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Use of RAPD markers to characterize commercially grown rust resistant cultivars of sugarcane

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Abstract

The brown leaf rust caused by *Puccinia melanaocephala* (Syd. & P. Syd.) is a major cause of reduction in sugar production and withdrawal of sugarcane cultivars from growing fields. In The present study, the eight commercially grown sugarcane cultivar was characterized under natural inoculation and divided into two discrete groups i.e. four rust resistant and four rust susceptible. The extracted genomic DNA was subjected against RAPD (Random Amplified Polymorphic DNA) markers. After screening of decamers, 7 were picked for further analysis on the basis of polymorphism, which is the most important application of DNA markers. These decamers generated 21 trackable loci with the range of 400-2500 bp and 100% polymorphic loci were recorded genomic DNA of eight sugarcane cultivars. From 7 primers, 3 generated 5 specific loci in 4 sugarcane cultivars, which is a potential use of RAPD-PCR for identifying *Saccharum spp*. hybrids and clones. Primer G-06 generated only one locus 1200 bp in the rust susceptible cultivar NSG-59 while J-05 had produced two loci i.e. 400 bp and 1100 bp in rust resistant cultivars NSG-555 and CP-77-400 respectively. Primer L-18 generated 700 bp and 1400 bp loci in rust susceptible cultivar CoJS-84. The homology tree was constructed using DNAMAN software based on binary data set. During cluster analysis, we found two major groups of cultivars with different minor groups. This study will be useful to characterize rust resistance in sugarcane and could be extended for the development of SCAR (Sequence Characterized Amplified Region) marker, which is specific, reliable and reproducible marker.

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Introduction

Sugarcane (*Saccharum* spp.) is an important multiuse perennial, tropical and subtropical commercial crop and is cultivated over 105 countries in the world. Recently, it is being cultivated to produce a bio-fuel ethanol as an energy cane. Although, Pakistan happens to be the world's 5th largest grower of sugarcane it ranks among the lower yield countries.

Sugarcane production is severely affected by a number of diseases. The most significant sugarcane diseases are red rot, sugarcane mosaic virus, smut, brown leaf rust, stem canker, leaf spot and pokkah boeng etc. The brown rust caused by *Puccinia melanocephala* (H. & P. Syd.) has a wide distribution and occurrence in almost all sugarcane- growing areas and the cause of withdrawal of cultivars (Comstock et al., 1982; Taylor et al., 1986; Ryan and Egan, 1989). The development of disease resistant cultivars could be the effective remedy. The development of durable brown rust resistance in commercially grown sugarcane cultivars is the major objective to minimize the brown rust effects on yield loss (Hoy and Hollier, 2009). The first requisite in this step is to map the location of gene for particular trait, e.g. resistance toward a disease.

Molecular markers can provide a prevailing tool to unravel the complex genome of sugarcane and enhance the determination of Mendelian bases for trait inheritance (Aitken et al., 2005). In recent years, the use of molecular markers in sugarcane crop has increased rapidly. With the use of molecular techniques, it has become possible to locate desirable genes for different traits. Polygenic characters which were previously very difficult to analyze using traditional plant breeding methods, are now easily being tagged using molecular markers (Casu et al., 2005). So, DNA profiling/marker may be used as marker-assisted selection (MAS) on the basis of presence/absence of a marker for selection of phenotype in crops to facilitate conventional breeding (Ribaut et al., 1997).

Recently, different attempts were made to find out association of molecular markers i.e. Random Amplified Polymorphic DNA (RAPD), Simple sequence repeats (SSR) and Amplified Fragment Length Polymorphism PCR (AFLP) with disease responses in sugarcane (Asnaghi et al., 2004; Muhammad et al., 2010; Hameed et al., 012; Srivastava et al., 2012). Le Cunff et al. (2008) reported map based cloning and isolation of rust resistance gene *Bru1* in sugarcane French cultivar R570. Due to genetic complexity of sugarcane, this species has received very little research investment despite its economic importance, and molecular resources have just recently begun to be developed (Grivet and Arruda, 2002). So, the selective breeding has been practicing to achieve desire goals and the gene pool exploitation is limited in traditional breeding programmes (Mariotti, 2002). However, due to the aneu-polyploidy nature of the sugarcane genome, it has been difficult to pinpoint exactly which genes are involved in disease responses.

The most economical, effective and justified method to enhance sugarcane yield is to breed and release of new varieties that are resistant to insects and diseases. New approaches that involve modern breeding technology need to be explored to develop sugarcane cultivars with durable resistant to rust. The main objective of our study was to explore and identify least expensive DNA marker i.e. RAPD (Random Amplified Polymorphic DNA) linked to rust resistance in sugarcane. This study further investigated genetic diversity of commercially grown sugarcane cultivars.

Material and Methods

Plant material

For the present study, eight cultivars grown in Punjab as resistant/susceptible to brown rust were selected (Table 1). All these sugarcane cultivars were evaluated for resistance/susceptibility to brown rust under natural inoculation at Shakarganj Research Institute (SRI), Jhang, Pakistan. The rating system recommended by The International Society of Sugar

Cane Technologists' (ISSCT) was used (Hutchinson and Daniels, 1972).

Extraction of total genomic DNA

Total Genomic DNA was isolated from eight commercially grown sugarcane cultivars using modified small scale DNA isolation CTAB protocol (Doyle and Doyle, 1990). The quality and quantity of extracted DNA were checked by using 1% agarose gel electrophoresis (AGE)/TBE and spectrophotometery. Different dilutions DNA i.e. 10ng, 30ng, 50ng and 100ng per µl were prepared in autoclaved ddH_2O for PCR optimization and amplification.

RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction)

A single arbitrary decamer primer is the novelty of the RAPD markers to amplify template DNA without prior knowledge of the target loci. Two hundred and sixty decamers of A to M series were obtained from BIONEER for initial screening of commercially grown sugarcane cultivars. Random amplified polymorphic DNA (RAPD) primers were used for screening of rust resistant and susceptible genotypes. After initial screening only those primers giving polymorphic bands were selected for further use.

Using Standard conditions, Polymerase Chain Reaction (PCR) was performed to amplify template DNA. Total volume of each reaction mixture was adjusted to 25 µL with sterilized distilled water in 200 µL sterile tubes. The amplifications were carried out in DNA Thermocycler Eppendorf Master Cycler AG22331 with a modified version of conditions used by William et al. (1990). It was programmed as single denaturation step of 5 min at 94 °C, followed by a step cycle program for 40 cycles of denaturation at 94 °C for 1 min, annealing at 34 °C (depending upon best amplification based on GC content of decamer primers) for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The retrieved PCR products were electrophoretically separated on 1.5% Agarose/TBE gels along with the DNA marker (Fermentas cat# SM0331) and gel photographed were visualized under UV lights and documented using "Uvitech" gel documentation system.

Scoring and Data processing

The amplification of PCR products was done twice or thrice for reproducibility of band scoring. The sizes of amplified RAPD-DNA fragments (bp) were estimated by reference to a known DNA marker. Those amplified fragments was compared and used in statistical processing which shared over the two or three PCR runs. The presence or absence of fragments was recorded as either 0 (absent) or 1 (present). The binary data set was recorded on spreadsheet for further processing. To draw dendrogram (homology tree) based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis, the DNAMAN 5.2.2.0 software was used.

Results and discussion

The main goal of this work was to determine the feasibility of using RAPD markers to differentiate between rust resistant and susceptible sugarcane cultivars from Pakistan. To achieve objective of this study, 4 highly rust resistant and 4 highly susceptible cultivars were selected and purified template DNA from these commercially grown sugarcane cultivars (Table 1) was subjected against random 10-oligonucleotide primers. After initial screening, 7 RAPD primers were chosen for further study based on polymorphism and specificity. Various parameters i.e. total bands (TB), polymorphic bands (PB), monomorphic bands (MP), percentage of polymorphism (PP) and rust resistant/susceptible cultivar (R and S) specificity were observed (Table 2).

The most important application of DNA marker is polymorphism, which can be used to discriminate the genetic basis of traits in crops. The seven selected primers produced 21 detectable amplicons with the mean of 3 loci per primer (Table 2) and showed 100% polymorphism among 8 cultivars of sugarcane from Pakistan to reveal the genetic similarity and genetic distances. Observed polymorphic loci could be used as a tool to evaluate rust resistance and susceptibility on the basis of presence and absence of specific locus (Fernandez et al. 1999; Kawar et al., 2009). Primer L-

18 was recorded the most informative. It produced 9 polymorphic loci in each 8 sugarcane cultivars with the range of 450-1500 bp.

Table 1. Trait specific characteristic and parentage of sugarcane cultivars.

Finally selected Pakistan cultivars_Rust Data _2005, 2006, 2007, 2008, 2009		
Cultivars	Parentage	Rust Responses
SPF-213	SP70-1006	R
CP-77-400	Not Known	R
CSSG-668	81-N289C×P74-2005	R
NSG-555	CP63-588 × MO/F	R
NSG-59	91W0510 × 82F0542	S
SPF-234	SP71-8210 × SP71-6180	S
CoJ-84	Co-1148x?	S
CPSG-2453	MQ87-1215 × 86A3626	S
R=Resistant, S=Susceptible		

In the present study, the number of specific loci with the approximate size range of 400 to 1400 bp were observed to identify sugarcane cultivars Three RAPD markers were seen as a genotype specific with 5 polymorphic loci ranging, which is a potential use of RAPD-PCR for identifying *Saccharum spp*. hybrids and clones (Nair et al., 2002; Pan et al., 2004; Khan et al., 2009). Primer G-06 produced 1200 bp, band, in the rust susceptible cultivar NSG-59, (Table 3) while one primer J-05 had amplified 400 and 1100 bp locus in rust resistant cultivars NSG-555 and CP-77-400 (Table 3), respectively and one primer L-18 had amplified 700 and 1400 in rust susceptible cultivar CoJ-84, (Table 3). Previously, Barnes and Botha (1998) had identified two RAPD marker linked to rust resistance in sugarcane variety NCo-376 on the basis of reproducible polymorphism of loci in rust susceptible clone. Many RAPD markers were identified in different crops linked to rust resistance for gene isolation and potential use in the breeding for crop improvement (Park et al., 2003; Mumtaz et al., 2009). Recently, Srivastava et al. (2012) developed a SCAR (sequence Characterized Amplified Region) marker from polymorphic RAPD locus to identify drought tolerance sugarcane cultivars. We are reporting various rust linked RAPD loci to identify rust resistant and susceptible cultivars of sugarcane grown in Pakistan. In our previous study, one RAPD marker OPN-06 had been identified linked to rust resistance in mapping population of sugarcane (Muhammad et al., 2010).

RAPD banding pattern were scored for the presence (scored 1) or absence (scored 0) of specific amplicon and recorded data were analyzed using DNAMAN 5.2.2.0 statistical software. We constructed a dendrogram showing the similarities among eight objects as determined with seven primers from 21 binary characters of the amplicon presence–absence (Saitou and Nei 1987). The Nei's (1978) genetic distance based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) classified eight sugarcane genotypes into two major groups based on 49 % homology.

Our study has further revealed that genetic diversity varied among eight hybrid cultivars of Pakistan and ranged from 61% to 81% which is commonly measured by genetic distances or genetic similarity (Figure 1). This study suggested that there was very limited genetic diversity within this group rust resistant (R) and rust susceptible cultivars, especially as the calculated values were skewed towards maximum variation. From the above mentioned results, it is concluded that high value of genetic similarity reflects that genotypes were from the some genetic parentage. Alvi et al. (2008) and Hameed et al (2012) recorded genetic similarity among 21 red rot resistant and susceptible sugarcane cultivars grown in Pakistan and Pan et al. (2004) also reported the similar results for genetic diversity of *Saccharum spontaneum* and elite accessions.

In present study, the cluster analysis among the eight Pakistani cultivars showed remarkable relationship with a specific character (rust resistance and susceptibility). In cluster analysis, the major groups further divided into different subgroups. The rust susceptible cultivars i.e. CoJ-84 and SPF-234 were

seen in one group along with resistant cultivars and similarly rust resistant cultivar i.e. CP-77-400 had shown homology 67% and clustered with other rust susceptible cultivars (Figure 1).

Table 2: The detail of polymorphic and monomorphic bands produced by 07 RAPD primers in eight sugarcane cultivars.

Primer Name	Sequence	TB	PB	MB	PP	Range of Loci (bp)
G-01	GGCACTGAGG	3	3	0	100	700-1500
L-02	TGGGCGTCAA	1	1	0	100	1500
F-19	CCTCTAGACC	3	3	0	100	600-2500
G-06	GTGCCTAACC	1	1	0	100	1200
G-08	TCACGTCCAC	2	2	0	100	750-2000
J-05	CTCCATGGGG	2	2	0	100	400-1100
L-18	ACCACCCACC	9	9	0	100	450-1500
Total		21				

Abbreviations: TB = Total Bands; MB = Monomorphic Bands; PB = Polymorphic Bands; PP = % of polymorphism. Overall total loci per primer = 3: Overall P.B loci per primer = 3

Table 3: Selective RAPD primers produced specific loci against rust resistant (R) and rust susceptible (S) groups of sugarcane cultivars.

Primer Name	Sequence	Bands/Loci (bp)	Specific to	R/S
G-06	GTGCCTAACC	1200	NSG-59	S
J-05	CTCCATGGGG	400-1100	NSG-555 and CP-77-400	R
L-18	ACCACCCACC	700-1400	CoJ-84	S

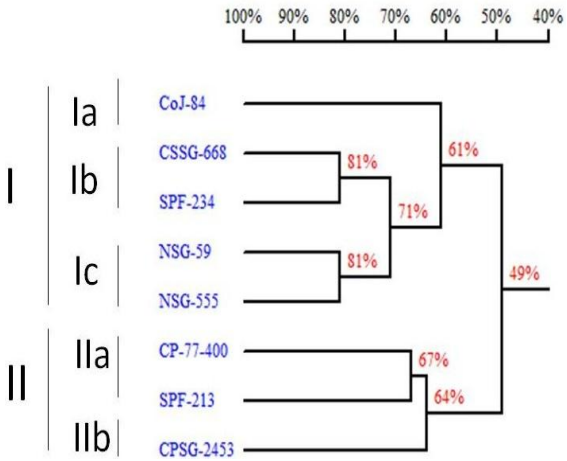

Fig. 1. Homology dendrogram constructed showing the genetic similarity among sugarcane cultivars from Pakistan by DNAMAN 2.2.2 version based on Nei's (1978) identities/distances.

It can be concluded that a relevant marker-fragment (loci) that can be used to compare only the continuous or only the discrete variable to discriminate the genotypes/cultivars and the success of a breeding programme depends on the understanding of the extent of variation existing in the available gene pool (Franco et al., 200; 1Selvi et al., 2003). So, this study will help to explore genetic basis of rust resistance and susceptibility in commercially grown cultivars, to identify genotypes of sugarcane to improve disease resistance and to develop sequence based specific DNA markers SCAR (Sequenced Characterized Amplified Polymorphic Region) for mapping of sugarcane cultivars.

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