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Full Length Research Paper

Developing markers for Sigatoka leaf spot disease (*Mycosphaerella musicola* Leach) resistance in banana (*Musa* spp.)

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Sigatoka leaf spot (*Mycosphaerella musicola* Leach) disease is a limiting factor in banana production in India and other places. Breeding for resistance is the most effective method to control *Musa* diseases. However, *Musa* improvement using conventional methods has been hampered due to lack of genetic variability, resulting to biotechnological approaches. In this regard, marker-assisted selection has become a reliable method to improve disease resistance in *Musa*. The objective of this study was to identify markers that may be linked to Sigatoka leaf spot disease in *Musa*, using RAPDs and converting such into sequence characterized amplified region (SCAR). Consequently, a total of 102 oligonucleotide OPERON primer pairs were used to screen genomic DNA from two resistant cultivars: Calcutta 4 (*Musa acuminate,* AA) and Manoranjitham (AAA), and two susceptible cultivars Anaikomban (AA) and Grande Naine (AAA) with only 11 (10.8%) of the primers being polymorphic. Eventually, OPK 01 and OPK 11 primers in Calcutta 4 were eluted, but only OPK 11 was sequenced and cloned using pGEM-2T vector, resulting to a band size of 4.3 KB, and the development of two SCAR markers. A FASTA search in the *Musa* genome database could not identify corresponding gene sequences that show homology with the sequenced PCR fragment. Finally, the SCAR marker was used to amplify genomic DNA from the segregating population which could not discriminate between resistant and susceptible samples. This may be due to amplification conditions, limited number of primers and most importantly, the absence of tight linkage with the gene of interest. In conclusion, it may be necessary to screen the segregating population with more reliable and reproducible amplified fragment length polymorphism.

Key words: Marker-assisted selection, disease resistance, *Musa*, random amplification of polymorphic DNA (RAPD), genetic improvement, SCAR.

INTRODUCTION

Banana and plantain (*Musa* spp. Linn) are important staple and cash crops that grow in the humid regions of Africa, America and Asia (Robinson, 1996). Worldwide annual production is estimated to be 400 million tons (Till et al., 2010). Approximately, 90% of the total production serves as food for domestic consumption. The impact of diseases and pests, especially Sigatoka leaf spots has been recognized as a serious constraint to *Musa* production in different parts of the world (Blomme et al., 2011). In particular, Sigatoka leaf spot (*Mycosphaerella musicola* Leach) has become prevalent especially in areas where black Sigatoka (*Mycosphaerella fijiensis* Morelet) is absent and this limits efficient production. Breeding for resistance has been credited as the most appropriate and effective method to control *Musa* diseases. However, long generation time (10 to 18 months), large space (6m^2 per plant), sterility of most edible cultivars and the lack of genetic variability have hampered the development of disease-resistant *Musa* by conventional breeding. Furthermore, plantain and

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banana phenotypes are a poor indicator of genetic constitution and this restricts the development of a reliable breeding scheme. This also hinders attempts to maximize heterosis and so, cannot reliably predict the specific combining ability of parental genotypes (Tenkouano, 2005). There is therefore a considerable need for accurate genetic information on which breeding strategies could be formulated to aid selection in the improvement of *Musa*.

PCR-based molecular markers are the most appropriate assays for molecular breeding applications because they are relatively simple to handle and can be easily automated (Rafalski and Tingey, 1993). Reports on the application of molecular markers in *Musa* studies have mainly concentrated on diversity analysis and genetic mapping (Ude et al., 2002a, b; Creste et al., 2004; Oreiro et al., 2006). Ferrreira et al. (2004) used RAPD markers to characterize banana diploids (AA) with varying levels of black Sigatoka and yellow Sigatoka resistance. Recently, a putative RAPD marker for Sigatoka resistance has been identified at the National Research Center for Banana (NRCB), India (Backiyarani et al., 2010). Hence, this study was undertaken to identify molecular markers that may be linked to Sigatoka leaf spot disease in *Musa*, a major production constraint in India, using RAPD technique and converting such into sequence characterized amplified region (SCAR) markers.

MATERIALS AND METHODS

Plant materials and DNA isolation

Genomic DNA was isolated from the cigar leaves of the test accessions (Table 1), using CTAB method (Gawel and Jarret, 1991) with minor modifications. DNA concentrations were determined with a spectrophotometer and its integrity was examined by 0.8% agarose gel electrophoresis.

RAPD PCR

A total of 102 random primers belonging to OPERON series (Operon Technologies Inc., Alameda, California) OPA, OPB, OPC, OPD, OPE and OPV were tested to analyze genetic diversity and phylogenetic relationships. PCR was carried in a 25 µl reaction containing 50 ng of genomic DNA, 1X PCR buffer (Genei, Bengaluru, India), 200 µM each of four dNTPs, 1.5 U of Taq polymerase (Genei, Bengaluru, India), 1 mM $MgCl_2$ and 0.2 µM of random primer. PCR program for RAPD consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, followed by extension at 72°C for 1 min, terminated by a final extension at 72°C for 15 min and incubated at 4°C.

Gel electrophoresis and data analysis

The amplified products were separated by gel electrophoresis on 1.5% (w/v) agarose gels using 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and the gels were stained with ethidium bromide (0.5 mg ml^{-1}). The banding patterns were documented in Alpha Innotech Image Analyser. The molecular weight of the amplified fragments was calculated by comparing the molecular weight of the marker (500 bp DNA ladder) using the software Alpha Imager Version 4.0 (Alpha Innotech Corporation, Santa Clara, California, USA).

DNA elution

Specific bands produced by the RAPD primers namely OPK 01 and OPK 11 in Calcutta 4 were excised from the gel under UV- Vis transilluminator (Hoefer MacroVue, Uvis-20, Amersham, Biosciences, USA). The sliced bands were used for DNA elution using MinElute (Qiagen, USA). The eluted bands were examined in 0.8% agarose gel and stored in the refrigerator (-20°C).

Approximately, 2 µg of the purified PCR product from the primer OPK 11 of size 1.3 kb from Calcutta 4 was cloned using pGEM-2T vector system (GeNei, Bengaluru, India). The clones were confirmed by the release of insert using NcoI restriction endonuclease and sequenced bi-directionally using T7 and SP6 (vector specific) primers. A FASTA search was conducted in the *Musa* genome database developed by the European Molecular Biology Laboratory (EMBL) (www.ebi.ac.uk/FASTA3/) to identify corresponding gene sequences that show homology with the sequenced PCR fragment.

Conversion of RAPD marker into SCAR marker

Primers for the putative diagnostic markers were designed based on the sequence result, using the software Primer 3 (Rozen and Skalestsky, 1998). The criteria used to design the primers were length of 218 to 250 bp, G : C content of 50 - 55%, Tm of 55 - 60°C and preference for G or C at the 3'- end of the primer. The annealing temperature for the SCAR markers was adjusted based on the melting temperature (Tm) of 55 to 60°C of the primer sequence and standardized at 58.6°C.

Validation of the developed SCAR marker

PCR amplification with SCAR primers was carried out in a 25 µl reaction with 1X PCR buffer, 2 U Taq DNA Polymerase (Sigma, St. Louis, Missouri, USA), 10 mM each of the dNTPs (dATP, dCTP, dGTP, dTTP), 1 mM $MgCl_2$, 50 ng of template DNA and 2 picomoles each of forward and reverse primers. PCR conditions were as follows: an initial denaturation at 95°C for 3 min, then 40 cycles of final denaturation at 94°C for 2 min, annealing at 58.6°C for 1 min, with initial extension at 72°C for 1 min 10 s and final extension at 72°C for 15 min followed by incubation at 4°C.The OPK 11 primer was used as control. The PCR amplicons were subjected to electrophoresis on 0.8% agarose gel, stained with ethidium bromide and visualized under UV illumination. The SCAR markers were tested on selected parents and hybrids, using Calcutta 4 and Manoranjitham, and Anaikomban and Grand Naine as resistant and susceptible controls, respectively.

RESULTS

DNA purification and amplification

The relation Â 260/280 for the extracted DNA samples were oscillated between 1.8 and 1.9, which indicates high purity and low protein contamination in the DNA

Table 1. List of progenies used in the validation of the SCAR developed for Sigatoka resistance.

Progeny number	Parentage	Breeding strategy
01/05	*Pisang Jajee* (AA w) x Matti (AA cv)	Elite diploid
02-1/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
02-2/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
02-3/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
02-4/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
02-5/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
02-6/05	Karpuravalli (ABB) x *P. Jajee* (AA w)	Triploidy
03-1/05	Matti (AA cv) x Anaikomban (AA cv)	Elite diploid
03-2/05	Matti (AA cv) x Anaikomban (AA cv)	Elite diploid
03-3/05	Matti (AA cv) x Anaikomban (AA cv)	Elite diploid
04-1/05	Matti (AA cv) x Cv. Rose (AA cv)	Elite diploid
05/05	Sannachenkadali (AA cv) x Lairawk (AA w)	Elite diploid
06/05	Anaikomban (AA cv) x *P. Jajee* (AA w)	Elite diploid
07/05	Anaikomban (AA cv) x *P. Jajee* (AA w)	Elite diploid
08/05	Sannachenkadali (AA cv) x Lairawk (AA w)	Elite diploid
09/05	Anaikomban (AA cv) x Lairawk (AA w)	Elite diploid
10/05	Anaikomban (AA cv) x *P. jajee* (AA w)	Elite diploid
11/05	Burro cemsa (ABB) x H3 (Rhodoclamys)	Triploidy
12/05	Manoranjitham (AAA) x Cv. Rose (AA cv)	Elite diploid
13-1/05	H3 x *Pisang Jajee* (AA w)	Intersectional
13-2/05	H3 x *Pisang Jajee* (AA w)	Intersectional
14-1/05	*Musa laterita* x *Pisang Jajee* (AA w)	Intersectional
14-2/05	*Musa laterita* x *Pisang Jajee* (AA w)	Intersectional

preparations. A total of 102 oligonucleotide primer pairs from kits A, B, C, D and K (Operon Technologies Inc., Alameda, California) were employed to screen genomic DNA extracted from the two resistant cultivars viz, Calcutta 4 (*Musa acuminata*) and Manoranjitham and two susceptible cultivars Anaikomban and Grande Naine. Although, many of the primers showed discrete banding patterns, only eleven (11) representing 10.8% were polymorphic. The size of the amplified fragments ranged from 400 to 2152 base pairs (Table 2).

Elution, cloning, sequencing and homology search

The fragment size of PCR products (Figure 1) were compared with eluted samples and found to be approximately 1.3 kb in both primers (Figure 2). The cloning of the OPK 11 fragment into the pGEM-2T vector generated a band of about 4.3 kb (Figure 3). The corrected and aligned insert sequence is indicated in normal font, while the vector DNA sequence data is marked in blue, with the cloning NcoI sites of the vector marked with pink (Figure 3).

Marker development and validation

Two SCAR makers were developed based on the sequence results. The forward 5´-GCAACACGGGCCTG TATAAT-3´ and reverse primer 5´-TTGGCAATGGAA CAATGG-3´ primers were synthesized and used to amplify DNA samples of the resistant and susceptible cultivars for validation. A combination of the forward and reserve primers generated a band of 245 base pairs when it was tested on Calcutta 4. This was within the range of the expected product size, based on the difference between the forward primer (541) and the reverse primer (314). However, when it was employed to validate the selected parents and hybrids, it was not able to discriminate the resistant and susceptible progenies. The sequenced RAPD product did not show any homology with genes in the *Musa* database. The FASTA search however, produced a 5.2 sequence close to the plant *Populus* on which basis the primer was designed.

DISCUSSION

Marker-assisted selection (MAS) is a useful and reliable method to improve disease resistance in crop plants (Josh and Nayak, 2010). In particular, PCR techniques such as RAPDs involve the design of arbitrary primers that can discriminate annealing sites (Innis et al., 1999; Williams et al., 1990). Markers that are linked to Sigatoka leaf spot disease resistance could expedite the development of resistant cultivars through adoption of

Table 2. Putative bands identified for Sigatoka using various RAPD primers.

S/N	RAPD primer	Resistant sample	Susceptible sample	Putative marker for Sigatoka (molecular weight in bp)
1	OPA 13	–	+	1230
2	OPB 01	–	+	2084
3	OPB 12	–	+	873
4	OPC 01	–	+	1371
5	OPC 04	+	–	1595
6	OPD 07	+	–	1296
7	OPD 12	+	–	2152
8	OPK 01	+	–	1309
9	OPK 11	+	–	1331
10	OPK 16	+	–	400
11	OPK 18	+	–	810

+ Present; - absent.

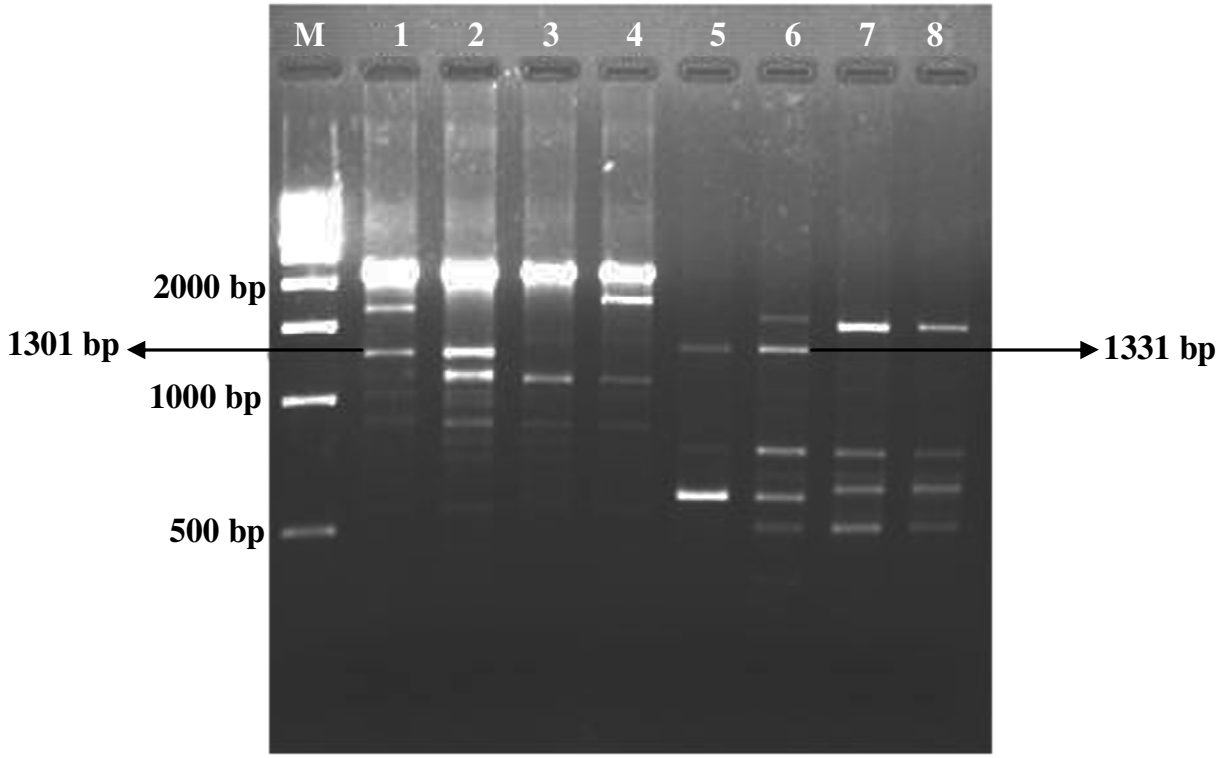

Figure 1. RAPD profiles generated from two resistant (Calcutta 4 and Manoranjitham) and two susceptible (Anaikomban and Grand Naine) *Musa* clones using primers OPK01 (lanes 1 - 4) and OPK11 (lanes 5 - 8). M: Marker, lanes: 1 and 5 – Calcutta 4, lanes: 3 and 7 – Anaikomban, lanes: 2 and 6 – Manoranjitham and lanes: 4 and 8 – Grand Naine.

appropriate MAS strategies. Screening with 102 random oligonucleotide primers resulted in 11 markers (10.8 %) that were polymorphic either in resistant or susceptible samples. Damasco et al. (1996) reported 28.8% polymorphism using RAPD technique to detect dwarf off-types in micropropagated Cavendish banana (*Musa* spp. AAA) and Williams, while Hamid et al. (2004) obtained 61.25% polymorphic bands in Cavendish banana using the same technique. The developed SCAR markers amplified a single DNA fragment of 245 bp in Calcutta 4 which was designated as SCAR 245. To further evaluate the possibility of using this SCAR marker to amplify genomic DNA from the segregating population with different genetic backgrounds, the results obtained showed that the SCAR primer could not detect polymorphisms in both resistant and susceptible samples.

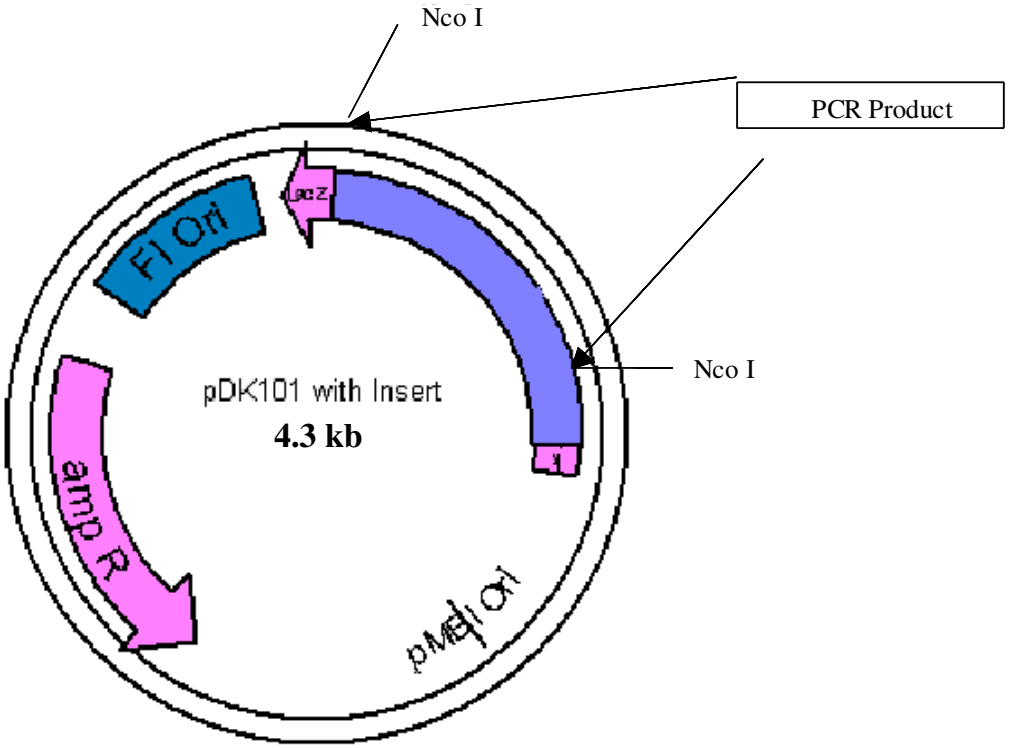

Figure 2. Putative marker for Sigatoka disease resistance identified using primer OPK 11 (1.3 kb) cloned in pGEM-2T vector and sequenced for conversion into SCAR marker.

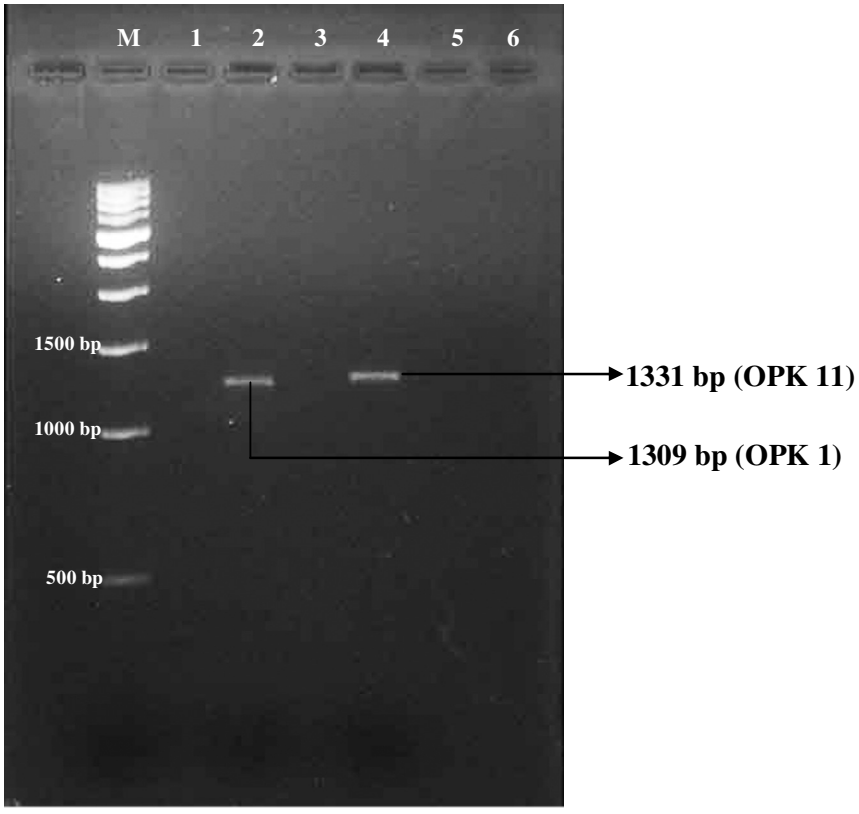

Figure 3. Eluted DNA of PCR amplified product of Calcutta 4 RAPD using RAPD primers OPK 01 and OPK 11.

This indicates that the putative band was not tightly linked to the gene of interest. Hence, the SCAR marker could not discriminate between resistant and susceptible samples. The PCR amplification that generates RAPD fragments is known to be very sensitive to specific conditions (Mutengwa et al., 2005). Usually, RAPD makers are inherited as dominant genetic markers which limit their use in downstream molecular applications. This is the reason behind the conversion of RAPD marker that is linked to a gene of interest into SCAR. Paran and Michelmore (1993) used SCAR to identify markers linked to downy mildew resistance in lettuce. RAPD analysis has also been used to develop DNA markers that are linked to disease and insect resistance in several crops (Williams et al., 1990; Mutengwa et al., 2005). This is yet to be achieved in Sigatoka leaf spot disease complex. Sigatoka leaf spot otherwise known as yellow Sigatoka is a major constraint to banana production in India and breeding for its resistance is a major priority. Marker-assisted selection is a key tool for achieving this objective, but is limited by the presence and number of available markers.

Some studies suggested that optimal number of SCAR markers is necessary for efficient improvement in MAS programs (Liang et al., 2004). This is because a designed primer may not necessarily result in polymorphism observed during agarose gel electrophoresis or direct sequencing (Slippers et al., 2004). In this study, only one out of the eleven primers that showed polymorphism was converted into SCAR markers. Umali et al. (2002) observed that two out of five SCAR markers were able to amplify scorable bands (fingerprints) in *Musa* AAA. Also, Liang et al. (2004) noted that MAS was more efficient with six markers than just using two markers. Thus, increasing the number of markers that are reproducible may be required for the development of SCAR marker for Sigatoka disease resistance in *Musa*.

Successful use of RAPD primers requires knowledge of the factors that influence PCR amplification conditions. According to Williams et al. (1993), G+C content of the primer and its length highly influence amplification products from RAPD. In this study, a primer length of 218 to 250 bp and G : C content of 50 to 55% may not have been optimum for the best amplification.

In conclusion, RAPD primers suffer from reproducibility, which accounts for why they are converted into robust and reliable SCAR markers. Such SCAR markers can detect a single locus and may be co-dominant. In this study, the RAPD-SCAR marker correctly amplified a fragment of Calcutta 4 but could not discriminate between resistant and susceptible samples. The failure may be attributed to amplification conditions, limited number of primers and most importantly, the absence of tight linkage with the gene of interest. Hence, screening the segregating population with more reliable and reproducible amplified fragment length polymorphism (AFLP) and attempt towards the development of SCAR for Sigatoka resistance is suggested.

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