Development of a thematic collection of *Musa* spp accessions using SCAR markers for preventive breeding against *Fusarium oxysporum* f. sp *cubense* tropical race 4

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ABSTRACT. Bananas are one of the most consumed fruits worldwide, but are affected by many pests and diseases. One of the most devastating diseases is *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp *cubense* (Foc). Recently, Fusarium tropical race 4 (Foc TR4) has been causing irreparable damage, especially in Asia and Africa where it has devastated entire plantations, including areas with Cavendish, which is known to be resistant to Foc race 1. Although this race is not yet present in Brazil, results obtained by Embrapa in partnership with the University of Wageningen, The Netherlands, indicate that 100% of the cultivars used by Brazilian growers are susceptible to Foc TR 4. In our study, 276 banana accessions were screened with sequence characterized amplified region (SCAR) markers that have been linked to the resistance of Foc TR 4. Two SCAR

primers were tested and the results revealed that SCAR ScaU1001 was efficient at discriminating accessions with possible resistance in 36.6% of the evaluated accessions. This is the first attempt to develop a thematic collection of possible Foc TR 4 resistant banana accessions in Brazil, which could be tested in Asian or African countries to validate marker-assisted selection (MAS), and for use in the preventive breeding of the crop to safeguard our banana plantations against Foc TR 4. We believe that this is an important step towards the prevention of this devastating disease, especially considering that our banana plantations are at risk.

Key words: Preventive breeding; Banana germplasm; SCAR markers; Foc TR 4

INTRODUCTION

Banana is a tropical fruit of great importance and a staple food for both urban and rural populations. Its cultivation is highly significant in agricultural systems, especially in tropical agro-ecological zones. Banana cultivation is one of the most economically and socially important agricultural activities in Brazil. Similar to several other cultivated species, banana cultivation is affected by various plant abiotic and biotic stresses. Fusariosis, or 'Panama disease', is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) and is one of the most devastating diseases in bananas (Ploetz, 2006; Haddad et al., 2011; Sutherland et al., 2013).

Foc is classified into one of three known physiological races: 1, 2, and 4, with race 1 infecting bananas of the Silk and Gros Michel varieties, race 2 infecting Bluggoe bananas and plantains (also known as plantain-type bananas), and race 4 infecting banana varieties susceptible to races 1, 2, and those of the Cavendish subgroup. Race 4 is divided into subtropical and tropical in order to differentiate populations that affect these varieties under subtropical and tropical conditions, and is considered to be one of the most devastating races threatening banana plantations worldwide (Ploetz, 2006). Foc TR 4 was described in the early 1990s in South Asia; however, it has not yet been found in Brazil (Matos et al., 2012). Although Foc TR 4 has not yet been found Brazil, it has caused irreparable damage in Asian countries (Moore et al., 1993; Molina et al., 2009) and most recently in Africa (IITA Press Release, 2014). The main banana cultivars planted in Brazil, such as Prata-Anã and Pacovan, are susceptible to Foc TR 4 and occupy almost 80% of the cultivated area.

The emergence of new Foc races is a constant cause for concern for the global banana market (Ploetz, 1990; Sutherland et al., 2013) because the pathogen can survive in soil for over 20 years through structures known as chlamydospores (Stover, 1990). Although bananas have worldwide importance, their cultivation is hindered due to a lack of productive commercial varieties that are resistant to major pests and diseases, such as Panama disease, which simultaneously exhibit desirable agronomic characteristics and market acceptance. Because of the many issues associated with the quality and resistance of bananas to various diseases, it is necessary to obtain varieties with desirable characteristics that may reduce losses associated with cultivation and still meet market demands.

An alternative that has successfully addressed these obstacles is the use of molecular marker technology, which enables the acceleration and monitoring of breeding programs and may promote major advances in the development of improved varieties (Guimarães et al., 2009). Among several classes of molecular markers, SCAR markers are of interest because they can

be amplified using specific primers based on sequences that have usually already been characterized and mapped (Paran and Michelmore, 1993). These primers are derived from the conversion of other markers, many of them from random amplified polymorphic DNA (RAPD) markers. Several studies in the literature have used different approaches with SCAR markers in bananas (Javed et al., 2004; Ramage et al., 2004; Suprassana et al., 2008; Nwauzoma and Saraswath; 2011; Wang et al., 2012). The research of Wang et al. (2012) was used as the basis for the present study. Those authors demonstrated that SCAR bands could be used for the early identification of 'Williams 8818' and Goldfinger cultivars with resistance to Foc TR 4 and its absence in Gros Michel and Grande Naine varieties, which are susceptible to Foc TR 4 (Wang et al., 2012).

Since Brazil is one of the four nations worldwide that has a renowned banana genetic breeding program, the possibility of precociously identifying resistant materials to be tested abroad is of great importance. The development of a thematic collection that highlights accessions with possible resistance to Foc TR 4 contributes relevant information that can be used by countries where the disease already causes devastating losses, as well as by Brazil, where the arrival of the disease is imminent.

Therefore, the present study aimed to screen 276 banana accessions from the germplasm collection of Embrapa Mandioca e Fruticultura using SCAR markers and to develop a thematic collection of possible accessions to be used in a banana preventive breeding program for Foc TR 4 resistance.

MATERIAL AND METHODS

Genetic material

A total of 276 banana accessions from the germplasm collection at Embrapa Mandioca e Fruticultura, including cultivated and wild diploid accessions, as well as tri- and tetraploids, were screened. Of note, this collection is one the most representative of the genetic variability of *Musa* spp in the world.

SCAR markers

The screening of potential accessions with tolerance to Foc TR 4 was carried out using two SCAR markers described by Wang et al. (2012). These SCAR primers were deposited in the GenBank database with the following accession No. HQ613949 and HQ613950; and termed ScaU1001 and ScaS0901, respectively. Specifications for the SCAR primers and the 18S rRNA primer used as the PCR control are presented in Table 1.

Table 1. Specifications for the SCAR primers and the 18S rRNA gene primer used as the PCR control.

SCAR	Ta (°C)	bp	Sequence (5'-3')	Reference
ScaU1001F	66.00	1694	AAC TCG GCA CTC GAA GAC ACA T	Wang et al. (2012)
ScaU1001R	63.00	-	ACC TCG GCA CTA TTA CCC ATC AT	Wang et al. (2012)
Sca0901F	57.00	1429	TCC TGG TCC CAG TAC AAA TAC	Wang et al. (2012)
Sca0901R	60.00	-	TCC TGG TCC CTC TGA ATT TTC	Wang et al. (2012)
18S rRNA gene	54.5	500	CAT CAC AGG ATT TCG GTC CT	Ramage et al. (2004)
18S rRNA gene	55.5	500	AGA CAA ATC GCT CCA CCA AC	Ramage et al. (2004)

DNA extraction and PCR

Genomic DNA was extracted from young banana leaves collected in the field using the cetrimonium bromide method previously described by Doyle and Doyle (1990) and quantified on a 1% agarose gel. The amplification reactions were performed in a final volume of 25 μL and each contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 100 μM each dNTP (dATP, dTTP, dGTP, and dCTP), 0.2 μM each primer, 20 ng genomic DNA, and 1U *Taq* DNA polymerase. The amplifications were performed in an Applied Biosystems Veriti 96-well Thermal Cycler. The amplification reaction included a denaturation step of 5 min at 94°C, followed by 35 denaturation cycles of 30 s at 94°C, annealing with the temperature specific for each primer, 1 min extension at 72°C, and a final extension of 14 min at 72°C.

A multiplex PCR was also performed with an additional primer that amplifies the 18S rRNA gene of *Musa acuminata*, which was used as a positive control for PCR amplification; its sequence is deposited in GenBank with the accession No. U42083.

Electrophoresis and detection of polymorphism

The fragments were separated on 1.5% agarose gels under standard conditions, and the amplification products were stained with ethidium bromide and visualized under ultraviolet light.

Data analysis

Gel images were scanned in a Vilber Lourmat image documentation system, and the SCAR-amplified fragments were analyzed for their presence (1) or absence (0). To analyze the confidence of each marker, it was assumed that the possible resistant accessions displayed the band (1) and the possible susceptible accessions did not display the band (0) predicted for the ScaU1001 and ScaS0901 primers, which are linked to the Foc TR 4 resistance gene (Wang et al., 2012).

RESULTS

Foc TR 4 is considered to be the most important threat to banana plantations worldwide. It is estimated that over 80% of cultivated bananas are susceptible to this *Fusarium* race. Thus, monitoring the introduction of Foc TR 4 in Brazil is extremely important for the sustainability of banana crops (Haddad et al., 2011; Matos et al., 2012). In the present study, two SCAR markers, Sca0901 and ScaU1001, which previously showed efficient discrimination between resistant and susceptible accessions to Foc TR 4 (Wang et al., 2012), were tested in 276 banana accessions from the banana germplasm collection at Embrapa Mandioca e Fruticultura.

ScaS0901

Screening with primer Sca0901 generated the predicted 1429-bp band in more than 90% of the accessions analyzed (data not shown). After multiple tests were performed to check for errors, this SCAR marker was eliminated from our screening.

ScaU1001

Amplification with the SCAR marker ScaU1001, which produced a 1694-bp fragment,

showed highly satisfactory results. Of the 276 accessions tested, the band was present in 36.6%. The presence of the band in 101 accessions revealed the possibility of creating a preventive thematic collection based on these results. The electrophoretic profiles of the ScaU1001 primer in 143 accessions of BAG-banana plants are shown in Figure 1.

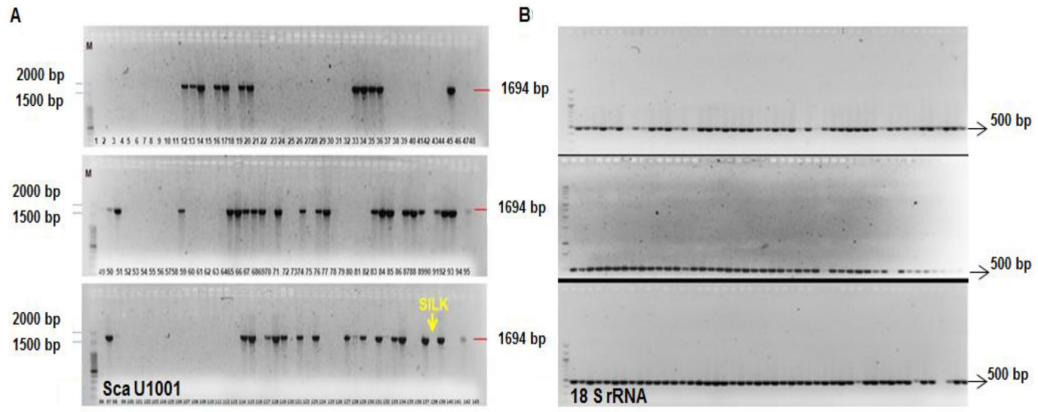

Figure 1. Electrophoretic profile of ScaU1001 primer on 1.5% agarose gel. Samples 1 to 145. Red arrow: ScaU1001 primer with the expected 1649-bp fragment size; Yellow arrow: 'Maça caule roxo' (Silk type banana) susceptible to Foc R1. *Lane M* = 100-bp ladder (Invitrogen™).

However, this band was not expected to appear in accessions belonging to the Cavendish subgroup (Nanicão Franco Rio Claro, Nanicão Taperão FRF, Grande Naine IAC FRF, and Grande Naine P. Formoso). These accessions are thought to be mutants of Nanica and Grande Naine bananas, respectively; and both are susceptible to Foc R1. This result, however, is consistent with the findings of Wang et al. (2012), who found that the SCAR band was present in a Williams-8818 (susceptible to Foc TR 4) mutant variety, Williams 8818-1 (resistant to Foc RT 4).

DISCUSSION

Use of the SCAR marker ScaU1001 will represent an innovative step for MAS in the breeding of *Musa* spp in Brazil once validated in plants inoculated with Foc TR 4. The screening of accessions in the banana germplasm bank with the ScaU1001 primer permitted the development of a "preventive thematic collection", which indicated possible accessions for use in banana breeding programs aimed at improving fruit quality, with the development of more productive plants resistant to fusariosis. Hence, this is the first attempt toward the prevention of one of the most devastating diseases in bananas worldwide.

Previous studies that have used SCAR markers in breeding programs have reported various levels of success. Srivastava et al. (2012) developed and validated the SCAR marker SCOPAK12 for use in predicting sugarcane genotypes that are tolerant to drought. According to those authors, it was possible to identify commercial and wild drought-tolerant genotypes. The authors suggest that this marker could be used to predict tolerant genotypes and may be useful in breeding programs aiming to develop more tolerant genotypes.

Carneiro (2014) worked with alleles resistant to Potato leafroll virus in the germplasm of *Solanum tuberosum* (potato) and observed that only 31% of the clones showed the band associ-

ated with viral resistance when using the SCAR marker RGASC850 in clones (potato); this result differs from the expected 86% of clones displaying the band because one of the parents has the resistance allele (RI_{adg}) under duplex conditions. According to the author, this result may have been caused by incorrect optimization of the PCR or by the lack of a band that could indicate false positives that would have occurred from possible recombination events. This is because there is a small possibility of exchange between the resistance gene and the marker.

In bananas, SCAR markers have been used for different purposes with mixed results. Ramage et al. (2004) performed PCR using the SCAR B1 and B2 markers in association with the 18S rRNA gene for the early detection of dwarfs in micropropagated plants (*Musa* spp AAA). According to the authors, the PCR/multiplex technique provided a reliable and reproducible method for the detection of dwarf bananas. However, they also emphasize that this method is highly sensitive to the type of material selected for analysis, with material from young plants being required to avoid failure of the PCR. Nwauzoma and Saraswath (2011) validated SCAR 245 in parents and hybrids selected for resistance to sigatokas in *Musa* spp, and observed that this marker was not able to discriminate between susceptible and resistant progenies because it could not detect polymorphism between individuals.

In bananas, the identification of SCAR markers is somewhat controversial because of their parthenocarpic nature and the insufficient number of seeds in the crossings of interest. This often hinders analyses in segregating populations, which could assist the more robust identification of markers related to genes of interest.

Also relevant is how most SCAR markers have been identified so far, where the relatively small number of accessions evaluated in terms of resistance or susceptibility to diseases (Nwauzoma and Saraswath, 2011; Wang et al., 2012) may not have been sufficient for reproducible results. This might have been the case with ScaS0901, which did not present satisfactory results in the present study.

Nwauzoma and Saraswath (2011) only used two resistant (Calcutta 4 and Manoranjitham) and two susceptible genotypes (Anaikomban and Grande Naine) of *Mycosphaerella musicola* to develop the SCAR marker. This small number of genotypes was probably not sufficient for the generation of more robust data, explaining why their SCAR marker was unsuccessful in the discrimination of banana genotypes resistant to yellow Sigatoka. The use of small numbers of genotypes in previous studies designed to identify SCAR markers limits the reproducibility of their results. According to Mutengwa et al. (2005), the generation of false positives and a lack of reproducibility implies that the band is not completely linked to the gene of interest. This might explain why primer ScaS0901 was not successful in the present study.

The primers used in this present study were based on those designed by Wang et al. (2012), and the scar markers were obtained from 500 RAPD markers in only seven genotypes (resistant and susceptible to Foc TR 4). Among these RAPD primers, primers RAPD OPU10 and OPS09 were the originators of the SCAR markers ScaU1001 and ScaS0901, respectively. These markers were efficient at obtaining bands from the resistant genotypes, 'Williams 8818-' and Goldfinger, and bands were absent in five genotypes, Williams 8818-(AAA), Grande Naine (AAA), Gros Michel (AAA), cv Brazilian (AAA), and cv Tinabao (AAA), which are susceptible to Foc TR 4. Since our breeding program uses 'Williams' and 'Goldfinger' in some crosses, we believe that these markers may be even more useful if this genetic background is enhanced in our germplasm collection.

It should be emphasized that a marker closely linked to the locus of interest improves the accuracy and efficiency of genotyping individuals for MAS and provides reproducible results; thus, it can be easily applied to plant breeding (Hittalmani et al., 1995).

These results represent the first step towards the development of methods that can be used to validate this information and consolidate the use of MAS in Banana Breeding Programs within Embrapa Mandioca e Fruticultura in Brazil since Foc TR 4 is not yet present.

Accessions of this thematic collection will be sent to the countries/institutions collaborating with Embrapa (Wageningen, The Netherlands, IITA-Nigeria, and Queensland, Australia) in order to validate this information following inoculation with Foc TR 4 virulent isolates, and they will subsequently be incorporated into the preventive breeding programs of *Musa* spp. Furthermore, given that the main banana cultivars planted in Brazil, such as Prata-Anã and Pacovan, are susceptible to Foc TR 4 and occupy almost 80% of the cultivated area, this becomes an important step towards the prevention of this devastating disease. Thus, this thematic collection is needed as an initial milestone to safeguard this crop before the introduction of this devastating disease in the country.

Conflicts of interest

The authors declare no conflict of interest.

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