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ORIGINAL PAPER

RAPD-based SCAR marker SCA 12 linked to recessive gene conferring resistance to anthracnose in sorghum [*Sorghum bicolor* (L.) Moench]

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Abstract Anthracnose, caused by *Colletotrichum graminicola*, infects all aerial parts of sorghum, *Sorghum bicolor* (L.) Moench, plants and causes loss of as much as 70%. F_1 and F_2 plants inoculated with local isolates of *C. graminicola* indicated that resistance to anthracnose in sorghum accession G 73 segregated as a recessive trait in a cross with susceptible cultivar HC 136. To facilitate the use of marker-assisted selection in sorghum breeding programs, a PCR-based specific sequence characterized amplified region (SCAR) marker was developed. A total of 29 resistant and 20 susceptible recombinant inbred lines (RILs) derived from a HC 136 × G 73 cross was used for bulked segregant analysis to identify a RAPD marker closely linked to a gene for resistance to anthracnose. The polymorphism between the parents HC 136 and G 73 was evaluated using 84 random sequence decamer primers. Among these, only 24 primers generated polymorphism. On bulked segregant analysis, primer OPA 12 amplified a unique band of 383 bp only in the resistant parent G 73 and resistant bulk. Segregation analysis of individual RILs showed the marker OPA 12_{383} was 6.03 cM from the locus governing resistance to anthracnose. The marker OPA 12_{383} was cloned and sequenced. Based on the sequence of cloned RAPD product, a pair of SCAR markers SCA 12-1 and SCA 12-2 was designed using the MacVector program, which specifically amplified this RAPD fragment in resistant parent G 73, resistant bulk and respective RILs. Therefore, it was confirmed that SCAR marker SCA 12 is at the same locus as RAPD marker OPA 12_{383} and hence, is linked to the gene for resistance to anthracnose.

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Introduction

Sorghum is a staple food for millions of people in semi-arid tropical areas of world (Doggett 1988). It is also utilized as fodder in the United States, Australia and other developed countries. Production of sorghum is adversely affected by biotic and abiotic stresses. Anthracnose, incited by *Colletotrichum graminicola* (Ces.) Wils, is one of the most destructive foliar diseases of sorghum and prevalent under warm humid conditions. It causes loss of as much as 70%. Damage by anthracnose ranges from grain deterioration to peduncle breakage, to stalk rot and foliar damage (Pastor-Corrales and Fredericksen 1980).

Marker-assisted selection (MAS) allows molecular marker-based approaches for crop improvement, as compared to selection solely based on phenotype. The development of molecular markers has enhanced the development of saturated genetic maps for major crops (Tanksley et al. 1989; Michelmore et al. 1992; Gardnier et al. 1993) including sorghum (Xu et al. 1994; Boivin et al. 1999; Klein et al. 2001). In addition, the use of different types of randomly dispersed molecular markers [RAPD, RFLP, STS, sequence characterized amplified regions (SCARs)] (Williams et al. 1990; Michelmore et al. 1992; Paran and Michelmore 1993) in combination with appropriate genetic material [di-haploids, bulks of F_2 individuals, recombinant

inbred lines or RILs, near-isogenic lines or NILs, aneuploids] has favoured the saturation of genetic regions near particular genetic loci (Beckmann and Soller 1983; Tanksley et al. 1989; Michelmore et al. 1992). MAS can be used to tag and map agronomically important genes such as genes for resistance to diseases.

Presence of tight linkage [<10 cM (centimorgan)] between the quantitative trait(s) and genetic marker(s) has proved useful (Kennard et al. 1994). Molecular markers are used to identify and tag desired genes. Several tightly linked RAPD markers for the anthracnose resistance gene in sorghum have been identified (Boora et al. 1998; Pandey et al. 2002). A RAPD-based SCAR marker linked to the anthracnose resistance gene specifically amplifying a 1,437-bp RAPD fragment in resistant lines has been recently developed (Singh et al. 2006). These markers will facilitate MAS in breeding for anthracnose resistance and map-based cloning of resistance gene(s).

In this paper, we report the identification and mapping of a RAPD marker linked to the anthracnose resistance gene in sorghum. The RAPD marker OPA 12_{383} was cloned and sequenced by primer walking. A SCAR marker SCA 12 was developed from the RAPD marker OPA 12_{383} closely linked to the anthracnose resistance gene in sorghum.

Materials and methods

Plant materials, fungal inoculation and scoring for disease resistance

Sorghum bicolor lines G 73, resistant to anthracnose caused by *C. graminicola*, and HC 136, agronomically superior, but susceptible to the pathogen were used. Parental lines HC 136 and G 73 were crossed to derive an F_2 population of 110 plants. The F_2 plants along with parental lines were scored for anthracnose resistance using a conidial suspension of a local isolate of *C. graminicola* under greenhouse conditions. Conidia were obtained from cultures of *C. graminicola* collected from diseased plants from the fields and cultured on casein lactose hydrolysate medium at 23°C under fluorescent light. Conidia were scraped off the plates and suspended in sterilized distilled water having a final concentration of 1×10^6 conidia/ml measured by using a haemocytometer. F_2 genotypes and 20 plants of each of the parents were inoculated with 1 ml conidial suspension (1×10^6 conidia/ml) into the leaf whorl of 4-week-old plants. Data were recorded three times at 10-day intervals after inoculation based on symptom expression of the anthracnose disease. F_2 plants were selfed until the F_8 generation by using single-seed descent method, to obtain RILs. Twenty plants of each recombinant inbred of the F_2 generation were evaluated for resistance by inoculating the same local isolate of the pathogen used previously to evaluate F_2 genotypes along with the parental lines. Plants were scored as resistant RILs when all the plants of F_8 progeny showed no disease symptoms (29 resistant) and susceptible RILs when all the plants had disease symptoms (20 susceptible). Segregation ratios for the disease reaction data from the F_2 population were tested using chi-square for goodness of fit to a Mendelian 3:1 genotypic ratio.

DNA extraction and bulked segregant analysis

A modified CTAB extraction protocol described by Xu et al. (1994) was used to isolate genomic DNA from lyophilized young leaves. The DNA samples were quantified using a UV absorbance spectrophotometer and diluted with 1 × TE buffer to a final concentration of 25 ng/μl. A RAPD marker closely linked to the anthracnose resistance gene in sorghum was identified by bulked segregant analysis developed by Michelmore et al. (1991). Equal volumes, i.e. 4 μg of standardized DNA from 20 susceptible and 29 resistant RILs from HC 136 × G 73 were pooled separately to make the susceptible and resistant bulks, respectively. RAPD analyses were done with 84 random sequence decamer oligonucleotide primers (Operon Technologies Inc., Alameda, CA, USA) having 60% or more G + C content. PCR reactions (20 μl) contained 50 ng of genomic DNA, 1 unit of Taq DNA polymerase (Genetix), 1 × Taq DNA polymerase buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 10% Triton × 100), 2 mM $MgCl_2$, 200 μM of each of dNTPs and 0.2 μM random primer. The thermal cycler was programmed for 3 min at 94°C and 45 cycles of 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C; final extension was at 72°C for 15 min. Amplification products on 1.0% (w/v) agarose gels in 1 × TBE were analysed by electrophoresis, revealed by ethidium bromide staining and photographed with a UV gel documentation system (Pharmacia Biotech). Primers showing polymorphism among the parents were evaluated with resistant and susceptible bulks to find markers closely linked to the anthracnose resistance locus.

Cloning vector

A pDrive Cloning Vector (supplied with the QIAGEN PCR cloning kit) was used for ligation of the PCR product. The vector contained several unique restriction

endonuclease recognition sites around the cloning site and a T7 and SP6 promoter on either side, allowing sequence analysis using standard primers. It allowed selection by ampicillin and kanamycin, as well as blue/white colony screening.

Sequencing of marker OPA 12_{383} and SCAR analysis

The amplified product OPA 12_{383} was purified from the agarose gel using a QIAEX II agarose gel extraction kit (Qiagen, Germany). The purified product was cloned into pDrive Cloning Vector using the QIAGEN PCR cloning kit ligation protocol and transformed into fresh competent cells of *Escherichia coli* EZ strain. A white colony positive for the desired insert was grown on a large scale, and plasmid DNA was isolated using a modified alkaline lysis method by Brinboim and Dolly (1979). The presence of insert was confirmed by restriction digestion of the recombinant plasmid with *Eco*RI restriction enzyme. This marker was sequenced in an automated DNA Sequencer, using ABI's Ampli Taq FS dye terminator cycle sequencing chemistry based on the dideoxy chain-termination method. Sequence homology searches were done using the BLAST algorithm at http://www.ncbi.nlm.nih.gov of the National Center for Biotechnology Information, with the programs BLASTN and BLASTX.

Based on the sequence of cloned RAPD product OPA 12_{383}, two oligonucleotides to be used as forward and reverse SCAR primers were designed using the MacVector program. Care was taken to avoid possible secondary structure or primer dimer generation and false priming, and also to match melting temperatures and to achieve appropriate internal stability while designing SCARs. Genomic DNA of parents, resistant bulks and RILs was amplified by using SCAR primers, and amplified products were resolved on 2.0% agarose gels.

Linkage analysis of RAPD marker OPA 12_{383}

Linkage was analysed by using a FORTRAN program to calculate the value of '*r*', i.e. map distance in Morgans (Burr et al. 1998). The map distance in Morgans '*r*' was calculated by using the following equation:

$$r = R/(2 - 2R)$$

where *R* represents the proportion of recombinants in a single meiosis.

The distance in cM was calculated using following formula (Haldane and Waddington 1931):

$$\mathrm{cM} = (-\ln(1 - 2r)) \times 50$$

Results

Genetic analysis of inheritance of anthracnose resistance in sorghum

F_1 of a HC 136 × G 73 cross was susceptible to an anthracnose biotype, indicating that resistance to anthracnose is controlled by a recessive gene. The symptoms associated with anthracnose include the appearance of small, circular, elliptical or elongated tan spots with reddish brown margins, studded with black dot-like acervuli on leaves. A total of 106 F_2 plants was evaluated and 79 plants were susceptible whereas 27 were resistant to anthracnose. A Mendelian segregation ratio of 3:1 was observed for susceptible and resistant genotypes of the F_2 population. This confirmed that resistance to anthracnose is controlled by a single recessive gene. These genotypes were further grown to create RILs, and resistance to disease evaluated in the F_8 generation. Twenty of the RILs were susceptible and 29 were resistant to anthracnose. Chi-square (χ^2) was used to test the segregation ratio for anthracnose reaction from the F_2 population. The observed value of χ^2 (0.012) was less than the expected value, showing that F_2 generation was segregated in a Mendelian 3:1 (susceptible:resistant) ratio.

Bulked segregant analysis

Of 84 random primers evaluated, 24 generated polymorphism among the parental genotypes HC 136 and G 73. A total of 46 polymorphic bands were amplified, with an average of 1.9 bands per primer. All of the 24 primers were evaluated with the resistant and susceptible bulks to identify the RAPD marker closely linked to the anthracnose resistance locus in sorghum. Marker OPA 12 amplified a 383-bp band unique to the resistant parent G 73 and resistant bulk (Fig. 1) indicating that marker OPA 12_{383} was closely linked to the anthracnose resistance gene in sorghum.

Sequencing and conversion of RAPD marker OPA 12_{383} into a SCAR marker

White colonies were selected as transformants with a 383-bp insert after transformation whereas blue colonies represented transformants with self-ligated plasmids. Plasmid DNA of recombinant clones was digested with *Eco*RI to produce two bands; one corresponding to the size of the vector (~3.85 kb) and the other to the size of the specific DNA insert (383 bp).

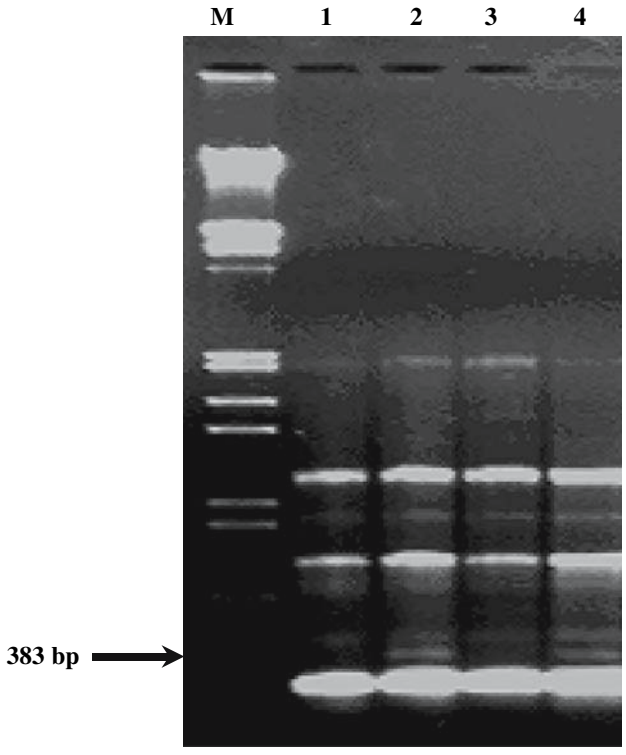

Fig. 1 PCR amplification products of parents and bulks using RAPD primer OPA 12. *Lanes M* Lambda DNA *Eco*RI/*Hin*dIII double digest marker, *1* HC 136, *2* G 73, *3* susceptible bulk, *4* resistant bulk. The *arrow* indicates the 383-bp polymorphic fragment linked to the anthracnose resistance locus

The sequence analysis of OPA 12_{383} revealed that the polymorphic RAPD fragment was bordered by the original ten bases of the primer OPA 12, i.e. TCGGCGATAG (Fig. 2). The GenBank accession number for the sequence of marker OPA 12_{383} is DQ299903. The sequence showed homology with that of several plant protein encoding genes. BLAST searches were made to determine if the sequenced fragment might identify a known gene or mapped sequence. While several sorghum and maize EST (expressed sequence tag) clones gave short runs of perfect matches, all of the *E* (expectation) values were >2, meaning that chance is a likely explanation for the homology seen. More extensive matches and much lower *E* values would be expected between the 383-bp fragment and its actual genomic homologue.

TCGGCGATAGTGCGATGTCCGGTCCCTTCCCTTCTTGGTAGGTGGTTCCCCT
GTAGAGGCGGTCCTTTCCTTATTGCCAGGCGATGGGATCCTTCTCGGGCGCC
GGCGCCCCCTTGTGGTGGCGCGGCTGTCATCCATGTTCCACCT**AAGATTAGT**
GCCTGAGTGTCGCTCTCAGAACTTTCTATTTCTTTTGTGGGGCCCCCGGATT
ACGTTGATCACCATCTGCCTCCCTTTTTGTGATTTGGCCATCTCGTAAA**CCA**
AGTTCTTTTTGCTTTTCTGGTGATCATACCCCGTCCACCTAGCAGCTTGGA
GGAATTTACAGAGATATCGATGTGTCATCATAATCTTGCGCTCCAAAACAGA
AAATCCCTATTGGCGAAAGG

Fig. 2 DNA sequence of OPA 12_{383}. The *underlined bold region* indicates the sequence for designing the SCAR markers SCA 12-1 and SCA 12-2. The *highlighted region* indicates the sequence of RAPD primer OPA 12

Based on the sequence of marker of OPA 12_{383}, a pair of specific forward and reverse SCAR markers (SCA 12-1 and SCA 12-2) was designed (Table 1). SCAR amplification produced a single specific band of predicted size, i.e. 135 bp in the resistant parent G 73 and resistant bulk (Fig. 3a), and also in most of the resistant RILs (Fig. 3b). Because this SCAR marker produced a null allele in the susceptible parent DNA, it was considered as a dominant marker for the recessive allele conferring resistance to anthracnose. Hence, SCAR primers amplified a specific product of the predicted size and showed the same segregation pattern as the original RAPD marker OPA 12_{383}, indicating that the RAPD and SCAR markers share a common locus linked to the anthracnose resistance gene.

Linkage mapping of marker OPA 12_{383}

Marker OPA 12 was evaluated with individual susceptible and resistant RILs to find the closeness of the marker to the anthracnose resistance locus. Marker OPA 12 produced a 383-bp unique band in all the resistant RILs except in three, but there was no amplification of this fragment in all susceptible RILs except in two. This showed a total of five cross-overs and this marker was found to be 6.03 cM away from the locus governing anthracnose resistance in sorghum.

Discussion

Genetic analysis of inheritance of the gene for resistance to anthracnose in a HC 136 × G 73 cross confirmed that anthracnose resistance is controlled by a single recessive gene. The F_1 of the HC 136 × G 73 cross was susceptible to anthracnose disease indicating that resistance to anthracnose is inherited as a recessive trait. A Mendelian segregation ratio of 3:1 was observed for susceptible and resistant genotypes of the F_2 population and confirmed by the chi-square test. The observed value of χ^2 was less than the expected value. However, the slight deviation of the observed value from the expected was due to chance error. Hence, anthracnose of sorghum is controlled by a single recessive gene. These reports are in agreement with the previous report of genetic inheritance of anthracnose resistance in sorghum (Boora et al. 1998).

Table 1 Characteristics of the SCAR primers derived from the sequence of RAPD marker OPA 12_{383}

SCAR locus	Sequence (5′ → 3′)	GC (%)	T_m (°C)
SCA 12	Forward (SCA 12-1): AAGATTAGTGCCTGAGTGTCGCTC 24 nt	50.0	55.8
	Reverse (SCA 12-2): CACCAGAAAAGCAAAAAGAACTTGG 25 nt	40.0	57.4

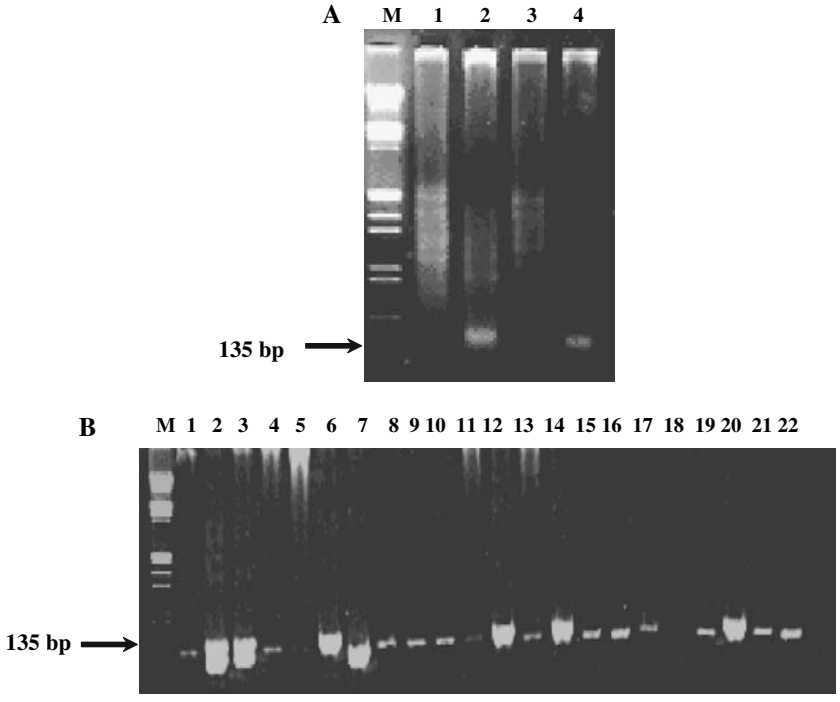

Fig. 3 Amplification of parents, bulks and resistant RILs using SCAR marker SCA-12. **a** SCAR amplification in parents and bulks, *1* HC 136, *2* G 73, *3* susceptible bulk, *4* resistant bulk. **b** SCAR amplification in resistant RILs (*lanes 1–22*). *M* Lambda DNA *Eco*RI/*Hin*dIII double digest marker. The *arrows* indicate a specifically amplified RAPD fragment linked to the anthracnose resistance locus

Recombinant inbred lines are considered useful as biological tools when searching for genetic markers, such as molecular markers, linked to an introgressed locus. In the present case, the F_8 generation was used as RILs. Markers closely linked to simple inherited traits can be easily identified by bulked segregant analysis if an appropriate F_2 population is available (Michelmore et al. 1991). A RAPD marker closely linked to the anthracnose resistance gene in sorghum was identified using bulked segregant analysis. In bulked segregant analysis, primer OPA 12 amplified a 383-bp band unique only in resistant parent G 73 and the resistant bulk. Hence, it was revealed that marker OPA 12_{383} was closely linked to the anthracnose resistance gene in sorghum. Segregation analysis of RILs showed that this marker was 6.03 cM away from the locus governing anthracnose resistance in sorghum. The identified RAPD marker showed 95% identity with the *Xho*I digested cDNA derived from mRNA isolated from mechanically wounded or/and jasmonate-treated and acid- and alkaline-treated leaves. Hence, this marker may encode for the gene which may be expressed under certain stress conditions. BLASTX results indicated that the marker showed significant alignment with the protein sequences in *Oryza sativa*, *Zea mays* and *Arabidopsis*. Most of such proteins were hypothetical with unknown function. The marker showed 42% alignment with kinase like protein in *O. sativa* and hence, it may encode for specific kinases involved in biosynthesis of phytoalexins, thus imparting resistance to fungal disease. Similarly, 36% identity with penicillin-binding protein in *Z. mays* revealed the marker may provide a binding site to antibiotics and thus be involved in resistance to microbial diseases. The significant alignment with nucleotide and protein sequences revealed that the marker OPA 12_{383} encoded for gene product that may be induced under stressed conditions, involved in biosynthetic pathways of phytoalexins, or involved in defensive mechanism.

Because RAPD technology might lead to irreproducibility among laboratories, the RAPD marker OPA 12_{383} was converted into a SCAR. This would increase the reliability and reproducibility of PCR assays. SCARs are advantageous over RAPD markers because they are codominant, detect only single genetically defined loci, identified as distinct bands in agarose gels, are easier to score, less sensitive to reaction conditions

and are more reproducible. In the present study, a pair of SCAR markers SCA 12-1 and SCA 12-2 was designed (Table 1). SCAR markers linked to disease resistance genes in sorghum have been identified (Boora et al. 1999; Singh et al. 2006). These SCARs would be useful for MAS and introgression of genes for resistance to disease (Chague et al. 1996). Therefore, these identified RAPD and SCAR markers can be used in a resistance-breeding program of sorghum anthracnose by marker-aided selection and isolation of the anthracnose resistance gene which can further be utilized for genetic improvement for resistance to disease.

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