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RESEARCH ARTICLE

DNA methylation patterns of banana leaves in response to *Fusarium oxysporum* f. sp. *cubense* tropical race 4

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Abstract

Fusarium wilt of banana, which is caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), is a serious soil-borne fungal disease. Now, the epigenetic molecular pathogenic basis is elusive. In this study, with methylation-sensitive amplification polymorphism (MSAP) technique, DNA methylation was compared between the leaves inoculated with Foc TR4 and the mock-inoculated leaves at different pathogenic stages. With 25 pairs of primers, 1 144 and 1 255 fragments were amplified from the infected and mock-inoculated leaves, respectively. DNA methylation was both changed and the average methylated CCGG sequences were 34.81 and 29.26% for the infected and the mock-inoculated leaves. And DNA hypermethylation and hypomethylation were induced by pathogen infection during all pathogenic stages. Further, 69 polymorphic fragments were sequenced and 29 of them showed sequence similarity to genes with known functions. And RT-PCR results of four genes indicated that their expression patterns were consistent with their methylation patterns. Our results suggest that DNA methylation plays important roles in pathogenic response to Foc TR4 for banana.

Keywords: banana, *Fusarium* wilt disease, *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), DNA methylation, methylation-sensitive amplification polymorphism (MSAP), epigenetics, disease defense genes

1. Introduction

Fusarium wilt, also known as Panama disease, is a destructive fungal disease that has a serious impact on banana production worldwide. The soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is categorized into four physiological races (race 1, 2, 3, and 4) based on variations in virulence to specific host banana cultivars (De Ascensao and Dubery 2000). Foc race 4 can infect almost 80% of banana cultivars worldwide, including the most important Cavendish groups (Ploetz 2006). Foc tropical race 4 (Foc TR4) is more virulent than the other strains and can cause wilt disease in Cavendish banana under almost all conditions (Guo *et al.* 2014).

To date, the molecular mechanism of banana-*Fusarium* wilt interaction remains unclear. Most previous studies focused on three aspects: pathogen, host banana, and the interaction between the pathogen and host (Wang *et al.* 2012). Identification of *F. oxysporum* is usually based on morphological characteristics, which requires detailed knowledge on *Fusarium* taxonomy, as well as molecular

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markers including internal transcribed spacer (ITS), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), and random amplified polymorphic DNA (RAPD) (Gurjar *et al.* 2009). Many studies have focused on bio-control of *Fusarium* wilt disease, such as isolating antagonistic bacteria for Foc (Ayyadurai *et al.* 2006), but there are few effective methods for controlling it, including chemical control methods (Zhao *et al.* 2013).

In plants, disease resistance occurs in a gene-for-gene manner *via* direct or indirect interaction between proteins encoded by plant resistance genes and pathogen avirulence genes. Such interactions trigger a cascade of defense responses to halt the spread of pathogen (Chisholm *et al.* 2006). Some *Fusarium* resistance genes have been isolated from other plants, such as tomato (Simons *et al.* 1998) and melon (Joobeur *et al.* 2004). To date, only two classes of resistance gene homologs have been isolated from banana, including nucleotide-binding site (NBS) and serine/threonine kinase (Peraza-Echeverria *et al.* 2007).

And some epigenetic variations might contribute to the host molecular response to pathogen infection, including hypermethylation on the genome-wide level, hypomethylation of resistance-related genes, and methylation of pathogen-responsive genes (Bender 2004; Dowen *et al.* 2012). For instance, when rice seedlings and adult plants were inoculated with the blight pathogen *Xanthomonas oryzae pv. oryzae*, methylation levels were higher in adult plants than in seedlings and the expression of methylated polymorphic fragments varied with the methylation pattern (Sha *et al.* 2005).

Cytosine DNA methylation was a conserved epigenetic silencing mechanism and involved in many important biological processes, including defense against transposon proliferation, regulation of genomic imprinting, and gene expression (Zhang *et al.* 2006). A relatively high degree of nuclear DNA methylation was a specific feature of plant genomes. Targets for cytosine DNA methylation in plant genomes include CG, CHG, and CHH (H is A, T, or C) sequences. More than 30% of total 5-methylcytosine (m5C) in plant DNA is located in non-CG sites (Vanyushin and Ashapkin 2011). Using the methylation-sensitive amplification polymorphism (MASP) technique, a total of 107 sites (23%) were found to be methylated at cytosine in micropropagated banana genomes, whereas no DNA methylation polymorphisms were detected in conventionally propagated banana plants (Peraza-Echeverria *et al.* 2001). However, there is not report on DNA methylation in banana till now.

The Banana Genome Project produced 523-megabase genome sequence of the *Musa acuminata* doubled-haploid genotype, providing a crucial stepping-stone for studying gene function and genetic improvement in banana (D'Hont *et al.* 2012). The interaction mechanism for banana roots and *Fusarium* wilt disease has been investigated and many differentially expressed genes were identified after high-throughput RNA-sequencing was developed (Li *et al.* 2013). In this study, DNA methylation patterns induced by Foc TR4 in banana leaves were investigaed with MSAP method, and the mechanism of banana leaves response to Foc TR4 was explored.

2. Materials and methods

2.1. Plant materials and pathogen inoculation

Banana plantlets, Brazilian (*Musa acuminata* L. AAA), were about 30 cm in height, which were obtained from the Tissue Culture Center of the Chinese Academy of Tropical Agricultural Sciences. The plantlets were planted in a growth room at 25°C, 80% relative humidity, and a photoperiod of 12 h light/12 h darkness.

Foc TR4, which was previously isolated and identified in our lab (Xie *et al.* 2010), was cultured in carboxymethyl cellulose (CMS) suspension (7.5 g L^{-1} hydroxymethyl cellulose ester, 0.5 g L^{-1} yeast extract, 0.5 g L^{-1} NH_4NO_3, 0.5 g L^{-1} KH_2PO_4 and 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$) for conidiospore production. The collected spores were diluted to 1×10^6 mL^{-1} with ddH_2O (Zhao *et al.* 2013). Healthy leaves were cut from banana plantlets and placed into a clean plastic box (40 cm×30 cm×10 cm) with absorbent paper and the stalks were also provided with enough water. Then the leaves were punctured six points per leaf by a fine needle and inoculated with 20 μL diluted spore solution at room temperature. The mock-inoculated leaves were got by inoculated with ddH_2O at the same conditions. The experiments were replicated three times, each replication with six leaves. Samples were collected at 0, 4, 12, 24 h, 3 and 6 d post inoculation. The samples of three replications were mixed and were immediately frozen with liquid nitrogen. Genomic DNA was extracted using a modified CTAB method (Manfioletti *et al*. 1988).

2.2. MSAP analysis

MSAP analysis was performed as described previously (Xiong *et al.* 1999). Briefly, one of 500 ng genomic DNA was double-digested with 5 U *Eco*RI and *Msp*I, and the other of 500 ng genomic DNA was double-digested with 5 U *Eco*RI and *Hpa*II, respectively, in 20 μL of 1Y$^+$/TANGO buffer (Sangon, Shanghai, China) for 2 h at 37°C. Then, 5 pmol *Eco*RI adapter and 50 pmol *Hpa*II-*Msp*I adapter were added in the 20 μL ligation solution, and incubated overnight at 37°C. The ligated mixture was diluted to 1:10