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Characterization of the genome of *Sclerospora graminicola*, the causal fungus of downy mildew of pearl millet

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A genomic library of the most virulent pathotype path-6 (7042S) of *Sclerospora graminicola*, the causal fungus of downy mildew in pearl millet, was constructed in the lambda gt11 vector. Repetitive DNA content was estimated at about 8% based on colony hybridization of total fungal DNA with a genomic library. Digestion with isoschizomeric methylation-sensitive restriction enzymes revealed partial methylation of GATC and CCGG sequences in the genome. The presence of retrotransposable elements in the genome was detected by amplification of part of the reverse transcriptase gene in a PCR reaction, using a specific set of primers designed for the conserved region.

Key words: Genomic library, pearl millet, repetitive DNA, retrotransposon elements, *Sclerospora graminicola*.

Downy mildew caused by *Sclerospora graminicola* (Sacc) Schroet is the most destructive and widespread disease of pearl millet (*Pennisetum glaucum* (L) R.Br) in the tropics and subtropics of the Asian and African continents. Pearl millet is an important cereal crop in these regions because of its ability to grow on soils of marginal fertility in semi-arid conditions. The fungus systemically colonizes stems, leaves and apical meristem. The infection leads to chlorosis, stunting and proliferation of floral parts and is also called 'green ear' disease (Ball 1983). Until recently, the downy mildews received very little attention due to difficulties of maintaining the isolates on the living host plants in controlled environments and long-term storage of viable inoculum. The diploid status of the mycelium and spores, heterothallism and the difficulty of recovering progeny from oospores make genetic studies difficult (Shaw 1983). The pathogen is highly variable and several host genotype-specific pathotypes have been reported (Thakur *et al.* 1992).

An in-depth knowledge of the genetics of the host–pathogen interaction is essential for the understanding of the mechanism of variability in the pathogen population and for the development of strategies to control the disease. As a first step in this direction, we demonstrated that DNA fingerprinting could be used to detect genetic variability in *S. graminicola* (Sastry *et al.* 1995). In this report genome analysis of the fungus with reference to the presence of repeat elements and retrotransposons is described.

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Materials and Methods

Fungal Material

Isolates of *S. graminicola* were maintained on seedlings of pearl millet genotypes by repeated inoculation with asexual spores in isolation chambers in a greenhouse. We analysed the isolates of six host genotype-specific pathotypes, namely Path-1 (NHB3), Path-2 (BJ 104), Path-3 (MBH 110), Path-4 (852B), Path-5 (700651), and Path-6 (7042S) (Thakur *et al.* 1992).

Genomic Library Construction

DNA was isolated from sporangia of a highly virulent pathotype (path-6) as described by Sastry *et al.* (1995). The DNA was digested extensively with *Eco*RI to obtain fragment sizes mainly less than 4 kb. The genomic DNA fragments generated by *Eco*RI were further purified and ligated to lambda gt11 (Stratagene, Germany) arms according to the manufacturer's protocol. Effectiveness of the ligation reaction was checked by electrophoresis through a 0.6% agarose gel. Recombinant lambda phage arms were packaged using Gigapack II Gold packaging extract

(Stratagene, Germany). These phage particles were adsorbed to the strain Y1090R and plated to check the packaging efficiency which was 1×10^7 recombinant plaques ml^{-1}.

Screening the Genomic Library

The original stock of lysate was adsorbed on to the bacterial strain 1090R. The adsorbed bacterial cells were plated on 150-mm NZY (HiMedia, India) agar media plates with NZY top agarose. The plates were incubated overnight at 37 °C and then at 4 °C for few hours. The plaques were transferred to nitrocellulose filters and colony hybridization was carried out (Sambrook *et al.* 1989). The plaques of interest were isolated and suspended in SM buffer [0.1 M NaCl, 0.015 M $MgSO_4$, 50 M Tris/HCl (pH 7.5), 0.01% gelatin] with a drop of chloroform. The plaques were further purified by subsequent screening.

Restriction Enzyme Digestion and Southern Hybridization

A number of restriction enzymes [*Alu*I, *Bam*HI, *Bgl*II, *Eco*RV, *Hae*III, *Hinf*I, *Hind*III, *Hpa*II, *Msp*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, and *Taq*I (Amersham, UK and New England Biolabs, USA)] were used to digest pathogen genomic DNA. Methylation-sensitive isoschizomeric enzymes such as *Msp*I, *Hpa*II, *Mbo*I, *Sau*3AI and *Dpn*I (Amersham, UK) were also used. Approximately 5–8 μg of DNA was digested and the DNA fragments were separated by electrophoresis through 0.8% agarose gels in TPE buffer (90 mM Tris–phosphate, 2 mM EDTA, pH 7.5) followed by transfer to nylon filters by Southern blotting. The filters were hybridized with different DNA probes labelled with [α-^{32}P]-dCTP (Sambrook *et al.* 1989), and detected by overnight exposure to X-ray film (Indu, India).

DNA Amplification through Polymerase Chain Reaction

Based on the conserved sequences identified by Xiong & Eickbush (1990) in box 1 and 5 of the reverse transcriptase gene sequence, Weichman & van den Bussche (1992) designed PCR primers to amplify a fragment of about 343–396 bp. The same set of primers was used to amplify part of the reverse transcriptase gene from the *S. graminicola* genome. Amplification reactions were performed with 50 ng genomic DNA, 200 μM of dNTPs (New England Biolabs, USA), 40 ng of each primer and 1 unit of Taq polymerase (Bangalore Genei, India) in a 25 μl reaction mix using an MJR Thermocycler (MJ Scientific, USA). Amplification was for 40 cycles with an initial denaturation at 93 °C for 4 min, followed by a temperature regime of 93 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplified products were analysed by electrophoresis through

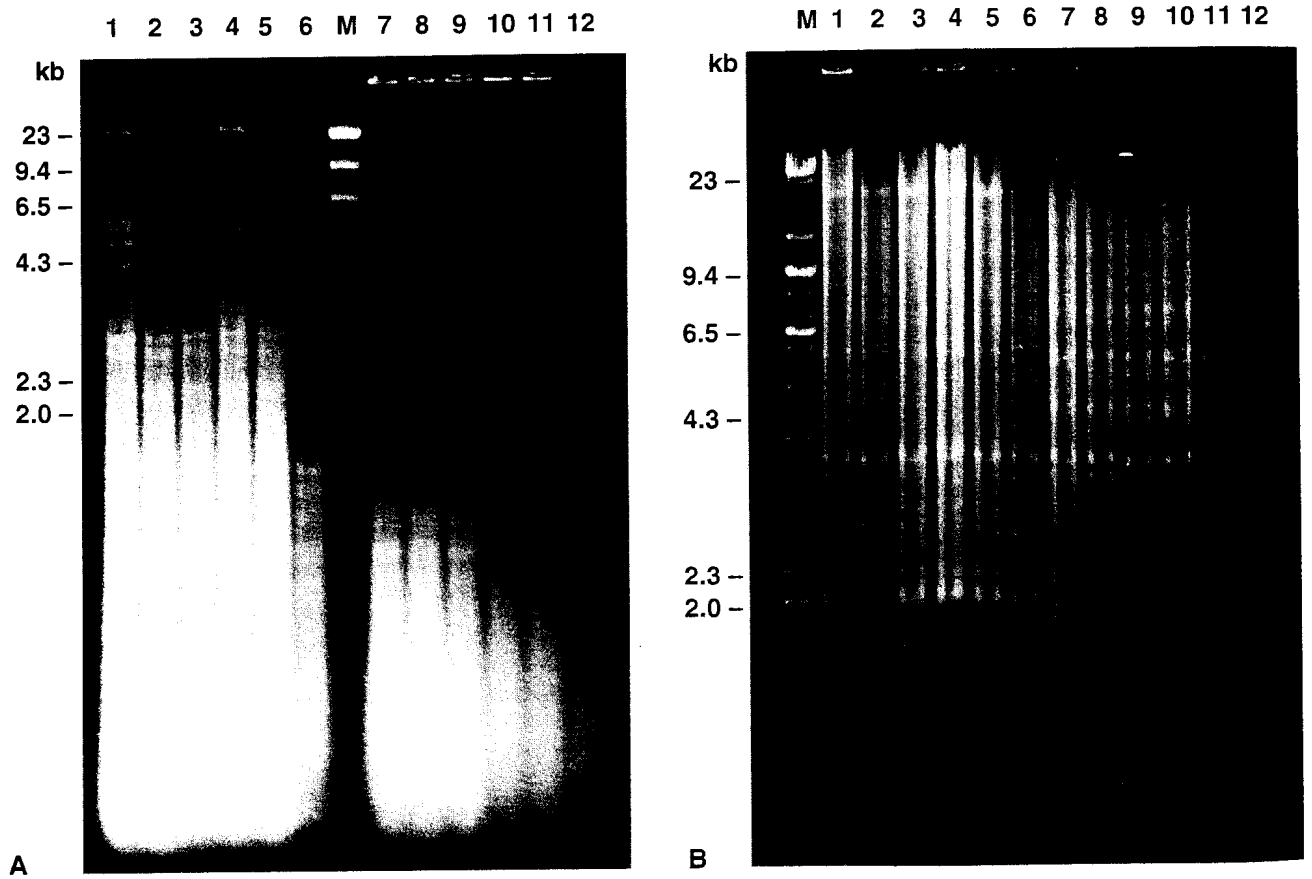

Figure 1. (A) Restriction enzyme digestion pattern of the six pathotypes digested with *Msp*I (lanes 1–6) and *Taq*I (lanes 7–12) electrophoresed through an agarose gel: lanes 1 and 7, Path-1 (NHB3); lanes 2 and 8, Path-2 (BJ 104); lanes 3 and 9, Path-3 (MBH 110); lanes 4 and 10, Path-4 (852B); lanes 5 and 11, Path-5 (700651); lanes 6 and 12, Path-6 (7042S); M, molecular weight marker: a mixture of lambda *Hind*III digest and ϕX 174 *Hae*III digest. (B) Restriction enzyme digestion pattern of the six pathotypes digested with *Bam*HI (lanes 1–6) and *Hind*III (lanes 7–12) and electrophoresed through an agarose gel: lanes 1 and 7, Path-1 (NHB 3); lanes 2 and 8, Path-2 (BJ 104); lanes 3 and 9, Path-3 (MBH 110); lanes 4 and 10, Path-4 (852B); lanes 5 and 11, Path-5 (700651); lanes 6 and 12, Path-6 (7042S); M, molecular weight marker: lambda *Hind*III digest.

1.2% agarose gels in 1 × TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide staining and u.v. light.

Cytofluorometric Analysis of the Sporangial Population

Sporangia were collected in ice-cold PBS (pH 7.4, without Ca^{2+} and Mg^{2+} salts) isolated from sporulated leaves and passed through a sieve (12–22 μm) to remove leaf debris. Sporangia were then treated with absolute ethanol followed by 70% ethanol for 30 min at 4 °C and resuspended in PBS. They were treated with RNAse (10 μg/ml) for 30 min and then washed with PBS. Spores were incubated with (6.9×10^{-5} M) propidium iodide, (2.5 μg/50 ml Hoechst 33256) and 0.1 μg DAPI (4'6-diamidino-2-phenylindole) (Sigma USA)/ml PBS for 30 min at 4 °C followed by centrifugation, washing, and resuspension in PBS to a final concentration of 1×10^6 cells/ml. These stained sporangia were analysed in a Fac Star Plus (Becton and Dickinson, USA).

Results and Discussion

Restriction Enzyme Analysis

Restriction enzyme digestion and agarose gel electrophoresis showed a large number of bands on a background smear of ethidium bromide staining. This pattern was more prominent with tetracutter enzymes, such as *Taq*I, *Msp*I and *Hae*III in the range 4 kb–500 bp than with hexacutter enzymes. *Bam*HI and *Hind*III gave some prominent bands in the high molecular weight range of 2 kb and 23 kb (Figure 1A and 1B) indicating the presence of repeat elements occuring in a specific organized pattern in *S. graminicola*.

Identification of Repetitive Elements

The total repetitive DNA content in *S. graminicola* path-6 was about 8% of which 2.0% was highly repetitive and 6.0% moderately repetitive. This estimate of repetitive DNA is in agreement with those observed in other oomycete obligate fungi (Hulbert *et al.* 1988). Moderate levels (8%) of repetitive DNA was found in rice blast pathogen, *Magnaporthe grisea* (Hammer *et al.* 1989), and high levels (26 and 29%) were found in *Septoria tritici* and *Phytophthora infestans* (McDonald & Martinez 1990; Goodwin *et al.* 1992).

Among the highly repetitive clones in the genome of *S. graminicola*, eight clones giving very intense signals were selected for characterization. These clones were further purified to homogeneity through three cycles of plaque hybridization, and hybridized with genomic DNA digested with a selection of restriction enzymes (*Bam*HI, *Hind*III, *Eco*RV, and *Sal*I). All eight of the repeat elements showed a number of bands in Southern analysis (Figure 2). Multiple hybridizing bands of various molecular weights for all eight repeat elements indicated that a few copies of each repeat element are clustered in tandem arrays which are dispersed throughout the genome. This clustered and dispersed arrangement of these

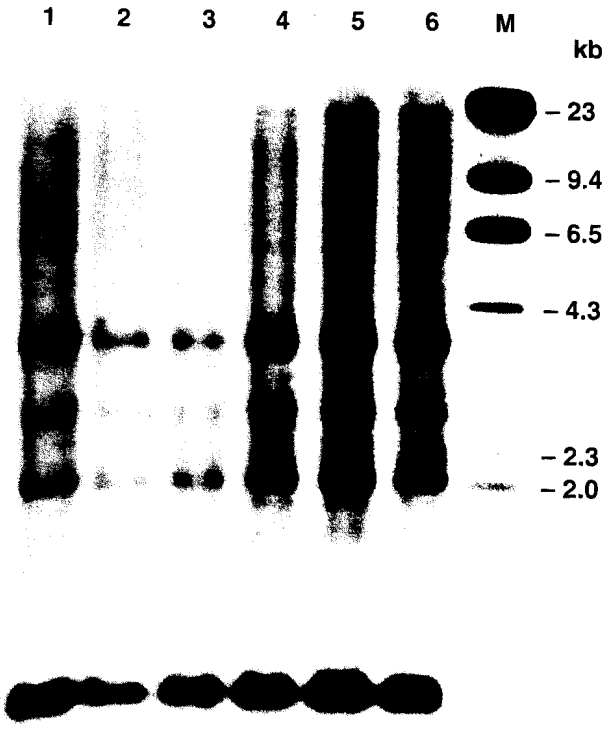

Figure 2. A representative autoradiogram showing the hybridization pattern of a 500 bp genomic repeat hybridized to *BamH*I-digested blot. Lane 1, Path-1 (NHB3); lane 2, Path-2 (BJ 104); lane 3, Path-3 (MBH 110); lane 4, Path-4 (852B); lane 5, Path-5 (700651); lane 6, Path-6 (7042S); M, molecular weight marker: Lambda *Hind*III-digest.

highly repetitive elements in *S. graminicola* was seen in all six pathotypes examined. In our earlier report (Sastry *et al.* 1995) we demonstrated the presence of microsatellites especially $(GATA)_n$ and minisatellites (PV47 and R18 like elements) in the genome of *S. graminicola*, confirming the clustered and dispersed arrangement of certain highly repetitive elements.

DNA Methylation

Although there was no difference between digestion patterns of enzymes *Hpa*II and *Msp*I, there were some differences in the repeat elements, indicating that CpC and CpG dinucleotide methylation is prominent in *S. graminicola* repeat elements (Figure 3A). Another isoschizomeric methylation-sensitive enzyme set, namely *Dpn*I, *Sau*3AI and *Mbo*I which recognizes the sequence GATC, showed that digestion by *Dpn*I was less extensive than by *Mbo*I and *Sau*3AI (Figure 3B). The band patterns observed in five isolates digested with *Mbo*I and *Sau*3AI were, however, almost identical. These data reveal that the adenine nucleotide in the GATC sequence of the genome is partially methylated, and that there is no cytosine methylation in the sequence GATC. The specific

variation in the electrophoretic pattern of repeat elements in the genomes digested with methylation-sensitive enzymes indicates the predominance of 'A' methylation, CpG and CpC methylation in GATC- and CCGG-containing sequences in *S. graminicola*.

Retrotransposable Elements

A prominent band of 400 bp was produced by the PCR (Figure 4) and was present even when the annealing conditions were changed. The P5 probe (McHale *et al.* 1989) which represents the conserved region of the reverse transcriptase of *Fulvia fulva*, hybridized to the PCR-amplified fragment, suggesting the presence of retrotransposon-like elements in the fungal genome (data not shown). Retrotransposons, mobile genetic elements, play an important role in genome evolution and constitute about 5–10% of the genome in eukaryotes (Bingham & Zachar 1989). Since these elements will be helpful in strain/race identification in *S. graminicola* (as already

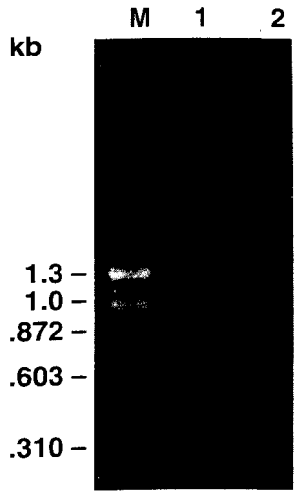

Figure 4. PCR amplified products from: lane 1, Path-1; lane 2, Path-6; M, φx174 *Hae*III digested molecular weight marker.

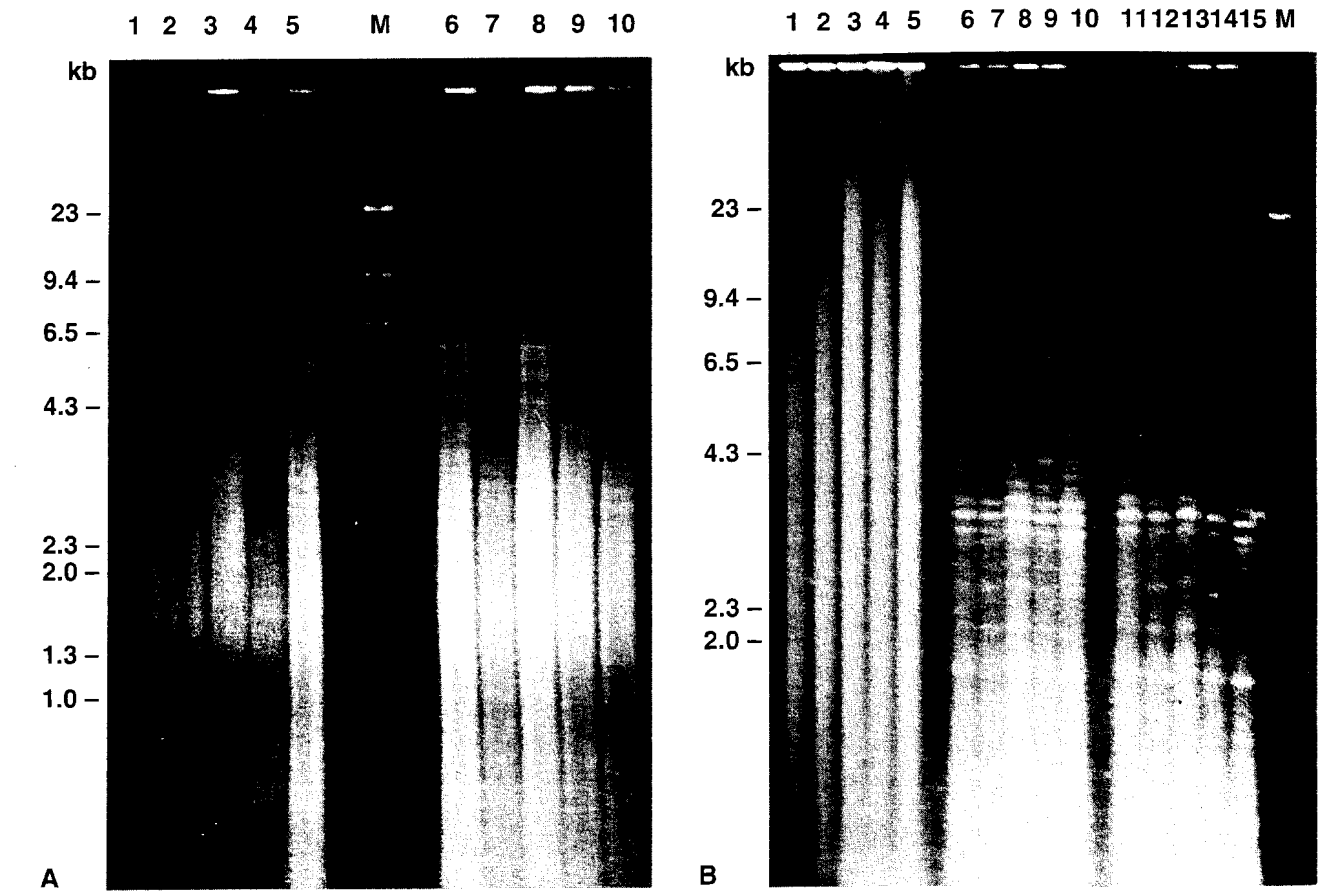

Figure 3. (A) Digestion pattern obtained with methylation-sensitive enzymes *Hpa*II and *Msp*I: lanes 1 and 6, Path-1 (NHB3); lanes 2 and 7. Path-2 (BJ 104); lanes 3 and 8, Path-3 (MBH 110); lanes 4 and 9, Path-4 (852B); lanes 5 and 10, Path-6 (7024S); M, Lambda *Hind*III-digested molecular weight marker. (B) Digestion pattern obtained with methylation sensitive enzymes *Dpn*I, *Sau*3AI and *Mbo*I: lanes 1, 6 and 11, Path-1 (NHB3); lanes 2, 7 and 12, Path-2 (BJ 104); lanes 3, 8 and 13, Path-3 (MBH 110); lanes 4, 9 and 14, Path-4 (852B); lanes 5, 10 and 15, Path-6 (7042S); M, Lambda *Hind*III-digested molecular weight marker.

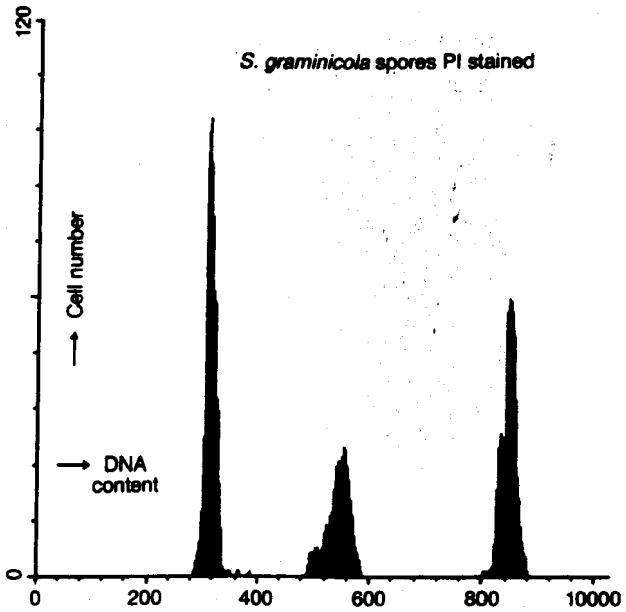

Figure 5. Cytoflurometric analysis of propidium iodide-stained sporangial cells. The x-axis represents DNA content (arbitary units) and the y-axis represents sporangia number.

shown in yeast (Pearson *et al.* 1995), sequencing the amplified products is in progress to confirm our findings.

Cytofluorometric Analysis of Sporangia

Propidium iodide-stained *S. graminicola* spores (Figure 5) revealed differences in ploidy level in the sporangial population; this included haploids, diploids and triploids, of which haploids and triploids were predominant. Cytological studies which yield direct chromosome counts are desirable to confirm the results. These ploidy differences in *S. graminicola* could be important in establishing population genetic diversity.

Fungal pathogens can evolve rapidly to overcome biotic and abiotic stresses. Obligate pathogens, such as *S. graminicola*, are more dynamic because of their ability of rapid host adaptation. Retrotransposons may generate variability which may be important for maintenance of virulence by fungal pathogens in response to the introduction of resistant varieties of crop species and the application of fungicides. Further studies will clarify the contribution of retrotransposons to evolution, virulence and adaptability of the *S. graminicola*.

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