA*) or inducible (*alcA*) (18). These constructs will be associated with a nonautonomous marked element, thus providing the basis for a two-component system as described for plants (6,24).

TABLE 3. Estimation of mean life time (MLT) for melon seedlings inoculated with the nonpathogenic strain FO47, the pathogenic strain FOM24, or any of the 26 revertants altered in their pathogenicity

Class[x]	Number of isolates[y]	MLT in days[z]
FO47	1	>72
1	2	>72
2	15	64.23 (0.73) a
3	9	56.64 (3.33) a
FOM24	1	38.87 (3.04) b

[x] Revertants were grouped in 3 classes according to speed of typical Fusarium wilt symptom development.

[y] Test performed on two sets of 12 plants.

[z] MLT was calculated based on a nonparametric estimation (Kaplan-Meier estimate) of the survival function of melon ($S(t)$) of each class. MLT could not be calculated for FO47 and revertants of class 1 because no failure occurred. Values followed by different letters are significantly different according to Logrank test ($P = 0.05$).

ACKNOWLEDGMENTS

Research supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST 40% "Genetica di funghi fitopatogeni") and by the Ministère de l'Education Nationale, de la Recherche et de la Technologie et du Centre National de la Recherche Scientifique.

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