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Induced Change in DNA Methylation of *Fusarium oxysporum* f. sp. *niveum* due to Successive Transfer

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Abstract : Changes in pathogenicity of old and successively-cultured isolates of *Fusarium oxysporum* f. sp. *niveum* have been observed and the concept that such cultures will become attenuated is generally accepted. However, the genetic basis for this phenomenon has not been studied. In an effort to identify a DNA marker closely linked to variations, DNA methylation was investigated both before and after the successive transfers of *F.* o. f. sp. *niveum* isolates on artificial media. A sector of mycelium in *F.* o. f. sp. *niveum* race 2 isolate (TX-XID) which showed variation in pigmentation and colonial morphology occurred after 18 successive weekly transfers on potato dextrose agar (PDA). The sector characteristics were stable and did not change after more successive transfers. It was shown that DNA methylation preexists in ribosomal RNA gene (rDNA) of *F.* o. f. sp. *niveum* and that additional changes in DNA methylation occurred during successive culturing.

Key words : DNA methylation, Fusarium wilt, isoschizomer, rDNA

Although there is a close relationship in *Fusarium oxysporum* f. sp. *niveum* between vegetative compatibility groups (VCG) and pathological races (Larkin *et al.*, 1990), the genes that control VCG (*vic* gene) are as complex as those controlling pathogenicity (Puhalla, 1984). However, no *vic* gene or any other simply-inherited closely-linked marker to a gene affecting pathogenicity has been found in *F. oxysporum.* Variations in pathogenicity of old and successively-cultured isolates of *Fusarium oxysporum* f. sp. *niveum* have been observed (R. D. Martyn, personal communication). However, the genetic basis for this phenomenon has not been studied. In an effort to identify a DNA marker closely linked to pathogenicity, DNA methylation was investigated both before and after the successive transfers of *F.* o. f. sp. *niveum* isolates on artificial media.

DNA methylation is involved in restriction-modification systems, in strand discrimination during mismatch repair, in the regulation of gene expression, and in the differentiation of specialized tissue (Magill and Magill, 1989; Razin and Cedar, 1991). Only four major bases (G, A, T, C) are incorporated during DNA replication and methylated or other modified bases are formed enzymatically on the DNA after replication. The known methylase uses S-adenosylmethionine (SAM) as a methyl group donor and has specific sites for modification. In those fungi and higher eukaryotes that have been tested, only 5-methylcytosine (5 mC) has been found in the DNA (Magill and Magill, 1989). Several studies have shown that 5 mC is mostly located in the CG dinucleotide (Antequera *et al.*, 1984; Wang *et al.*, 1996) and that most CG dinucleotides are in CCGG and CGCG sequences (Whittaker and Hardman, 1980; Zolan and Pukkila, 1985). Specht *et al.* (1984) have shown that methylated CCGG sequences are not randomly dispersed throughout the genome but often occur in repeated sequences such as the rRNA genes (rDNA) of *Schizophyllum commune*. Similar results were obtained in *Neurospora crassa* (Russell *et al.*, 1985). However, no complete methylation of all methylatable sequences has been found in eukaryotic DNA. In order to detect DNA methylation, several methods have been empolyed including paper chromatography (Evans and Evans, 1970), high pressure liquid chromatography (HPLC) (Russell *et al.*, 1985), bisulfite treatment followed by sequencing (Frommer *et al.*, 1992) and restriction enzyme analysis (McClelland, 1981). Restriction enzyme analysis of DNA methylation is now the most commonly used procedure and requires isoschizomers which differ in cleavage only by methylation of a base within the cleavage site. Therefore, methylated DNA will show differences in the isoschizomer-digested patterns which would generally require an appropriate probe to be detected.

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Many changes in the physiological state of a fungus are known to be associated with the mitochondrial DNA (mtDNA) genome or vice versa. These include the petite mutation in yeast (*Saccharomyces cerevisiae*) (Dujon, 1981), the stopper mutation of *N. crassa* (Collins and Lambowitz, 1981), and the ragged mutation of *Aspergillus amstelodami* (Lazarus and Kuntzel, 1981). Likewise, the senescence phenomena in kalilo strains of *N. intermedia* (Bertrand *et al.*, 1985) and *Podospora anserina* (Wright *et al.*, 1982) are known to be caused by the insertion of a nuclear plasmid into mtDNA and by the excision of a DNA fragment from the mtDNA, respectively.

In the present study, DNA methylation of *F. o.* f. sp. *niveum* isolates was examined both before and after successive transfers on artificial media using methylation-sensitive isoschizomers with cloned rDNA and mtDNA probes.

Materials and Methods

Fungal isolates

The two most aggressive race 2 isolates [TX-XID and IS-59] of *F. o.* f. sp. *niveum* were chosen for analysis. Cultures of each isolate were single-spored, amplified and stored in sterile soil tubes at room temperature until used. The first active cultures (T0) were obtained by plating a small aliquot of soil culture on potato dextrose agar (PDA, Difco, USA) and incubating at 25°C for 1 week. A small agar plug from the actively growing hyphal region was cut from the agar and transferred to fresh PDA to make "the first transfer isolate (T_1)". Likewise, successive transfers of each isolate to the fresh PDA were made at weekly intervals for 28 weeks (T_{28} isolate). Every fourth-transfer isolate (T_0, T_4, T_8, T_{12}, T_{16}, T_{20}, T^{24}, and T_{28} isolates) was amplified in potato dextrose broth (PDB, Difco, USA), filtered through Miracloth (Calbiochem, San Diego, USA), and the fungal mycelial mat was frozen in liquid nitrogen and stored at -70°C until used for DNA extraction. In addition, bioassay was carried out at every fourth transfer to determine if any changes in pathogenicity occurred.

Bioassay

Inoculation of plants was conducted using the root dip method (Martyn and Bruton, 1989). Fungal isolates were incubated under continuous light in 50 ml of fusarium liquid culture (FLC) for 3-4 days on a rotary shaker (110 rpm) at room temperature (Martyn and Bruton, 1989). Contents of the flasks were filtered through eight layers of sterile cheese cloth, and the suspension which was predominantly microconidia was calibrated to 1×10^6 microconidia/ml for use as inoculum. The watermelon cultivar, Calhoun Gray which is resistant to race 0 and 1 but susceptible to race 2, was used to determine any changes in the pathogenicity of the successively-transferred isolates. Individual seedlings (10 to 14 days old) were dipped into the inoculum for 30 seconds and immediately transplanted into soil in 15-cm diameter pots. There were six replicated pots (five seedlings per pot) for each inoculum. Each trial included five treatments: H_20 control, IS-59 Tn, TX-XID Tn, IS-59 T_0, and TX-XID T_0.

Restriction enzyme analysis of DNA methylation

DNA was extracted from every fourth-transfer isolate according to the minipreparation method as described previously (Kim *et al.*, 1991). Two pairs of isoschizomers, *MboI/Sau*3AI and *MspI/HpaII* (Promega, Madison and BRL, Gaithersburg, USA) were selected for the analysis. The recognition sequences of the enzymes and sensitivity to the methylation are described below: *MboI* cleaves DNA sequences of GATC or GATmC where mC indicates methylation of cytosine on the 5C in the pyrimidine ring (5-methylcytosine). *Sau*3AI recognizes the same base sequence, GATC, but does not cleave the phosphoester bonds when the terminal C is methylated as in GATmC. *MspI* and *HpaII* cleave CCGG, however, *MspI* and *HpaII* do not cleave mCCGG and CmCGG, respectively.

DNA (1.5 μg) was completely digested and the restriction fragments were separated by 1.2% agarose gel electrophoresis using 1×TBE buffer with a potential gradient of less than 5 V/cm/h. Restriction fragments were visualized by ethidium bromide staining followed by UV transillumination (Maniatis *et al.*, 1982) and Southern blotted onto nylon hybridization membrane. The clone pRW614a containing the transcribed sequence (all coding regions of the repeat unit) of rDNA from *Neurospora crassa* was kindly provided by Peter J. Russel, Biology Dept., Reed College, Portland, Oregon and used along with the *F. o.* f. sp. *niveum* mtDNA-polyprobe, pFON2a-pFON8b, described previously (Kim *et al.*, 1991). Each clone was labeled with [α-^{32}P] dATP using the randomly primed DNA labeling procedure of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). Southern hybridization was conducted at 65°C according to the supplier's manual.

Results

Pathogenicity tests

After the 18th transfer, a sector of mycelium occurred in the TX-XID isolate which showed distinctive characteristics of variable pigmentation within the sectored area and an irregularity in the colony margin (Fig. 1).

The sectored mycelium was selected from the parental TX-XID isolate and successively transferred on PDA weekly. The transfer numbers of sectored isolates matched the numbering of the parental TX-XID isolate, i.e. T_{20} through T_{28}. Morphology and other characteristics of the sector were stable and consistantly appeared throughout successive transfers of the sectored isolate.

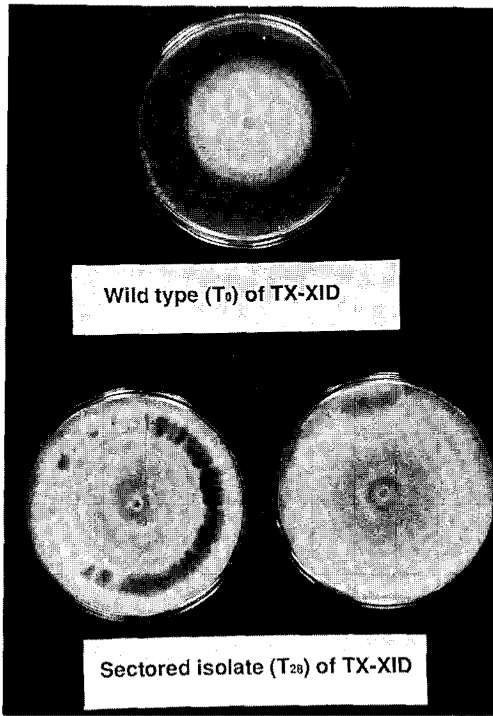

Fig. 1. Isolate of *F. o.* f. sp. *niveum* (TX-XID) after the 28 th transfer on PDA. Sectoring, as induced by different types of pigmentation within a single colony and an irregular margin of the colony are depicted.

Table 1. Pathogenicity of successively-transferred isolates of *F. o.* f. sp. *niveum* race 2 to the watermelon cultivar Calhoun Gray

weekly		days after inoculation			
transfers (Tn)	isolates	12	16	18	24
T4	IS-59	(6^a : 8^b)	(18 : 25)	(26 : 29)	(29 : 29)
	TX-XID	(3 : 10)	(23 : 26)	(29 : 29)	(29 : 29)
T8	IS-59	(12 : 29)	(20 : 29)	(24 : 30)	(29 : 30)
	TX-XID	(9 : 3)	(13 : 6)	(20 : 22)	(27 : 30)
T12	IS-59	(0 : 29)	(20 : 30)	(30 : 30)	
	TX-XID	(9 : 6)	(15 : 22)	(24 : 28)	
T20	IS-59	(3 : 13)	(13 : 14)	(23 : 20)	(25 : 23)
	TX-Sec.	(1 : 7)	(7 : 26)	(14 : 27)	(22 : 27)
	TX-XID	(12 : 7)	(19 : 26)	(22 : 27)	(26 : 27)
T24	IS-59	(19 : 22)	(27 : 26)	(27 : 30)	(28 : 30)
	TX-Sec.	(15 : 13)	(24 : 21)	(24 : 22)	(24 : 23)
	TX-XID	(19 : 13)	(27 : 21)	(27 : 22)	(27 : 23)
T28	IS-59	(29 : 28)	(30 : 30)		
	TX-Sec.	(26 : 30)	(27 : 30)		
	TX-XID	(28 : 30)	(30 : 30)		

[a]Data represent the number of dead plants out of 30 plants by T_n inoculum.

[b]Data represent the number of dead plants out of 30 plants by T_0 inoculum as a control for comparison to transfer isolates.

All successively transferred isolates including the sectored isolate were tested for pathogenicity up through the 28th transfer and none of them showed significant changes (Table 1) when compared with the soil-culture inoculum of T_0 from both isolates. However, pathogenicity of the sectored isolate caused a delay in symptom expression of disease and was possibly less aggressive, although ultimately there was no significant change in pathogenicity from T_0.

Restriction enzyme analysis of methylation with rDNA and random probe

Ethidium bromide-stained gel containing *Mbo*I-digested DNA from T_0, T_8, T_{20}, and T_{24} isolates showed several discrete bands along with a large smeared band (Fig. 2). The largest discrete band (6.0 kb) from TX-XID (T_0) was excised from the gel, cloned into pUC19 vector, named as pDK-m1 and ^{32}P-labeled for further Southern analysis.

The hybridization pattern of *Mbo*I and *Sau*3AI-digested DNA to the rDNA probe is shown in Fig. 3. There were differences in the 0.9 kb fragment between *Mbo*I and *Sau*3AI digestions. In the *Sau*3AI digest, a 0.9 kb

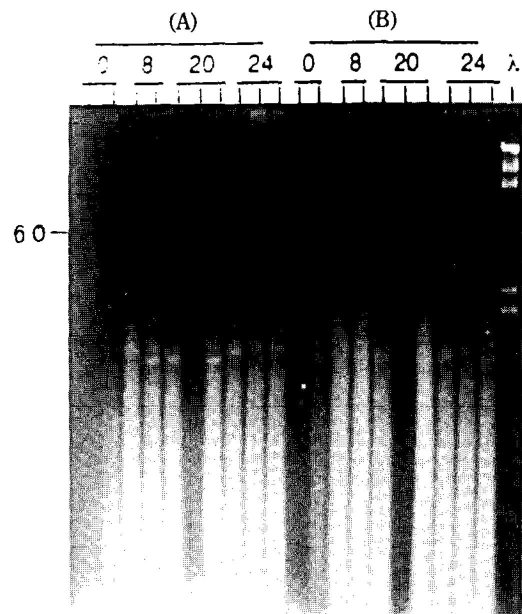

Fig. 2. Ethidium-bromide stained gel containing DNA digested with *Mbo*I (panel A) and *Sau*3AI (panel B). The numbers at the top of each bar represent the number of transfers. The order of the isolates in the lanes is IS-59 and TX-XID for T_0 and T_8. Beginning with T_{20}, the order is IS-59, TX-XID, and TX-XID (sectored). Numbers on the left refer to fragment sizes (kb). The λ lane contains *Hin*dIII-digested λ DNA.

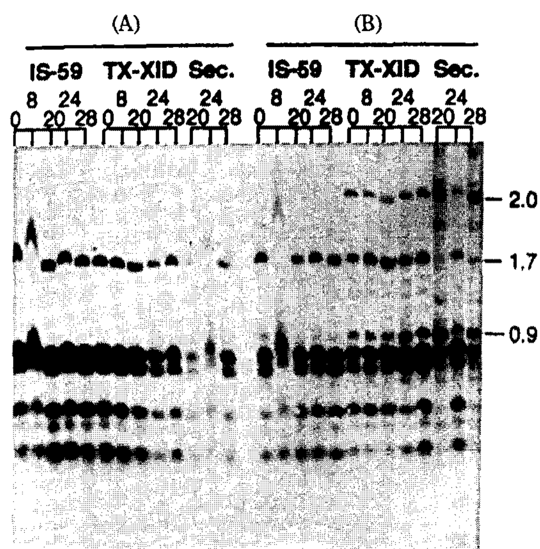

Fig. 3. Hybridization pattern of *Mbo*I and *Sau*3AI-digested DNA to rDNA probe. *Mbo*I as well as *Sau*3AI hybridization patterns are represented at panel A and B, respectively, and original isolates are shown at the top. The numbers on the bar represent the number of transfers and numbers on the right refer to fragment sizes (kb).

band was present in all strains whereas this fragment was missing in the *Mbo*I digestion. There was a restriction fragment length polymorphism between TX-XID and IS-59 isolates in that Sau3AI-digested TX-XID and the sectored strain had a hybridizing band at 2.0 kb whereas the IS-59 transfers did not. Again, neither the 2.0 kb nor 0.9 kb hybridizing bands were detected in *Mbo*I-digested DNA which indicated DNA methylation preexisted in rDNA. In the *Mbo*I digestion, there were no differences in the hybridization pattern among the transfer-strains.

DNA from T_0 and T_{28} isolates were digested with *Msp*I and *Hpa*II followed by hybridization to the rDNA probe. The hybridization pattern is shown in Fig. 4. The *Msp*I hybridization pattern showed differences in the upper bands of 1.5 kb and 1.2 kb fragments between T_0 and T_{28} isolates. The 1.5 kb fragments appeared in T_0 but disappeared in T_{28} transfer of all isolates which suggested DNA methylation in rDNA was induced during successive transfer. In addition, the 1.2 kb fragment disappeared from T_{28} TX-XID as well as TX-XID (sectored) isolates whereas IS-59 isolate in T_{28} transfer showed the discernible, but faint, band at 1.2 kb. Those of 1.2 kb hybridization patterns among isolates indicated that methylation sites at rDNA were multiple and methylation capability differed among isolates, which were in concert with the fact that the capability of phenotypic variations depends on isolates used. These 1.5 kb and 1.2 kb bands were not present in the *Hpa*II digestion, which, with the result of *Mbo*I and *Sau*3AI analysis, confirmed

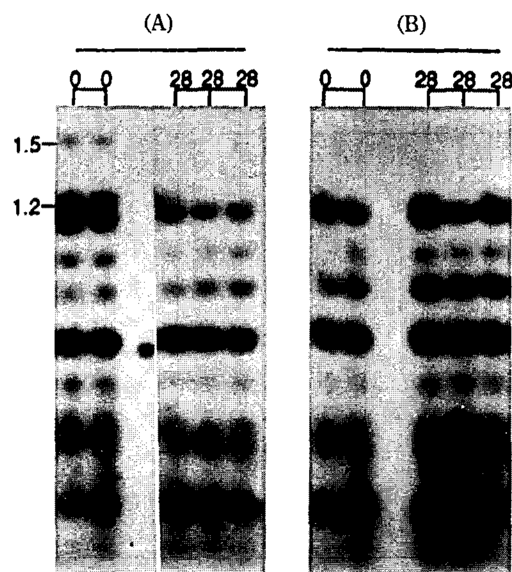

Fig. 4. Hybridization pattern of *Msp*I and *Hpa*II-digested DNA probed with rDNA. *Msp*I and *Hpa*II hybridization patterns are represented at panel A and B, respectively. The numbers at the top of each bar represent the number of transfers. The order of the isolates in the lanes is IS-59 and TX-XID for T_0 and beginning with T_{28}, the order is IS-59, TX-XID, and TX-XID (sectored). Numbers on the left refer to fragment sizes (kb).

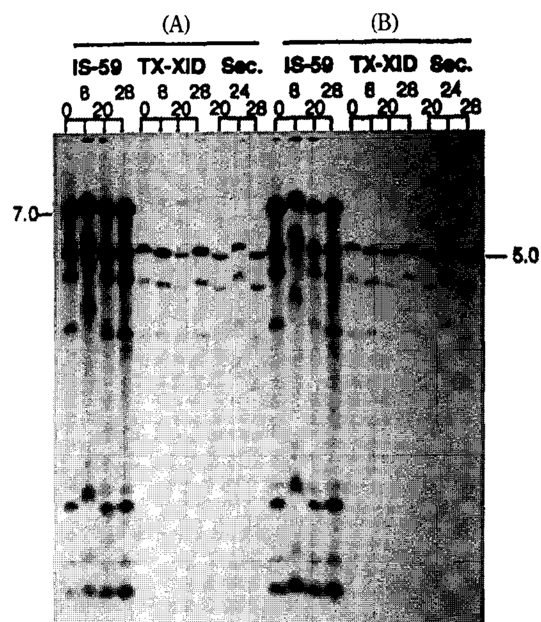

Fig. 5. Hybridization pattern of *Msp*I and *Hpa*II-digested DNA to the 6.0 kb probe. *Msp*I as well as *Hpa*II hybridization patterns are represented at panel A and B, respectively, and the original isolates are shown at the top. The numbers on the bar represent the number of transfer and numbers on left and right refer to fragment sizes (kb).

DNA methylation existed in rDNA of *F. o.* f. sp. *niveum*. Similarly, there were no differences in the hybridization pattern in any of the transfer isolates and no differences

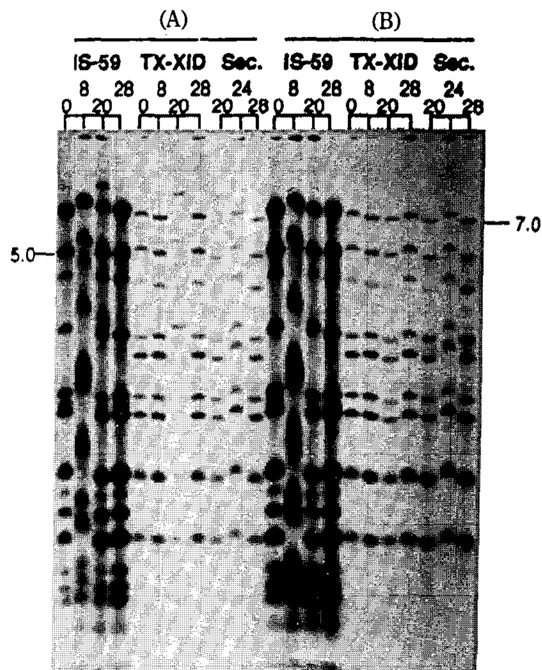

Fig. 6. Hybridization pattern of *Msp*I and *Hpa*II-digested DNA to mtDNA polyprobe. *Msp*I as well as *Hpa*II hybridization patterns are represented at panel A and B, respectively, and the original isolates are shown at the top. The numbers on the bar represent the number of transfers and numbers on the left as well as right refer to fragment sizes (kb).

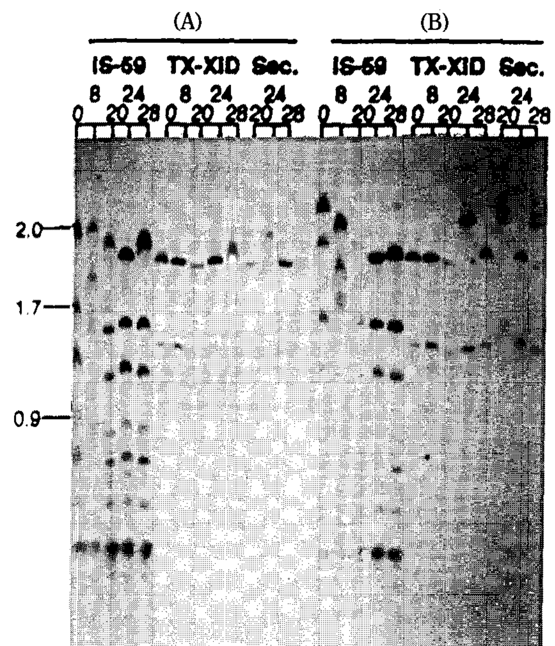

Fig. 7. Hybridization pattern of *Mbo*I and *Sau*3AI digested-DNA to the mtDNA polyprobe. *Mbo*I as well as *Sau*3AI hybridization patterns are represented at panel A and B, respectively, and the original isolates are shown at the top. The numbers on the bar represent the number of transfers and numbers on the left refer to fragment sizes (kb).

were seen between the IS-59, TX-XID, and the TX-XID sectored isolates. Since *Msp*I and *Hpa*II digestions revealed the changes in DNA methylation during the successive transfer, MspI and HpaII digestions of DNA from successive transfers were further analyzed with the anonymous 6.0 kb probe and the hybridization pattern is shown in Fig. 5. Different restriction fragment patterns were detected in 7.0 kb fragment between IS-59 and TX-XID transfer and the sectored isolate. However, there were no differences in the hybridization pattern between *Msp*I and *Hpa*II digestions. No differences among the transfers of each isolate were detected either.

Restriction enzyme analysis of methylation with *F. o.* f. sp. *niveum* mtDNA probe

The hybridization patterns of transferred isolates were also examined with the *F. o.* f. sp. *niveum*-mtDNA probe. Hybridization patterns of *Msp*I and *Hpa*II digestions and *Mbo*I and *Sau*3AI digestions are shown in Fig. 6 and Fig. 7, respectively. Again, different restriction fragment patterns were detected between the transfer strains that originated from IS-59 and TX-XID; however, there were no differences in the hybridization pattern between digestions by either pair of isoschizomers or among the transfers, which suggested that neither induced methylation nor DNA methylaltion occurred in mtDNA.

Discussion

A decrease in the pathogenicity of fungi caused by successive culturing and long term storage has been observed with many fungi. However, the mechanism by which the decrease in pathogenicity occurs has not been elucidated. Although no changes in pathogenicity were detected in this experiment, it does not necessarily mean that a decrease in pathogenicity can not occur. It is possible that a change in DNA methylation during successive transfers may result in one of many possible changes of functions involved in the control of fitness or aggressiveness of the original isolate. However, proof of this hypothesis will require more accurate quantitative assays for pathogenicity and a longer period of successive transfers as well as many other assays of fungal physiology.

Numerous species of fungi show variability in their morphological or physiological characters; some of these changes have been attributed to mutation or to heterokaryosis. Some of the reported variability has peculiarities suggesting that a nuclear phenomenon is not responsible, but rather, is of cytoplasmic origin. This is particularly the case in the sudden sectoring phenomena of fungi in culture (Daboussi-Bareyre and Parisot,

1986). *Nectria haematococca*, the sexual stage of *F. solani*, has been shown to sector in culture and it was reported to be the result of nucleocytoplasmic interactions, i.e., both nuclear and cytoplasmic inheritance were involved (Daboussi-Bareyre and Parisot, 1986). Based on the fact that the sectoring characteristics of *F. o.* f. sp. *niveum* were passed to the next generation, it was considered important to examine the mtDNA by methylation-sensitive isoschizomers. However, no differences between sectored isolates and nonsectored strains of TX-XID were detected. Therefore, the sectoring phenomenon did not directly involve rDNA or any gene sequence probes used in this study.

The hybridization pattern of *Sau*3AI digested-DNA to the rDNA probe differed from that of *Mbo*I-digested DNA. Similarly, differences in the hybridization pattern of T_0, and T_{28} transfers of IS-59 and TX-XID to rDNA were detected in *Msp*I and *Hpa*II digestions. Therefore, DNA methylation at specific sites in the rDNA gene families of *F. o.* f. sp. *niveum* occurs naturally and are induced due to successive culturing as well.

Restriction enzyme analysis of DNA methylation is a good tool to examine the regulation of a specific gene; however, it is hard to determine the overall methylation pattern. In this study, three different kinds of probes were used and only the rDNA probe detected DNA methylation and subsequent individual changes during successive transfers. Although there were no changes detected in pathogenicity among the transfers or in DNA methylation between a sectored strain and its original isolate, the fact that changes in DNA methylation occurred is still significant and considered as a phenomenon for further research.

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