Research Article

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SCAR marker for the identification of *Alternaria alternata* causing leaf spot disease in rubber (*Hevea brasiliensis*)

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Abstract

The objective of the present work was to develop reliable sequence characterised amplified region (SCAR) marker to identify *Alternaria* causing leaf spot disease in rubber. The pathogens *Alternaria* and *Corynespora* cause similar disease symptom on rubber and was found to occur during the same season of the year. Two pairs of SCAR primers, derived from unique RAPDs, were successfully used in diagnostic PCR to identify *Alternaria*. This technique could detect the pathogen when the fungal DNA concentration was as low as 1 ng in standard PCR reaction volume of 25 µl. DNA samples from diseased lesions (0.2 to 0.5 cm diameter) were successfully used in identification of the pathogen using SCAR markers. This molecular tool appears to be very useful for epidemiological studies on *Alternaria* leaf blight in rubber

Key words: *Alternaria*, *Corynespora*, *Hevea brasiliensis*, SCAR markers

Introduction

Molecular markers have proven to be useful for detection of economically important plant pathogens, which are difficult to differentiate using conventional techniques. Among the PCR-based markers RAPD markers are most widely used. Although RAPD markers are usually dominant markers, they are sensitive to minor changes in reaction conditions during PCR amplification, which can result in irreproducible results (Rugienius *et al*., 2006). To improve the reliability of RAPDs and to convert them to co-dominant markers, Paran and Michelmore (1993) developed a technique known as sequence characterized amplified region (SCAR), which is based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that specifically bind to the RAPD fragment. SCAR markers are more advantageous than RAPD markers because they usually detect only a single locus and are therefore, more specific and stable (Ye *et al*., 2006; Akkurt *et al*., 2007). Their PCR amplification is less sensitive to reaction conditions; they are more likely to be co-dominant markers and are therefore reproducible (Kethidi *et al*., 2003).

The leaf disease caused by *Alternaria* was recently identified on rubber (Roy *et al*., 2006), which has symptoms similar to that of the devastating Corynespora leaf disease caused by *Corynespora cassiicola* on rubber. Hence a sensitive and quick method for its identification was essential to discriminate between *Alternaria* and *Corynespora* for developing efficient disease management strategies. This paper reports the development and evaluation of two SCAR markers for the identification of *Alternaria* on rubber.

Materials and Methods

Leaf samples showing symptoms such as circular/ irregular spots surrounded by a yellow halo, shrivelling of leaf tips, railway track symptom etc. were collected from different clones of rubber during the disease season (January to April). The pathogen was isolated by plating surface-sterilized pieces of diseased tissue on potato dextrose agar (PDA) medium. All isolates were purified, maintained on PDA and stored at room temperature.

For extraction of genomic DNA, six mycelial plugs (5 mm diameter) were taken from the advancing margins of 7-day-old culture of each isolate, transferred

to potato dextrose broth (PDB) and incubated at 25°C on an orbital shaker (100 rpm) for 4 days. Extraction and purification of the total genomic DNA were carried out following the modified CTAB protocol (Saha *et al*., 2000).

Twenty arbitrary decamer primers (Operon technology Inc., USA) were selected based on our earlier studies (Roy *et al*., 2006) for PCR amplifications of *Alternaria* and *Corynespora* cultures. Amplifications were performed in a total volume of 25 µl by mixing 50 ng of template DNA with 10 picomoles of single primer, 0.2 mM of each dNTPs, 0.7 units of *Taq* DNA polymerase (GE Heathcare, UK) and 2.5 µl of 10x DNA polymerase buffer. Amplifications were performed in a thermal cycler with an initial denaturation step at 94°C for 3 min., followed by 40 cycles of 30 sec. at 94°C, 1 min. at 37°C and 2 min. at 72°C with a final extension at 72°C for 7 min. Amplified products were analysed along with a DNA marker, as molecular size reference, by electrophoresis on a 1% agarose gel in 1x TBE buffer, stained with ethidium bromide (0.5 g/ml), visualized with ultraviolet light and photographed using Gel-documentation system (Bio-Rad Laboratories Inc., USA).

Two isolates each of *Alternaria* and *Corynespora* were chosen to develop pathogen-specific SCAR marker for *Alternaria*. After performing RAPD-PCR with selected operon primers, the amplified product was electrophoresed and bright, consistent and specific bands existing only in *Alternaria* was selected for conversion into Alternaria-specific SCAR marker Based on differential banding pattern between *Alternaria* and *Corynespora*, nine RAPD primers were selected and used for further analysis. Six RAPD fragments $OPA17_{800}$, $OPA17_{900}$, $OPA19_{1100}$, $OPAB11_{1000}$, $OPAC5_{700}$ and $OPAF17_{1200}$ were selected for conversion to SCAR markers (the names of the fragments chosen indicate the primer with which the fragment was amplified and the size of the amplified product is given as subscript). The intensity of the amplification products and their good separation from neighbouring RAPD bands facilitated their isolation and cloning.

The selected RAPD marker bands from *Alternaria* were excised from the agarose gel using sterile blades and purified using GFX-Gel band purification kit (GE Healthcare, UK). The purified fragments were cloned using the pGEM-T Eazy vector system (Promega Corporation, USA). Subsequently, the positive clones were selected by blue white screening and confirmed by colony PCR using vector directed primers. This was followed by sequencing of the positive clones in both forward and reverse orientations. From each sequenced RAPD marker sequence, oligonucleotides to be tested as SCAR primers were designed based on the forward and reverse sequences. The presence of the RAPD primer was checked at both ends of the sequences and primers were designed consisting mainly of the 10 bases of the RAPD primer completed by the following 3' base sequence to design a 20-23 mer primer with an approximate Tm value of 60°C. Primers were custom synthesized by MWG Biotech, India (Table 1).

Genomic DNA from two representative isolates of *Alternaria* and *Corynespora* were used as templates to optimize annealing temperature. PCR thermal programme was as follows: initial denaturation at 94°C for 3 min. followed by 35 cycles of 94°C for 30 sec., annealing at 58-62°C for 1 min. depending on the Tm of the primers and 72°C for 2 min. with a final extension at 72°C for 7 min. Each PCR reaction contained 50 ng of genomic DNA template, 0.7 units of *Taq* DNA polymerase, 0.2 mM of dNTP, 10 pmol each of forward and reverse SCAR primers, 2.5 µl of 10x PCR buffer with 1.5 mM $MgCl_2$ in a final volume of 25 µl. The products were visualized and photographed as described earlier. The primer-pairs were tested for both sensitivity and specificity. To determine sensitivity, serial dilutions of the *Alternaria* genomic DNA were prepared with TE (10:0.1) buffer and added at concentrations of 1 to 50 ng per reaction to check for amplification of the fungal DNA at its least concentration. To determine specificity,

Table 1. Details of SCAR markers developed from the RAPD primers for identification of *Alternaria*

SCAR marker for the genus	RAPD fragment converted to SCAR	SCAR primers	Nucleotide sequence (5' to 3')	Tm(°C)
Alternaria	$OPAB11_{1000}$	SCAR-1F	GTGCGCAATGGGTGTTGTAGC	61.8
	$OPAB11_{1000}$	SCAR-1R	GTGCGCAATGACGGTTAACAGT	60.3
	$OPA17_{800}$	SCAR-14F	GACCGCTTGTGGAGGGCATG	63.5
	$OPA17_{800}$	SCAR-14R	GACCGCTTGTTACCTACCCATT	60.3
	$OPA17_{900}$	SCAR-15F	GACCGCTTGTCAATTAACTGAC	58.4
	$OPA17_{900}$	SCAR-15R	GACCGCTTGTGGTATGAGCA	59.4

the primer-pairs were tested with the fungal DNA from both the pathogens *Alternaria* and *Corynespora*.

Results and Discussion

Two highly variable representative isolates each of *Alternaria* and *Corynespora* were chosen based on our earlier studies for the development of pathogen-specific SCAR marker for *Alternaria*. After RAPD-PCRs, DNA electrophoresis revealed polymorphic bands between these organisms using nine primers namely, OPA4, OPA7, OPA8, OPA17, OPA19, OPA20, OPAB11, OPAC5 and OPAF17. Intense, unique bands of size 0.5 to 1.5 kb, specifically present only in *Alternaria* were selected (Fig. 1) as they were expected to serve as marker. Six RAPD fragments $OPA17_{800}$, $OPA17_{900}$, $OPA19_{1100}$, $OPAB11_{1000}$, $OPAC5_{700}$ and $OPAF17_{1300}$ were selected for conversion to SCAR markers.

Fig. 1. Amplification patterns obtained with RAPD primers using template DNA from two isolates each of *Alternaria* (A) and *Corynespora* (C).
Arrow indicates polymorphic band that was cloned and sequenced for the development of *Alternaria*-specific primer pair.
M: Molecular weight marker (l-DNA *Eco* RI + *Hind* III double digest).

Using the sequence information of the cloned specific RAPDs, six pairs of putative SCAR primers were designed and tested for their specificity in amplification. Three pairs of primers produced multiple bands and did not reveal polymorphism between the two fungi. Multiple bands produced by these primer-pairs could be attributed to the fact that the original polymorphisms with the 10-base RAPD primers were due to small mismatches at the priming sites resulting in no amplification products at this site, whereas the longer SCAR primers (20-23 bases) were not affected by small mismatches at the priming sites. The positional shifts observed for these markers as compared to the original RAPD markers are due to the higher experimental stringency applicable to SCAR markers. They were therefore deemed to be unsuitable for discrimination (Fig. 2). However, the additional band could be avoided by increasing the annealing temperature. Finally, two SCAR primer-pairs generating SCAR markers: SCAR1 and SCAR14, derived from RAPDs $OPAB11_{1000}$ and $OPAF17_{1300}$ respectively, showed unique amplicons of expected sizes matching perfectly with the original RAPDs from which they were derived (Table 1; Fig. 3). These two SCAR markers were therefore successfully used to detect *Alternaria* causing leaf spot disease in rubber.

Fig. 2. SCAR products amplified using the SCAR primers revealing the specific amplification of the desired band in *Alternaria* (A) isolates along with a few minor bands in *Corynespora* (C) isolates.
M – Molecular weight marker (l-DNA *Eco* RI + *Hind* III double digest).

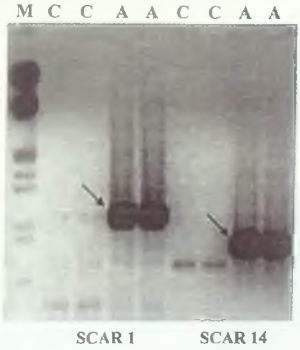

Fig. 3. Banding pattern of *Alternaria* isolates showing a distinct and reproducible band only in *Alternaria* (A) and not in *Corynespora*
M. Molecular weight marker (l-DNA *Eco* RI + *Hind* III double digest)

Sensitivity tests with serial dilutions of total *Alternaria* DNA proved that the limit of detection (*i.e.*, the lowest concentration of total genomic DNA from which the target fragment got amplified) was 1 ng in a standard PCR reaction volume of 25 µl (Fig. 4). This could indicate that these primers would detect the pathogen by PCR, even in their early stage of symptom development. Hence, this PCR test could also be of great importance to check nursery plants as a means of preventing the spread of the pathogen into disease-free areas. Specificity of the SCAR primers with genomic

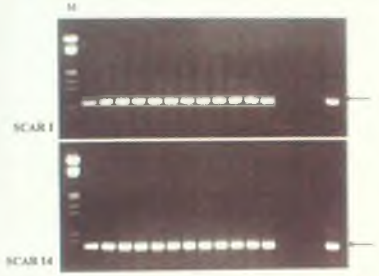

Fig. 4. Limit of detection of the *Alternaria* SCAR primers with varying concentrations of *Alternaria* DNA.
Positive control with *Alternaria* DNA alone as well as negative control with *Corynespora* DNA are also shown. Amplifications performed directly with the diseased sample also indicated the efficiency of the SCAR primer. Arrow indicates specific SCAR marker.
M: Molecular weight marker (l-DNA *Eco* RI + *Hind* III double digest)

DNA from both the pathogens also showed that the SCAR markers directed the amplification of the specific fragment only in *Alternaria*. The primers were tested against host genomic DNA for its interference with the amplification of the fungal DNA. It was observed that the SCAR primers efficiently amplified the fungal DNA even in the presence of plant DNA. This observation is considered to be significant as the diseased sample can directly be used in the detection of these pathogens without establishing pure cultures of the respective pathogen. Due to the specificity and sensitivity of the primers, they could be useful in the bio-PCR technique by speeding up identification of plant material infected by these pathogens.

The present work proves SCAR marker to be an efficient way for identification of *Alternaria* causing leaf disease in rubber. The results encourage the application of this PCR-based identification method in setting up a reliable diagnostic assay for their detection from infected rubber plantations as early detection is essential for controlling the spread of the pathogen thereby making this technique appropriate for diagnostic purposes.

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