A Detection Method for Tropical Race 4 of the Banana Pathogen *Fusarium oxysporum* f. sp. *cubense*

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Abstract

***Fusarium oxysporum* f. sp. *cubense* (Foc) is the causal agent of Fusarium wilt, the devastating disease that ruined the 'Gros Michel' (AAA)-based banana production in the first half of the 20th century. The occurrence of a new variant in Southeast Asia that overcomes the resistance in Cavendish clones such as 'Grand Naine' (AAA) is a major concern to current banana production worldwide. The threat posed by this new variant, called tropical race 4 (TR4), may be overcome by the introduction of resistant cultivars. However, the identification of new resistant sources or breeding for resistance is a long-term effort. Currently, the only option to control the disease is to avoid or reduce the spread of the pathogen by eradication of infected plants and isolation of infested plantations. This requires sensitive and highly specific diagnostics that enable early detection of the pathogen. A two-locus database of DNA sequences, from over 800 different isolates from multiple formae speciales of *F. oxysporum*, was used to develop a molecular diagnostic tool that specifically detects isolates from the vegetative compatibility group (VCG) 01213, which encompasses the Foc TR4 genotype. This diagnostic tool was able to detect all Foc TR4 isolates tested, while none of the Foc isolates from 19 VCGs other than 01213 showed any reaction. In addition, the developed diagnostic tool was able to detect Foc TR4 when using DNA samples from different tissues of 'Grand Naine' plants inoculated with TR4 isolates.**

INTRODUCTION

Fusarium wilt of banana, also known as Panama disease, wiped out the 'Gros Michel'-based banana industry in Central America in the middle of the past century. The disease, caused by the soilborne pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), can hardly be controlled by chemical or cultural methods. Once soil is infested with Foc, susceptible cultivars cannot be successfully replanted for up to 30 years (Stover, 1962). Over time, three physiological races of Foc have been identified in banana: race 1 that caused the epidemics on 'Gros Michel' (AAA), race 2 that affects 'Bluggoe' (ABB) and other cooking bananas, and race 4 that affects all cultivars susceptible to races 1 and 2 as well as cultivars from the Cavendish subgroup (AAA), plantains and many other genotypes (Su et al., 1986). Before 1990, isolates classified as race 4 only caused serious losses in Cavendish genotypes in subtropical regions of Australia, the Canary Islands and Taiwan (Su et al., 1986; Pegg et al., 1996). However, since then, a new variant, which infects Cavendish cultivars in the tropics, has been identified in South Asia (Ploetz, 2006). Thus, two phenotypes of Foc attacking Cavendish cultivars have been designated, i.e. subtropical race 4 (ST4) and tropical race 4 (TR4). The occurrence of TR4, which overcomes the resistance in Cavendish clones such as 'Grand Naine' (AAA), is a major concern to current banana production worldwide. The threat posed by TR4 could be overcome by the introduction of resistant cultivars. However, the identification of new resistant sources or breeding for resistance is a long-term effort. At the moment, the two best options to control the disease are eradicating infected plants and isolating infested

plantations, to avoid or reduce the spread of the pathogen. This requires a sensitive and highly specific diagnostic that enables early detection of the pathogen. As TR4 isolates are grouped in vegetative compatibility group (VCG) 01213 or in the VCG complex VCG 01213/16, VCG tests appear to be useful for TR4 diagnosis. However, it requires the availability of testers for all the Foc VCGs and the generation and characterisation of *nit* (nitrate-nonutilising) mutants (Correll et al., 1987), which is very time consuming. Here, we report the development of a molecular diagnostic specifically detecting isolates from VCG 01213, which encompasses the Foc TR4 genotypes.

MATERIALS AND METHODS

A total of 82 Foc isolates originating from different banana production areas around the world and comprising 20 VCGs were analysed. Samples from geographic regions known to be infested by TR4 were received as dried pseudostem strands, which were sectioned in pieces, placed on Komada's medium (Komada, 1975) and incubated at 25°C. Isolated colonies were examined by light microscopy for the presence of macroconidia and microconidia that are diagnostic of *F. oxysporum*. Positive samples were transferred to plates with potato-dextrose agar (PDA) and stored for further analyses (for details, see Dita et al., 2010).

For DNA isolation, a single-spore culture of each isolate was grown in Petri plates containing PDA and incubated at 25°C for 5 days. Total genomic DNA was extracted from mycelia and spores using the Wizard Magnetic DNA Purification System for Food kit (Promega, Madison, USA) according to the manufacturer's instructions. The translation elongation factor 1α (TEF-1α) and the intergenic spacer (IGS) region of the nuclear ribosomal operon were amplified as described by O'Donnell et al. (1998, 2009). The TEF-1α gene and the IGS regions were sequenced using the aforementioned primers. Sequences were manually edited using the SeqMan module of DNASTAR 6.0 to generate a consensus sequence. DNA sequences of the IGS region and the TEF-1α gene from the 82 Foc isolates were used for comparative analyses, both as individuals and as a combined data set, with sequences from 848 *F. oxysporum* isolates (O'Donnell et al., 2009). Eventually, we used the IGS dataset to design primer set FocTR4-F/FocTR4-R for specific detection of TR4 (Dita et al., 2010).

For PCR method validation, hardened 3-month-old tissue culture-derived banana plants of 'Grand Naine' were inoculated with one of three TR4 isolates or with one race 1 isolate as described by Dita et al. (2011). Rhizome and pseudostem samples collected at 40 days after inoculation (dai) were used for DNA extraction, using the above-mentioned methodology, and PCR amplification.

RESULTS AND DISCUSSION

All TR4 isolates were successfully recovered from symptomatic rhizomes of field samples assayed on Komada's medium. VCG numbers were assigned to most of the isolates coming from areas where TR4 is reported and were suspected of belonging to VCG 01213. High-quality genomic DNA was obtained from all isolates, and the primers and amplification conditions resulted in high-quality DNA sequences of the TEF-1α gene and IGS regions. Phylogenetic analyses of IGS and TEF-1α revealed polymorphisms between the Foc isolates, but for the TEF-1α gene, these were insufficient to allow a reliable discrimination of VCG 01213 from other VCGs (data not shown). Comparative analysis of the IGS region showed a higher SNP frequency and was, therefore, used for primer design. This confirms results of Fourie et al. (2009) who showed that restriction fragment length polymorphisms of the IGS region (IGS-RFLP) were more powerful than three other genome regions, including TEF-1α, for the discrimination of Foc lineages. In addition to the rich genetic diversity present in IGS, it is a multi-copy region that increases the sensitivity of PCR-based diagnostics. The diagnostic TR4 primer set produced only one amplicon in VCG 01213 isolates, whose predicted length of 463 bp was confirmed by gel electrophoresis (Fig. 1). Moreover, all isolates coming from the different TR4-infected areas were typed as VCG 01213. However, in other studies, TR4

isolates have been typed also as VCG 01216 or grouped in the complex VCG 01213/16 (Fourie et al., 2009). However, VCG 01216 isolates may just represent strains in VCG 01213 that show less pronounced complementation in *nit* assays than the 'true' VCG 01213 isolates. As this required further investigation, we successfully used our diagnostic on the VCG 01216 strains, confirming its specificity for Foc TR4.

DNA from infected plants was successfully used for PCR amplification using the TR4 diagnostic primers. No amplicons were observed from samples of non-inoculated 'Grand Naine' plants (Fig. 2). All the samples reacted with the plant actin primer set used as positive control for DNA quality (Fig. 2). In the future, a duplex PCR might be designed by using these two primers sets in one single reaction.

CONCLUSIONS

Considering the Fusarium wilt history (Stover, 1962; Ploetz, 2006) and the Cavendish dependence on export trades, TR4 is currently a major threat to the global banana industry. TR4 is still restricted to South and South-East Asia, but if it reaches the major banana plantations in Latin America, the Caribbean and West Africa, a multibillion dollar production and export industry will be facing devastation. Moreover, the food security of millions of people depending on small-holder production will be in danger. In the absence of resistant cultivars, delimiting the dissemination of the disease is a top priority that relies on accurate diagnosis. Here, we describe the development of a rapid and reliable PCR diagnostic for Foc TR4/VCG 01213 that also can be used for in planta detection. In comparison with the traditional methods of agar plating and pathogen purification from infected samples, VCG analysis and pathogenicity tests, which may take weeks or months, the in planta detection method described here provides a receipt-to-result efficiency of about 6 hours. Application enables monitoring of the disease and supports management and eradication strategies.

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Figures

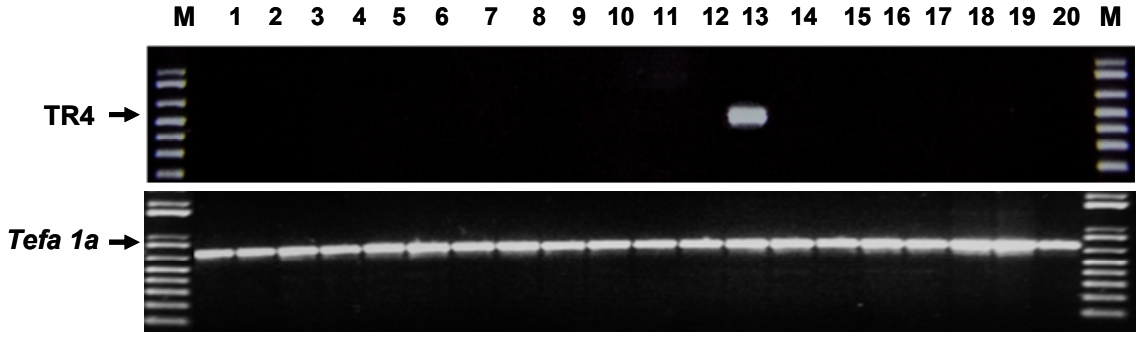

Fig. 1. Amplification of PCR products (TR4 = 463 bp; Tefa 1a = 648 bp) of 20 representative vegetative compatibility groups (VCG; in parentheses) of *Fusarium oxysporum* f. sp. *cubense* (Foc) using primer set FocTR4-F/FocTR4-R (upper panel), and elongation factor 1α (lower panel). 1 - NRRL36101 (0120); 2 - NRRL36102 (0121); 3 - NRRL36103 (0122); 4 - NRRL36104 (0123); 5 - NRRL36105 (0124); 6 - NRRL36106 (0125); 7 - NRRL36107 (0126); 8 - NRRL36111(0128); 9 - NRRL36110 (0129); 10 - NRRL26029 (01210); 11 - NRRL36109 (01211); 12 - NRRL36108 (01212); 13 - NRRL36114 (01213); 14 - NRRL36113(01214); 15 - NRRL36112 (01215); 16 - NRRL36120 (01218); 17 - NRRL36118 (01221); 18 - NRRL36117 (01222); 19 - NRRL36116 (01223); 20 - NRRL36115 (01224); M - molecular marker 1-kb DNA ladder plus.

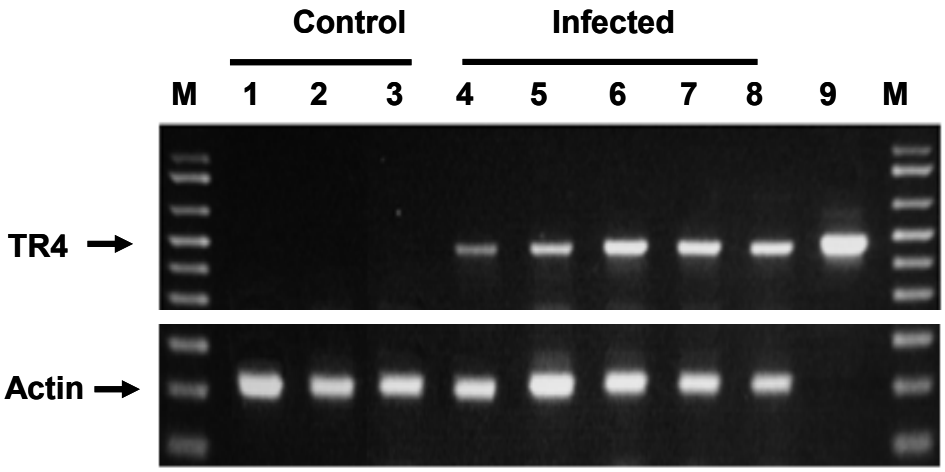

Fig. 2. PCR amplification products using the diagnostic *Fusarium oxysporum* f. sp. *cubense* (Foc) TR4 primer set (463 bp, upper panel) and the banana actin primer set (217 bp, lower panel) on inoculated or noninoculated (control) banana samples. 1 - 'Pisang Mas'; 2 - 'Grand Naine' (leaves from tissue-culture plants); 3 - Rhizome from non-inoculated 'Grand Naine plants'; 4-6 - Infected rhizomes from 'Grand Naine' plants inoculated with Foc TR4 isolates NRRL36114 - BPS3.4 and II-5; 7-8 - Infected pseudostems from 'Grand Naine' plants inoculated with Foc TR4 isolates NRRL36114 and BPS3.4; 9 - Positive control using DNA from a pure of culture of isolate NRRL36114. M - molecular marker 1-kb DNA ladder plus. Specific DNA bands for Foc TR4 and the banana actin gene are indicated on the left.