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Development of SCAR marker for screening Sigatoka-leafspot resistance in banana genotypes

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Abstract

Sigatoka leaf spot is a major fungal disease of banana worldwide, particularly, in pacific island, from where this pathogen originated. Not so many resistant genotypes are reported against this. Initially, RAPD fragments correlating with lower percentage of disease index (PDI) were identified involving fifteen banana cultivars collected from diverse zone of West Bengal. Four correlating RAPD fragments from each two independent genotypes were cloned, sequenced and submitted into GenBank. It had been observed that sequence information was dissimilar even though same RAPD primer amplified equal sized amplicon from two different varieties. Even fragment from two different clones of a variety, was also dissimilar. Based on sequence information, eight pairs of SCAR primers were designed. Only one SCAR primer based on the sequence of $OPA13_{640}$ from Kanthali Clone1 showed reproducible dominant banding pattern like RAPD. Identified SCAR marker when validated among additional 26 diverse genotypes, collected across India, showed strong correlation with the Sigatoka resistance lines. SCAR markers identified and validated in this study will be useful in screening Sigatoka resistance lines in future banana breeding program.

Key Words: Banana, Sigatoka disease, SCAR, Kanthali clone 1

Introduction

Banana (*Musa* species) is one of the important fruit crops in the world, both as a source of livelihood security as well as an export commodity for many tropical countries. It is widely grown in India with great socio-economic significance and is interwoven in the cultural heritage of the country. In India banana is grown in an area of 721.79 hectares with an annual production of 24.86 million tones. Most of the edible bananas in India are triploid and come from inter specific crosses of the wild diploid, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). Sigatoka disease (SD), caused by *Mycosphaerella musicola*, is one of the most important diseases affecting the majority of the commercial cultivars of banana (Jones 2009). Sigatoka leaf spot disease causes yield loss of 11% to 80% in India (Shanthiyaa et al. 2013).

In general, the first symptom is the appearance on the upper leaf surface of pale yellow streaks, 1-2 mm long which enlarge to form necrotic lesions with yellow haloes and light grey centers. Lesions can coalesce and destroy large areas of leaf tissue which results in reduced yields and premature ripening of fruit. Although systemic fungicides provide effective control of the disease in large orchard but their effects on the environment are cause for concern. Genetic resistance to SD is clearly the best long-term goal for disease control especially for smallholders who cannot afford to purchase chemicals. But source of resistance is absent in widely used cultivars like 'Cavendish' or 'Grand Naine'. However, several genotypes were already identified as moderately resistant. The banana genetic breeding program is based mainly on the improvement of (AA) diploids and subsequent crosses with AAB triploids Prata and Silk types generating AAAB tetraploids, agronomically superior and resistant to diseases (Vuylsteke et al. 1993).

Identification of molecular markers correlated with Sigatoka disease will expedite the selection process more accurately. Although molecular markers have been developed for use in banana improvement,

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focusing on germplasm characterization (Bhat et al. 1995) and identification of A and B genomes (Lagoda et al. 1998; Creste et al. 2005; Buhariwalla et al. 2005), little has been devoted to the development of DNA markers for important banana pathogens such as Sigatoka disease. Recently, a subset of 95 potential defense-related gene-derived simple sequence repeat (SSR) loci were identified by comparing whole transcripts from the infected leaves of two contrasting M. *aciminata* accessions, Calcutta4 and Grand Naine (Passos et al. 2013). Another attempt was made earlier (Nwanzoma et al. 2011) for identification of RAPD-SCAR marker from a resistant parent, Calcutta 4, but unable to validate between resistance and susceptible samples. In this study, numbers of SCAR markers were developed from polymorphic RAPD fragments and one has shown a significant correlation between resistance and susceptible genotypes when further validated among a large number of samples.

Materials and methods

Plant materials and disease scoring

Banana genotypes belonging to three genomic groups as shown in the Table 1 were selected for this study. These were maintained in the banana resource conservation center under all India Co-ordinated Research Project (AICRP) of tropical and sub-tropical fruits, Bidhan Chandra Krishi Viswavidyalaya, India. The percent disease index (PDI) for SD was calculated from the six months old leaves for consecutive two years following the standard severity scoring system, (0, no symptom; 1, <1% of lamina with symptoms (only streaks and/or up to 10 spots); 2, 1 to 5% of lamina with symptoms; 3, 6 to 15%; 4, 16 to 33%; 5, 34 to 50% and 6, 51 to 100% of lamina with symptoms) modified by Gauhl (1993).

Table 1. Percent Disease Index (PDI) of genotypes, their genome symbol, state of collection and status of the amplification of newly designed SCAR marker; yes (Y) or no (N)

Genotype	Genome	Place of cultivation	PDI (%)	$OPA13_{640}$
Local landraces				
Hill banana (1)	AAB	West Bengal	8.6-9.4	N
Krishna Vazai (24), Kalibhog (15)	AAB	Andhra Pradesh	9.1	Y
Alpan (25)	AAB	Kerala	9.7	Y
Madhranga Bale (37)	AAB	Tamil Nadu	10.1	Y
Baish Chhana (2), Kanthali Clone-1 (6), Behula (7), Baisha (9), Bagda (13), Purulia Colln (14), Kanchkel (16), Thonte (17), Kanthali Clone-II (21), Deshi Kanthali (36)	ABB	West Bengal	8.3-10.1	Y
Green Bombay (22), Paunsia Banthal (39)	ABB	Andhra Pradesh	9.7	Y
Karpoorvalli (23), Simolu (32)	ABB	Tamil Nadu	8.9-9.3	Y
Pantharaj (29)	ABB	Bihar	9.9	Y
Onchini (41)	ABB	Kerala	9.9	Y
Jurmuney Kanthali (18)	ABB	West Bengal	9.3	N
Champa (12), Sabri (10), Deshi Malbhog (11), Matta Poovan (8), Martaman (4)	AAB	West Bengal	9.2-42.6	N
Patakapura (30)	AAB	Tamil Nadu	37.5	N
Amritpani (34)	AAB	Andhra Pradesh	38.7	N
Karpurachakkarkeli (28)	AAB	Kerala	37.2	N
Robusta Clone-III (35), Srimanti (40)	AAA	West Bengal	41.5	N
Mahalaxmi (31)	AAA	Tamil Nadu	40.3	N
Jahaji Clone-I (33), Sinduri Harichal (38), Lacaton (27)	AAA	Andhra Pradesh	36.7-43.5	N
Cultivars				
Grand Naine (3), Dwarf Cavendish (5), Amrit Sagar clone-I (19), Giant Governor (20)	AAA	West Bengal	39.4-45.3	N
Gross Michel (26)	AAA	Kerala	42.4	N

Note: Sl. No. in parenthesis

DNA isolation and RAPD reaction

Genomic DNA was isolated from the banana leaves following the method as described earlier (Prakash et al. 2009) with the additional addition of 2% polyvinylpyrrolidone in the extraction buffer. The concentration and purity were determined from the A_{260}/A_{280} ratio using a spectrophotometer and its integrity was examined by 1.0% agarose gel electrophoresis. A total of 20 decamer primers belonging to OPERON series like, OPA, OPB, OPC, OPD, OPH, OPM and OPN were tested to identify correlating RAPD-fragments with the PDI value against Sigatoka. PCR was carried in a 25 µl reaction containing 50 ng of genomic DNA, 10X *Taq* DNA polymerase buffer, 5.0 µl of 2.5 mM dNTP mix, 1 U of *Taq* DNA polymerase (Genei, Bangaluru), 2.0 mM $MgCl_2$, 0.15 µM of decamer primer. PCR program for RAPD consisted of an initial denaturation at 94^{o}C for 1 minute, followed by 35 cycles of denaturation at 92^{o}C for 30 second, annealing at 38^{o}C for 30 second and extension at 72^{o}C for 1.5 minute and finally extended for 7 minutes.

Gel electrophoresis and data analysis

The amplified products were separated by gel electrophoresis on 1.2% (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 visualized and captured on UV transilluminator and documented using Gel Documentation system (UVP Ltd, UK). Each band in the RAPD fingerprint was considered as a separate putative locus. Only distinct, reproducible, well-resolved fragments were selected and scored for presence (1) and absence (0) of a band. Fragments which are correlating with the lower percent of disease index (PDI) value were identified using single point ANOVA analysis (Microsoft excel) where p value is below 0.05.

SCAR marker development and validation

Correlating fragments amplified by OPA13, OPC01, OPD07 and OPD08 were excised from the selected banana cultivars (Table 2) under UV-Vis trans-illuminator (Genei, Bangaluru). DNA was eluted using spin elution kit (Qiagen, Germany). The eluted bands were examined on 1.0% agarose gel and were reamplified in order to increase the concentration using corresponding RAPD primers. These were further cloned into pGEM-T (Promega) vectors. The presence of the respective bands were confirmed by the restriction endonuclease digestion using NcoI and Pst 1 and sequenced bi-directionally using T7 and SP6 (vector specific) primers from the commercial source (GCC Biotech, Kolkata). A Basic Local Alignment Search Tool (BLAST) was conducted (www.ncbi.nlm.nih.gov/BLAST/) to identify corresponding gene sequences that with the studied sequence, putative diagnostic primers were designed using the software, Fast PCR 6.5 (*www.primerdigital. com/fastpcr.html*). The criteria used to design the primers were G:C content above 50%, T_m-55-60^{o}C, absence of dimerization capacity and oligonucleotides between 18 to 24 bases. The newly designed SCAR primers were tested for validation not only within fifteen genotypes where RAPD markers was validated but also twenty six additional genotypes was considered where PDI score was known. Validation was carried out in a 20 µl reaction with 10 µl of PCR master mix (Ampli taq gold, Invitrogen, USA), 50ng of template te DNA, 1 µl each of newly designed forward and reverse primers (15n moles) and 6 µl of nuclease free water. PCR conditions were as follows: initial denaturation at 94^{o}C for 5 minute, 35 cycles of denaturation at 92^{o}C for 45 seconds, annealing at 55^{o}C for 45 seconds and extension at 72^{o}C for 1 minute followed by final extension at 72^{o}C for 7 minutes.

Table 2. Accession number of the sequence correlated with the RAPD fragments and designed primer pairs for SCAR

RAPD fragment	Source	Accession number
$OPA13_{640}$	Kanthali Clone-1	KJ801649
$OPA13_{640}$	Baisha	KJ801650
$OPC01_{696}$	Martaman	KJ801517
$OPC01_{696}$	Deshi Malbhog	KJ801399
$OPD07_{800}$	BaishChhara	KJ810518
$OPD07_{800}$	Behula	KJ810521
$OPD08_{710}$	BaishChhara	KJ810520
$OPD08_{710}$	Behula	KJ810519

Results

Identification of co-segregating RAPD fragments

Disease reaction was scored following the severity of Sigatoka leaf spot disease. Disease index recorded less than or almost equal to ten was considered as moderately resistance, and above ten as susceptible. First fifteen genotypes as described in Table 1 were

considered initially for identifying RAPD fragments correlating with the SD resistance. None of the genotypes showed complete resistance or immune against this disease. However, ten out of fifteen genotypes exhibited as moderately resistance whereas five were considered as susceptible as described in Table 1. A total of 20 decamer primer pairs from Operon series were employed for fingerprinting using genomic DNA, extracted from the fifteen cultivars, mainly collected from the farmer's field surrounding the Kolkata (Table 1). These primers generated clear, simple, and reproducible polymorphic fragments. The size of the amplified products ranged from 170 to 2500 bp. A total 125 reproducible bands were generated by these twenty decamers, out of which 85 bands showed polymorphism. Four RAPD fragments, $OPA13_{640}$ (Fig. 1a), $OPC01_{696}$, $OPD07_{800}$ (Fig. 1b) and $OPD08_{710}$, showed significant correlation with lower Sigatoka PDI value ($p < 0.05$) as observed from single point ANOVA analysis.

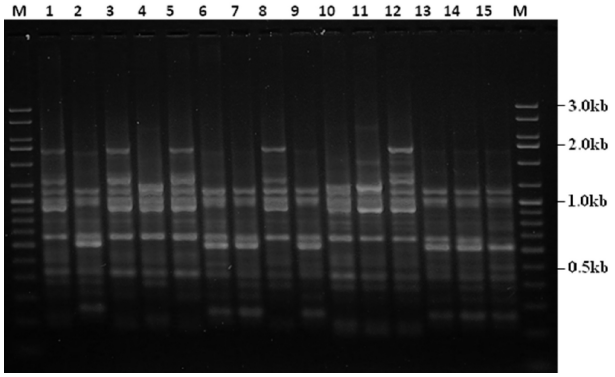

Fig. 1a. Agarose gel electrophoresis of PCR products with RAPD primer, OPA13; Lane 1-15: Different banana genotypes as mentioned in Table1

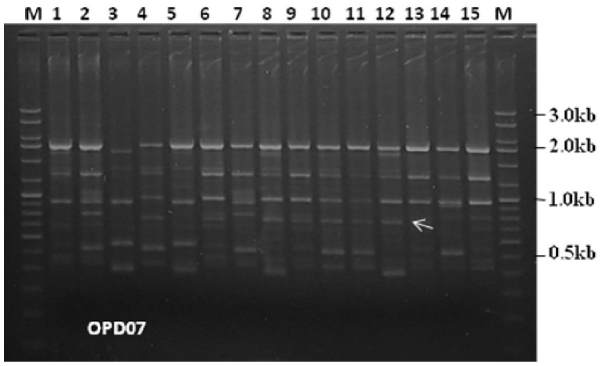

Fig. 1b. RAPD profile of OPD07 showing target fragment (shown with arrow) for cloning, sequencing and SCAR primer development

Designing of SCAR primers and validation

Each correlated band was eluted from two independent genotypes, like, $OPA13_{640}$ fragment was eluted from both Kanthali Clone-1 and Baisha; $OPC01_{696}$ fragment was eluted from both Martaman and Deshi Malbhog and re-amplification was done using same RAPD primers. The fragment size of the PCR products were compared with eluted samples and found to be same in both the cultivars. Thus four correlated fragments from two independent genotypes i.e., total eight fragments were cloned into pGEM-T cloning vector (Promega, USA). Sequencing of all these clones was done by di-deoxy chain termination method in both directions, and submitted to the gene bank and multi-alignment was carried out for their similarity analysis. The sequence of $OPA13_{640}$ between Kanthali Clone1 (KJ801649) and Baisha (KJ801650) showed cent percentage similarity. But sequence of two independent clones from the same genotypes, like, Baisha (KJ801650 and KJ810522) was dissimilar. Similarly, $OPC01_{696}$ from Martaman (KJ801517) and Deshi Malbhog (KJ801399) showed completely dissimilarity although equal size of fragment was cloned. Sequence of remaining two fragments, $OPC01_{696}$ and $OPD07_{800}$, from two independent genotypes showed cent percent similarity. SCAR primers were designed from all these sequences (Table 2). Forward, MS1, 5´-GCACCCACCAACAGTTTGA-3´ and reverse primer, MS2 5´-CAGCACCCACTT CCAATT-3´ was developed based on the sequence of 640 bp fragment of Kanthali Clone 1 (Fig. 2). A combination of the forward and reserve primers amplified a band of 640 base pairs when it was tested not only on Kanthali Clone-1 but from all the genotypes those amplified $OPA13_{640}$ fragments by RAPD. Contrarily, primers designed from OPD_{710} and $OPD07_{800}$ gave multiple and non-specific bands respectively. Therefore, SCAR primers designed from OPA_{640} of Kanthali Clone1 amplified same banding pattern among the fifteen genotypes as par initial RAPD analysis but other primer pairs failed. Like RAPD, newly designed SCAR primer failed to amplify the 640bp fragment in two moderately resistance genotype like, Matta Poovan and Champa (Fig. 3). A fragment of 670bp was amplified from a susceptible cultivar, Grand Naine. However, when newly designed SCAR primer pair was employed to validate among the additional twenty six genotypes, it produced a 640 bp fragments from all the moderately resistant genotypes (Fig. 3) except Jurmney Kanthali. Therefore, identified SCAR marker developed in this study is highly correlated with Sikatoga disease of banana.

Fig. 2. Sequence of OPA13$_{640}$ with RAPD primers on two terminals represented by bold letters. Anchoring sites of SCAR primer pair are shown by arrow heads

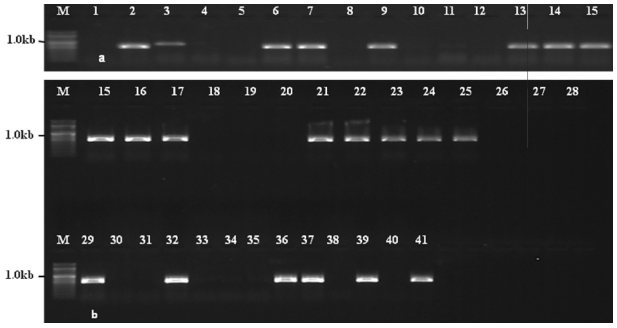

Fig. 3. Agarose gel electrophoresis of PCR products with the newly designed SCAR primer pair, Lane 1-41: Different banana genotypes as mentioned in Table 1

Discussion

Most banana that produce edible fruits evolved from either intra or interspecies crosses comprising of the wild diploid species, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). It is an internationally important crops but several biotic threats like Sigatoka leaf spot, wilting of root, Panama wilt etc. cause significant yield loss every year. Only a few genotypes have shown resistance against this disease. In this study, genotypes which exhibit moderate resistance reaction against Sigatoka, carry at least one B genome which serve as donor for the resistance genes against several biotic stresses as shown earlier (Pillay et al. 2002). On the other hand, all ten genotypes with AAA genome had shown susceptible reaction. Reproducibility of amplification patterns was very consistent; identical RAPD patterns were obtained under the same amplification conditions for the 2-3 replicates. RAPD fragments which showed significant correlation with the low PDI values were considered for SCAR marker development. Genotype Kanthali Clone1 is one of the most popular and oldest cultivar of West Bengal which is highly resistance against several biotic and abiotic stresses.

The 640bp fragments amplified by RAPD primers among two genotypes are not similar in sequence. Hence, OPA13$_{640}$, in different varieties comprised of several fragments of similar size as confirmed by sequencing analysis. Why RAPD markers were not reproducible can easily be concluded from the sequence data. Same size of fragment amplified by a primer from two different varieties, were dissimilar, not only that, even fragment from two different clones of a same variety, Baisha, was also dissimilar in case of OPC01$_{696}$. That was the reason for developing new primers where annealing temperature would be at least 55^{o}C for getting reproducible and uniform result. So, mapping of RAPD markers might not be effective for linking with the desirable character until it is converted into a reproducible marker like SCAR marker. When newly developed SCAR primer pair was employed for validation among forty one genotypes, all twenty four resistance genotypes did not amplify the expected 640 bp fragment. Although, Matta Poovan, Champa and Jurmuney Kanthali (DSI 10.0, 9.2 and 9.3) showed moderate resistance against Sigatoka but did not amplify the expected 640 bp fragment by newly developed SCAR primer. Part of Genomic DNA used for designing primer pair for SCAR markers may be dissimilar for these three varieties. In earlier study, RAPD-SCAR marker amplified a desired fragment from the resistance genotype, Calcutta 4, but was unable to discriminate between resistance and susceptible sample (Nwanzoma et al. 2011). In this study, RAPD fragments which showed positive correlation with PDI value, were considered for SCAR development. Out of eight primer pairs only one RAPD-SCAR primer pair designed from the Kanthali Clone-1 showed significant correlation with the Sigatoka resistance. Initially, fifteen genotypes collected from different parts of West

Bengal, were considered for identifying RAPD marker associated with Sigatoka resistance. Additional twenty six genotypes, collected from different parts of India were employed for validation of the newly developed SCAR marker. So, forty one genotypes used in this study were relatively diverse. Thus SCAR marker developed in this study had shown stronger association with Sigatoka resistance even among the diverse genotypes. Only one out of the eight primers that showed polymorphism was converted into SCAR markers. Some studies suggested that optimal number of SCAR markers is necessary for utilization in MAS programs and this is because a designed primer may not necessarily result in polymorphism observed during agarose gel electrophoresis or direct sequencing (Moreau et al. 1998). Umali et al. (2002) observed that two out of five SCAR markers were able to amplify scorable bands in *Musa* AAA. Recently, based on comparative transcriptomic between immune genotype, Calcutta4 and susceptible cultivar, Grand Naine, fewer transcriptomes were suggested for their role in imparting resistance against Sigatoka (Passos et al. 2013). Sequence of the target fragment OPA_{640} did not show any similarity with published Musa genome. It may be due to lack of data for B genome. Instead of sequence dissimilarity of OPA_{640} fragment among two genotypes (Kathanli Clone-1 and Baisha), cent percent similarity was observed among the sequences of DNA fragment generated by newly developed SCAR marker.

In conclusion, RAPD primers suffer from reproducibility, which accounts for why they are converted into robust and reliable SCAR markers. In this study, the RAPD-SCAR marker correctly amplified the desired fragment from 22 moderately resistant genotypes with diverse origin. An identified dominant marker may be useful for marker assisted pyramiding of Sigatoka resistance allele in banana breeding program in addition to tagging and isolation of resistance allele from Calcutta 4 genome.

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