Research Note

Gain of Virulence Caused by Insertion of a Pot3 Transposon in a *Magnaporthe grisea* Avirulence Gene

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The avirulence gene *AVR-Pita* in *Magnaporthe grisea* prevents the fungus from infecting rice cultivars carrying the disease resistance gene *Pi-ta*. Insertion of Pot3 transposon into the promoter of *AVR-Pita* caused the gain of virulence toward Yashiro-mochi, a rice cultivar containing *Pi-ta*, which demonstrated the ability of Pot3 to move within the *M. grisea* genome. The appearance of Pot3 in *M. grisea* seems to predate the diversification of various host-specific forms of the fungus.

Additional keywords: *Pyricularia grisea*; race variation.

Rice blast caused by *Magnaporthe grisea* (Hebert) Barr. (anamorph *Pyricularia grisea* Sacc.) is one of the most economically devastating crop diseases worldwide (Zeigler et al. 1994). Management of rice blast through the breeding of blast-resistant cultivars has had only limited success as a result of the frequent breakdown of resistance under field conditions (Zeigler et al. 1994). The frequent appearance of new races or pathotypes of the fungus has been proposed as the principal mechanism involved in the loss of resistance. Because the compatibility of interaction between *M. grisea* and rice follows the gene-for-gene model (Bryan et al. 2000; Jia et al. 2000; Orbach et al. 2000; Zeigler et al. 1994), it has been hypothesized that loss-of-function mutations of the avirulence gene could lead to the generation of new races. Cloning avirulence genes from *M. grisea* showed that such mutations frequently occur in some isolates (Orbach et al. 2000; Sweigard et al. 1995). Analysis of the spontaneous mutants of *M. grisea*, which gained virulence on weeping lovegrass, showed that *PWL2*, an avirulence gene preventing the fungus from infecting this plant, had been deleted in these mutants (Sweigard et al. 1995). The *AVR-Pita* gene, which corresponds in a gene-for-gene fashion to the disease resistance

gene *Pi-ta*, was cloned and characterized (Orbach et al. 2000).

The *AVR-Pita* gene is located entirely within a 1.5-kb telomeric fragment at one end of chromosome 3, and it encodes a putative metalloprotease (Orbach et al. 2000). Spontaneous mutants of *M. grisea* that had gained virulence specifically toward Yashiro-mochi, a rice cultivar carrying *Pi-ta*, exhibited diverse mutations in *AVR-Pita*. Three of the 11 mutants characterized by Orbach et al. (2000) contained point mutations in the *AVR-Pita* coding sequence and seven contained deletions of *AVR-Pita* gene sequences. The remaining mutant, CP1632, contained a 1.8-kb insertion located between the *SacI* and *EcoRI* sites of *AVR-Pita* (Fig. 1). In this report, we describe the cloning and characterization of this insertion.

Southern analysis of CP1632 with several restriction fragments between the *SacI* and *EcoRI* sites as probes showed that the insertion occurred between the *ApaI* and the *HindIII* site nearest to the open reading frame (ORF) of *AVR-Pita* (Fig. 1). Genomic DNA of CP1632 was amplified by polymerase chain reaction (PCR) with primers F3 and F4 flanking the *HindIII* and *ApaI* sites, respectively (Fig. 1). A 2-kb fragment amplified by PCR hybridized to the 500-bp probe A, which spans the insertion site (Fig. 1), confirming the successful amplification of the desired product. The amplified fragment hybridized to a large number of bands in CP1632 and other rice pathogens of *M. grisea*, suggesting that the insertion corresponds to a repetitive DNA element. The fragment was treated with T4 DNA polymerase in the presence of deoxynucleoside triphosphates to make its ends blunt, then digested with *HindIII* and subsequently cloned into a *SmaI*–*HindIII*-digested pBluescriptSK (Stratagene, La Jolla, CA, U.S.A.).

Sequence of the insertion in CP1632 showed that it belongs to the transposon family Pot3. The insertion site of Pot3 in *AVR-Pita* was located 304 bp upstream from the initiation codon of the *AVR-Pita* ORF. Orbach et al. (2000) used complementation analysis to show that subclones containing at least 477 bp of the 5' sequence were active in conferring avirulence to Yashiro-mochi when transformed into a virulent fungal strain, but neither the telomeric *HindIII* fragment with 219 bp of the 5' sequence nor the telomeric *ApaI* fragment with 389 bp of the 5' sequence was active in conferring avirulence (Fig. 1). Therefore, sequences essential for the gene function were located upstream from the insertion site. To rule out the possibility that an additional mutation in *AVR-Pita* caused the loss-of-gene function in CP1632, the 1,273-bp

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region of *AVR-Pita* between the telomeric repeat and the *Hind*III site (Fig. 1) was amplified by PCR and sequenced. The resulting sequence was identical to the wild-type allele. Thus, the insertion of Pot3 between the *Apa*I and *Hind*III sites is the only change detected relative to the functional *AVR-Pita* gene, and this insertion physically separated critical promoter sequences from the rest of the gene by 1.8 kb, causing the disruption of the avirulence gene expression.

Pot3 was identified originally as part of MGR586 (Farman et al. 1996a; Hamer et al. 1989), a repetitive DNA probe used extensively for population analysis of *M. grisea* (Levy et al. 1991; Zeigler et al. 1994). Pot3 contains two inverted terminal repeats (ITR) and one ORF exhibiting significant homology to transposases encoded by TC1/Mariner transposons (Farman et al. 1996a). The copy of Pot3 inserted in *AVR-Pita* (GenBank accession no. AF333034) was nearly identical to the previously sequenced copy (Farman et al. 1996a), with the following differences. As a result of one additional base pair near the 3' end of the ORF in the former, it (556 amino acids) is slightly larger than the latter (554 amino acids), and their carboxy termini (approximately 50 amino acids) are completely different. Transposition of Pot3 into *AVR-Pita* duplicated the target site (5'-TA-3'), which has been observed in other families of TC1/Mariner-like transposons, including Pot2 in *M. grisea* (Kachroo et al. 1994) and Fot1 in *Fusarium oxysporum* (Daboussi et al. 1992). To investigate whether any sequence preference exists for target sites of Pot3, eight independent cosmid clones carrying a single copy of Pot3 were isolated from a library of 4375-R-6 (the wild-type strain of CP1632). We determined the sequence of their ITR and flanking genomic DNA with primers P3 and P4 (Fig. 1). The ITR of six

of these Pot3 elements was identical to that of the copy inserted in *AVR-Pita*. The remaining two copies differed from the rest by either a point mutation in its 5' ITR or the absence of one copy of the direct repeat in its 3' ITR (not shown). Sequences flanking these elements did not exhibit significant similarity, except for the duplicated 2-bp target (Fig. 2).

On the basis of the nearly ubiquitous presence of sequences homologous to MGR586 among isolates from diverse hosts, it was hypothesized that the appearance of Pot3 in the *M. grisea* genome may predate the diversification of various host-specific forms of the fungus (Shull and Hamer 1994). To test this hypothesis rigorously, we surveyed 50 isolates from more than 20 host species other than rice for sequences homologous to Pot3 (Fig. 3). Southern analysis of the *Eco*RI-digested genomic DNAs of these isolates was carried out with probe B

Locus	Target Sequence
<i>AVR-Pita</i>	5'-TAATGCAGATAAT TA CACTAGCAAAAT-3'
Cosmid 1	5'-GCTGTGGTAGACT TA CTGGGTATTAGAC-3'
Cosmid 2	5'-TGCCCCATATAT TA TTCGTTAAAGGCGT-3'
Cosmid 3	5'-CAAACAAACAAC TA TTCCAAAGCTTT-3'
Cosmid 4	5'-TAACCATTATAAT TA TGGATTAAATCA-3'
Cosmid 5	5'-TTAGAACAATGACT TA TGTGTTTCTGCAT-3'
Cosmid 6	5'-CCTTTACCAAGGAT TA GAATAAAACGTC-3'
Cosmid 7	5'-AGGCTTTTTTCACT TA ATAAGGAACAGAT-3'
Cosmid 8	5'-TTGACATTAAGCAT TA TATTGTCCGCGCG-3'

Fig. 2. Comparison of the genomic sequences flanking nine copies of Pot3. The sequence duplicated by insertion of Pot3 (5'-TA-3') is in bold.

Fig. 1. Restriction map of the *AVR-Pita* locus in CP1632. Restriction sites for *Apa*I (A), *Eco*RI (E), *Eco*NI (En), *Hind*III (H), *Nco*I, and *Sac*I (Sc) are marked. The telomeric repeat sequence (5'-AACCCT-3') was denoted by (AACCCT)_n. The open reading frame of *AVR-Pita* is shown below the restriction map. Two arrowheads below the restriction map of the *AVR-Pita* locus indicate the primers used to amplify the inserted sequence in CP1632: F3 (5'-TTATTATTTTCAGCTTGACT-3') and F4 (5'-CATGAATTATGTAGTTATT-3'). Probe A was used to confirm the successful amplification of the inserted DNA. The *Sac*I-*Eco*NI fragment underneath the restriction map of Pot3, labeled probe B, was used to survey the distribution pattern of Pot3 (Fig. 3). Location of the two primers for determining the sequences flanking Pot3, P3 (5'-GCTTCGCAATTTGATGATTC-3') and P4 (5'-GATGTACATATATCCGATTG-3'), is shown underneath the restriction map of Pot3. The location and sizes (in base pairs) of three subclones of the *AVR-Pita* locus, which were used to determine the sequences required for the gene function (Orbach et al. 2000), are indicated below the restriction map. A 1,531-bp telomere end fragment was active (+) in conferring avirulence toward cultivar Yashiro-Mochi, but the 1,443-bp *Apa*I fragment and the 1,273-bp *Hind*III fragment were inactive (-) (Orbach et al. 2000).

(Fig. 1) under high-stringency conditions. Because there is an *Eco*RI site in the middle of probe B, the copy number of Pot3 is likely to be about one-half of the number of bands observed. All the isolates except two, G-227 from ginger and G-229 from *Cyperus* spp., contained one to 50 bands that hybridized to the probe. This result confirms that Pot3 is nearly ubiqui-

tous in *M. grisea*, thus supporting the hypothesis of Shull and Hamer (1994).

An isolate from barley, G-197, and three isolates from *Penisetum* spp., G-222, G-223, and G-224, had a high copy number of Pot3, comparable to that of isolates from rice. Isolate G-197 has a mitochondrial DNA type that is identical to

Fig. 3. Distribution patterns of Pot3 among isolates of *Magnaporthe grisea* from diverse host species other than rice. Genomic DNA of these isolates was digested with *Eco*RI, separated by electrophoresis on a 0.7% agarose gel, and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia, Buckinghamshire, U.K.). Hybridization of the membranes with labeled probe B (Fig. 1) and subsequent washings were performed in highly stringent conditions: 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt's reagent, and 0.1% sodium dodecyl sulfate (SDS) for hybridization, followed by a 30-min. wash at 65°C in 1× SSC and 0.1% SDS and an additional 30-min. wash in 0.2× SSC and 0.1% SDS. The genus (or common name) of the host plant from which individual strains were isolated is shown on the top of each figure. Detailed information on the origin of these strains is in Kang et al. (1995).

isolates from rice and infects rice and barley (B. Valent, *unpublished*), suggesting that G-197 is highly related to rice pathogens. Isolates from *Pennisetum* spp., however, are genetically distinct from rice pathogens (Shull and Hamer 1994) and are not pathogenic on rice. The high copy number of Pot3 in such isolates has resulted from amplification events independent of the amplification observed in the genome of isolates pathogenic on rice. A survey of isolates from *Oryza* spp. showed that two strains (O-312 and O-315) isolated from two wild rice species, *Oryza glaberrima* and *Oryza longistaminata*, respectively, contained less than 10 copies of Pot3 (S. Kang, *unpublished results*). These results indicate that the presence of a high copy number of Pot3 is not necessarily a unique characteristic of strains infecting rice.

It has been suggested that genetic changes caused by transposons may be one of the mechanisms responsible for race variation (Shull and Hamer 1994; Talbot et al. 1993; Valent and Chumley 1991). Recombination between members of the same family may cause such mutations as deletion, inversion, duplication, and translocation, depending on the relative orientation and position of the members that are recombined. In addition, some transposons are mobile and, as a result of insertion or excision, can cause mutations. Different types of transposons have been identified in *M. grisea* (Dobinson et al. 1993; Farman et al. 1996a; Farman et al. 1996b; Hamer et al. 1989; Kachroo et al. 1994; Kachroo et al. 1995; Sone et al. 1993; Valent and Chumley 1991). These include retrotransposons that transpose through an RNA intermediate and DNA transposons that directly transpose by a cut-and-paste mechanism. Two of the retrotransposons, MAGGY and MGL, have been shown to transpose (Nishimura et al. 2000; Shull and Hamer 1996). Our work demonstrates that the DNA transposon Pot3 can transpose and provides evidence that its transposition can change the virulence spectrum of *M. grisea*.

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