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Short Communication

Mobilization of a retrotransposon in 5-azacytidine-treated fungus *Fusarium oxysporum*

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Abstract It is well known that DNA methylation is involved in the control of transposable elements in eukaryotic cells. Recent studies indicate that demethylation of DNA in a mutant of *Chlamydomonas* and *Arabidopsis* causes transcriptional activation and mobilization of transposons. In this report, transposition of a retrotransposon was investigated in the phytopathogenic fungus *Fusarium oxysporum* treated with 5-azacytidine (5azaC), a reagent that causes reduction in the DNA methylation level. The results showed elevated transposition frequency in 5azaC-treated isolates when they were incubated for a long time. However, increase of retrotransposon transcripts was not observed, suggesting that the retrotransposon was mobilized by a mechanism other than its transcriptional activation.

Key words: 5-azacytidine, *Foxy*, *Fusarium oxysporum*, retrotransposon, transposition.

Fusarium oxysporum is a soil-born fungal pathogen that causes wilt disease in a variety of crops. The fungus enters the plant root directly, using penetration hyphae, and colonizes the cortex by intra-and intercellular growth (Pietro et al. 2003). Once it reaches the vascular tissue, it spreads rapidly upwards through the xylem vessels, provoking characteristic wilt symptoms (Rodriguez-Galvez 1995).

In a previous report, we analyzed the effect of 5-azacytidine (5azaC) on the pathogenicity of *F. oxysporum* f. sp. *cucumerinum* strain F9 (Akiyama *et al*. 1997). This cytidine analogue is an inhibitor of DNA methyltransferase (for review, see Santi *et al*. 1983), and it causes demethylation of DNA and heritable alteration of gene expression in various biological systems (Tamame et al. 1983; Podger 1983; Sano et al. 1990). After treatment of fungal mycelia with 5azaC, we obtained nonpathogenic isolates with a frequency up to 8%. These results suggest that demethylation of DNA affects expression of unknown gene(s) involved in the process of fungal infection and disease development, leading to the loss of pathogenicity.

DNA methylation regulates a number of biological functions in eukaryotic cells, including chromatin structure (Robertson et al. 2002), X-chromosome inactivation in females (Goto et al. 1998) and silencing of gene expression (Attwood et al. 2002). In addition, it plays a crucial role in the control of transposable elements. Transposition of the *Activator* element of maize is suppressed by DNA methylation of its terminal regions (Ros and Kunze 2001). Salzberg and colleagues analyzed methylated DNA sequences in *Drosophila melanogaster* and suggested that DNA methylation is involved in the silencing of transposons and repetitive elements (Salzberg et al. 2004).

In order to elucidate the mechanism for the 5azaC effect on the fungus, we investigated transposition of transposons in *Fusarium* isolates. Ten transposons have been reported in *Fusarium* species (for review, see Wostemeyer and Kreibichl 2002), and one of them, *Foxy* (Mes et al. 2000), was used in this experiment. *Foxy* is a short interspersed element (SINE) found in *F. oxysporum* f. sp. *lycopersici* strain Fo1007. This retrotransposon consists of 664 nucleotides and contains two putative RNA polymerase binding sites. The genome of the strain Fo1007 contains more than 160 copies of *Foxy*. *Foxy* in *F. oxysporum* strain F9 was cloned by polymerase chain reaction (PCR) under conventional conditions using total cell DNA and primers 5′-CTGGGTGGCTGTAACGAAGCC-3′ and 5′-GGAATTTTGAGAGAGTTCGCCT-3′. These primers were synthesized based on the terminal sequence of *Foxy* in the strain Fo1007 (Mes et al. 2000). The cloned fragment consisted of 546 nucleotides and it contained two putative RNA polymerase binding sites (data not shown). There were 9 base substitutions as compared to

Abbreviations: 5azaC, 5-azacytidine; PCR, polymerase chain reaction; PS, potato sucrose; SINE, short interspersed element.
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the *Foxy* sequence in Fo1007.

Using the *Foxy* fragment as a probe, Southern blot analysis was performed to investigate *Foxy* transposition in 5azaC-treated fungal isolates. Fungal mycelia were grown in liquid potato-sucrose (PS) medium and total cell DNA was prepared as described previously (Jain et al. 2005). DNA was digested with a restriction enzyme, subjected to agarose gel electrophoresis and hybridized with *Foxy* probe according to standard protocols by Sambrook and Russell (2001). The hybridization signal was detected with the chemiluminescence reaction using AlkPhos direct labeling kit (Amersham Pharmacia Biotech).

We first analyzed the strain F9 and five 5azaC-treated isolates; A1, A2, A3, which are nonpathogenic, and B1, B2, which are pathogenic. As shown in Figure 1, a new fragment with altered mobility could be detected in A3 as compared to the strain F9 suggesting transposition of *Foxy* in this isolate. But the possibility could not be excluded that 5azaC caused demethylation of the methylated cytosine residue in the recognition site of the restriction enzyme, leading to alteration of the hybridization pattern. The other four isolates showed no difference in the hybridization pattern.

Then we analyzed *Foxy* transposition using DNA prepared from cells cultured for a long time. The strain F9 and 5azaC-treated isolates A2, B1 were inoculated respectively in five culture bottles containing PS medium and grown for three months. During culture, cells were inoculated into a new medium every week. Then bud cells (yeast-form cells of *F. oxysporum*) were collected from each culture and plated on the PS plates. A single colony was isolated from each plate and used as subclones derived from strain F9, A2 and B1. Total cell DNA was extracted from these subclones and subjected to hybridization analysis using the *Foxy* probe. As shown in Figure 2A, five subclones from F9, F9-1 to F9-5, showed the same pattern of hybridization as the parental strain F9, suggesting no appreciable transposition of *Foxy*. On the other hand, two out of five subclones from A2, A2-3 and A2-4, showed one additional band with altered mobility (Figure 2B). Three out of five from B1, B1-1, B1-2 and B1-5, also showed one additional band

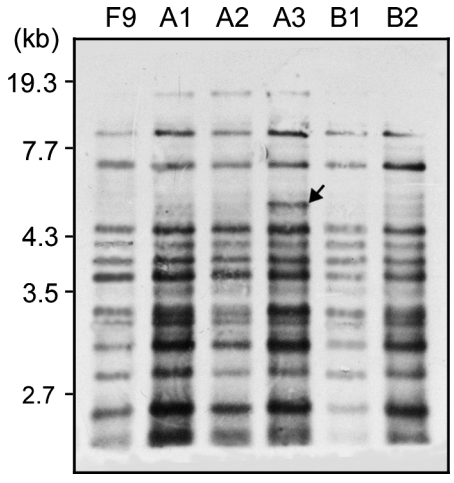

Figure 1. Southern blot analysis using the *Foxy* probe in the strain F9 and 5azaC-treated isolates A1, A2, A3, B1 and B2. One microgram of total cell DNA was digested with the restriction enzyme *Hinc*II, loaded onto the agarose gel and probed for *Foxy*. The arrow indicates a new fragment with altered mobility in the nonpathogenic isolate A3. DNA length markers are shown at left in kilobases.

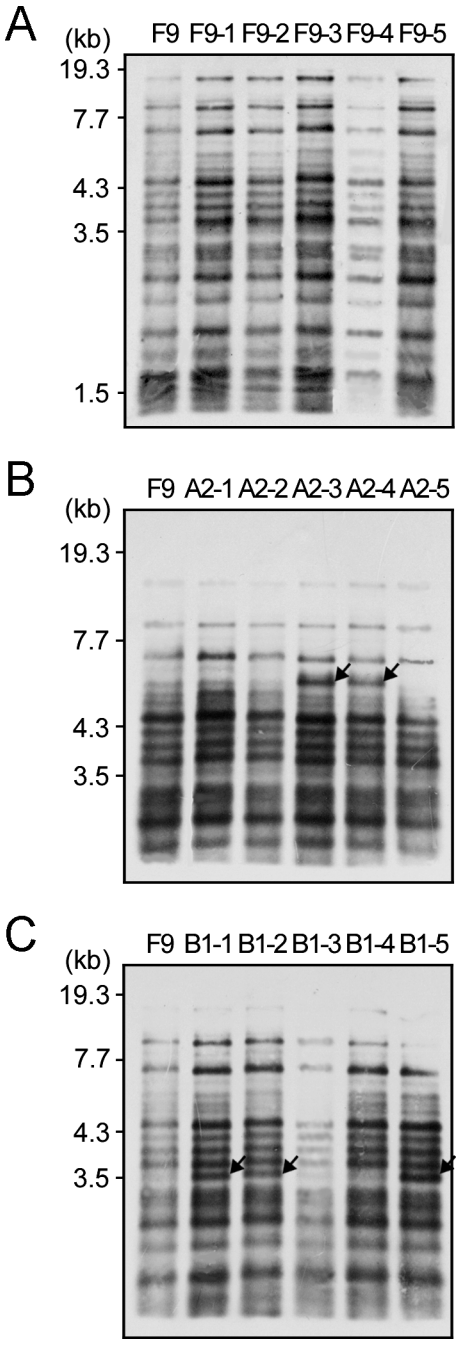

Figure 2. Southern blot analysis of *Foxy* transposition in five subclones derived from F9 (A), A2 (B) and B1 (C) after 3 months incubation. One microgram of total cell DNA was digested with *Hinc*II and probed for *Foxy*. The arrow indicates a new fragment in subclones from A2 and B1. DNA length markers are shown at left in kilobases.

(Figure 2C). These results strongly suggest that the transposition frequency of *Foxy* is enhanced in two 5azaC-treated isolates when they are cultured for several months. It is unlikely that alteration of the hybridization pattern is due to demethylation of the recognition sequence of the restriction enzyme, since the new band was observed in some of the subclones only after three months incubation in the absence of 5azaC. It should be noted that fungal strains were stored at −50°C after 5azaC treatment without long-term incubation. Since *Foxy* transposition was observed in both nonpathogenic and pathogenic isolates, there would be no direct relation between *Foxy* transposition and pathogenicity. Surprisingly an additional fragment observed in A2-3 and A2-4 seems similar in length, and the same is true in B1-1, B1-2 and B1-5. We also analyzed transposition of other *Fusarium* transposons, *Fot1* (Daboussi et al. 1992) and *Impala* (Langin et al. 1995). No transposition of these transposons was detected in Southern blot analysis (data not shown).

Mobilization of transposons is often associated with transcriptional activation. The amount of transposon transcript was increased in a *Chlamydomonas reinhardtii* mutant that showed elevated transposition frequency (Wu-Scharf et al. 2000). Kakutani and colleagues showed that transposon *CAC1* was transcriptionally activated and it increased in the copy number in an *Arabidopsis* mutant with hypomethylated DNA (Miura et al. 2001). We also examined the expression of *Foxy* in 5azaC-treated isolates. Total cellular RNA was extracted from fungal mycelia and subjected to Northern blot analysis using the *Foxy* probe according to the standard protocols. As shown in Figure 3, strain F9 gave a clear signal upon hybridization, suggesting that *Foxy* is actively transcribed as reported before (Mes et al. 2000). We detected no obvious difference in the hybridization signal in five 5azaC-treated isolates as compared to the strain F9. The results suggest that there is no change in the transcription level of *Foxy* in these isolates.

The results presented in this experiment show elevated transposition frequency of the retrotransposon *Foxy* in 5azaC-treated *F. oxysporum* isolates, especially when they are grown for a long time. On the other hand, there is no appreciable increase in *Foxy* transcript in these isolates, suggesting that elevated *Foxy* transposition is not due to the activation of *Foxy* expression. Koga and colleagues showed that 5azaC treatment of medaka fish promotes excision of the transposon *Tol2*, but similarly to our results they could not detect any change of *Tol2* expression level (Iida et al. 2006). Thus the transposition frequency would be increased by mechanism(s) other than elevated expression of *Foxy*. One possible mechanism would be that 5azaC treatment causes demethylation of the DNA sequence involved in *Foxy* insertion, leading to high transposition frequency. This notion is supported by the fact that an additional fragment observed in hybridization analysis seems similar in length in subclones from A2 and B1 respectively. The possibility could not be excluded, however, that 5azaC affects the expression of other genes involved in *Foxy* transposition.

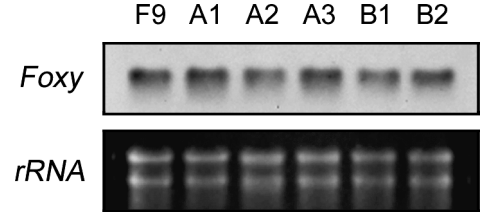

Figure 3. Northern blot analysis of *Foxy* transcription in the strain F9 and 5azaC-treated isolates. Ten micrograms of total RNA were loaded per lane and probed for *Foxy* (upper panel). The length of the hybridizing RNA is approximately 700 nucleotides. Ethidium bromide stained rRNA is shown indicating equal loading of total RNA (lower panel).

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