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DNA BASED GENETIC VARIATION FOR RED ROT RESISTANCE IN SUGARCANE

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Abstract

Genetic difference between twelve red rot resistant and five susceptible genotypes of sugarcane cultivated in Pakistan were studied using Random Amplified Polymorphic DNA (RAPD) markers. Initial screening was done using 300 markers and four genotypes (two resistant and two susceptible for red-rot). From these 300 markers, 24 were selected and further applied to all 17 genotypes. A total number of 182 loci were generated by these 24 primers. Of these 156 loci were polymorphic and 26 were monomorphic, whereas 10 loci were genotype specific. Moreover, the number of monomorphic loci for the resistant and susceptible genotypes was 29 and 52 respectively. However, none of the loci could be solely linked to either resistance or susceptibility against red-rot. The mean genetic similarity among the genotypes recorded was 74.37% which shows that a large part of the genome is similar. This may be due to the lack of parental diversity. This study reveals that there is possibly more than one genetic reason for resistance or susceptibility against red rot in sugarcane genotypes of Pakistan.

Introduction

Sugarcane is an economically important crop and is one of the most important field crops grown in the tropics and sub-tropics. It is grown over 1.07 million ha in Pakistan producing about 53.4 million tonnes of cane annually. The average yield being around 50 tonnes/ha (Govt. of Pakistan, 2005). About 73 sugar mills in different parts of the country depend on sugarcane for production of white sugar, ethanol and power generation (from bagasse) (Sugar Mills Directory, Pakistan, 2005).

Red rot disease, caused by the fungus *Colletotrichum falcatum* Went (Perfect state; *Glomerella tucumanensis* (Speg.) Arx a Muller), is one of the major production constraints for sugarcane production (Alexander & Viswanathan, 1996). In Pakistan, during the years 2003-2006, the greatest loss to sugarcane industry was due to red rot. The loss in cane weight was recorded to be about 29.07 % and resulted in 30.8 % loss in sugar recovery (Hussnain & Afghan, 2006). The disease is also responsible for the deterioration of sugarcane cultivars and continues to be a problem in other countries such as USA, Bangladesh, India, Australia, Thailand and Taiwan and the world wide loss in cane yield and sugar recovery was about 5-10 % (Viswanathan & Samiyappan, 2002). Red rot occurs in various parts of the cane plant but it is usually considered a stalk and a seed-piece disease. However, the first external symptoms appear mostly on the leaves (Sundara, 1998; Raid, 2006).

Various fungicides were tested for the management of the disease, but limited success was achieved under field conditions (Singh & Singh, 1989). Moreover, most of

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the newly released cultivars to replace the susceptible ones succumb to the pathogen almost as soon as they become popular due to the frequent emergence of new variants of the pathogen. Management of the disease by the use of disease-free seed canes for planting is impractical due to the difficulty in diagnosing the dormant infections of the fungus in seed canes under field conditions (Viswanathan & Samiyappan, 2002). It is, therefore, important to explore other possibilities for the management of red rot in sugarcane. In this context, better understanding of the genes involved in conferring resistance against it is being considered an important necessity at present.

Modern sugarcane cultivars are known to have one of the most complex genomes. Since most traits in sugarcane are multigenic and/or multi-allelic, and are quantitatively inherited, they thus represent a particular challenge for breeding, genetics and gene cloning purposes (Butterfield *et al.,* 2001). In recent years, considerable progress has been made in the mapping of the sugarcane genome and its progenitors (Aitken *et al.,* 2005). The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers (Korzun, 2002). Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. Extensive research using DNA markers is in progress in many institutions all over the world. RAPD markers have been successfully used to measure the genetic relationships of some cultivated species such as sugarcane, cotton and wheat (Leon *et al.,* 2001). PCR amplified DNA has been used to identify varieties (Piperidis *et al.,* 2004) and understanding the genealogy of sugarcane varieties in breeding programmes (Garacia *et al.,* 1997). Moreover, RAPD markers have proved useful in determining the genetic relationships among cultivars (Leon *et al.,* 2001).

Many markers tagged and mapped with specific genes have been identified. Rust was the first well characterized Mendelian trait described in the complex genomic context of sugarcane (Daugrois *et al.,* 1996; Asnaghi *et al.,* 2001). The first gene to be localized was *Bru1* (for brown rust) and is currently the focus of a map-based cloning project (D'Hont *et al.,* 2001; Asnaghi *et al.,* 2004). Recently two more genes: a gene controlling the red stalk colour and a new brown rust resistance gene were localized and labelled to two AFLP markers in sugarcane (Raboin *et al.,* 2006). These are the only other major genes to be identified in sugarcane so far.

The present research work aims at finding genetic differences in the genomes of red rot resistant and susceptible genotypes of sugarcane using Random Amplified Polymorphic DNA (RAPD) molecular markers. With the identification of molecular differences, screening of sugarcane germplasm and tissue cultures for these traits may prove an important advantage in future research work. Moreover, it may lay the foundations for the possibilities of gene therapy in the form of transgenic plants.

Materials and Methods

Different known and characteristically studied genotypes of *Saccharum* spp. hybrids were obtained from Shakarganj Sugarcane Research Institute (SSRI), Jhung and cultivated in the field of School of Biological Sciences (SBS), Punjab University, Lahore. Grouping of sugarcane cultivars was made on the basis of their resistance and susceptibility against red rot in Pakistan as notified by the SSRI (Table 1).

Genomic DNA was extracted according to standard protocol using CTAB (cetyl trimethyl ammonium bromide (Doyle & Doyle, 1990). Quantification of extracted DNA was done using spectrophotometer at O.D. 260nm and confirmed by agarose gel electrophoresis. Dilutions of 100ng/10ul of DNA were prepared in autoclaved distilled water.

In order to identify the red rot resistance genes Random Amplified Polymorphic DNA (RAPD) technique was applied using 300 random decamer primers of the S-series (BIO tools), A, B, C, D, E, F, G, H, I, J, K, L and M- series (BIO Neer). PCR conditions were optimized for the best amplification of primers by the genomic DNA under study. Initially the 300 RAPD primers were applied to two red rot resistant (CSSG-668 and SPSG-79) and two susceptible (SPSG-114 and SPF-234) genotypes of sugarcane. Twenty-four primers giving comparable results were then applied to all 17 genotypes. Amplification of polymorphic DNA was observed on 1% (w/v) agarose gel with ethidium bromide staining. Similar and differentiating bands between resistant and susceptible varieties were recorded.

PCR amplification was carried out in 50ul reaction mixtures containing 1mM $MgCl_2$, 0.2mM dNTP's, 30ng DNA template, 50pmoles of primer and 2.5U of *Taq* DNA polymerase.PCR reactions consisted of initial denaturation at 94^{o}C followed by 40 cycles of 94^{o}C for 50sec, 35^{o}C / 37^{o}C for 1min and 72^{o}C for 2min with a final primer extension cycle of 72^{o}C for 5min in a Applied Biosystems, GeneAmp^{R} PCR System 2700 thermal cycler. 25 µl of the amplification products were electrophoresed on 1% Agarose gels in 1x TAE buffer. The gels contained 0.5ug/ml ethidium bromide. The gels were observed under a UV transilluminator in a Dolphin Gel Documentation system and photographed. Clearly resolved bands were scored as present (1) or absent (0).

The data of 182 loci generated by the 24 primers used on all 17 genotypes was selected for statistical analysis using Minitab (version 13.0 for windows) software using

Table 1: List of Red Rot Resistant and Susceptible Sugarcane Genotypes Obtained from Shakarganj Sugarcane Research Institute (SSRI), Jhung

Genotypes	Male Parent	Female Parent	Trait
SPSG-114	N 5679	SP70-1143	Susceptible
SPF-234	SP71-8210	SP71-6180	Susceptible
HSF-242	SPSH89-2085	Poly cross	Susceptible
Coj-84	Co-1148	Not known	Susceptible
Co-1148	Co-301	P-4383	Susceptible
CSSG-668	81-N289	CP74-2005	Highly Resistant
SPSG-79	SP70-1143	SP73-5368	Highly Resistant
HSF-240	CP43-33	Open pollination	Highly Resistant
CPF-237	86P-19	CP70-1133	Highly Resistant
CSSG-676	ROC-1	CP74-2005	Highly Resistant
NSG-555	CP63-588	MO/F	Highly Resistant
NSG-39	84F-2753	MO/F	Highly Resistant
NSG-311	N19	MO/F	Resistant
SPSG-26	SP73-5368	SP70-1143	Resistant
SPF-213	SP70-1006	Open pollination	Resistant
CPF-236	86P-19	CP70-1133	Resistant
CP-77/400	Not known	Not known	Resistant

multivariate cluster analysis through group linkage method. The genetic distance between the genotypes was calculated as the decreasing function of the similarity. For better understanding a homology tree of the genotypes was constructed. Monomorphic loci, polymorphic loci and genotype specific bands were also recorded.

Results

A total of 182 bands were generated by the 24 primers used on all 17 genotypes. 156 of these bands were polymorphic while 26 were monomorphic. Thus, the average number of bands produced by each primer was 7.58. The size of the amplification products ranged from 150 bp to 2.1 kb.

The maximum number of bands (13) was produced by the primer F-7 (5'–3' CCGATATCCC), while the minimum number (4) was produced by the primers B-9 (5'–3' TGGGGGACTC), C-16 (5'–3' CACACTCCAG) and G-12 (5'–3' CAGCTCACGA). Among the genotypes, NSG-311 gave the total maximum number of bands and maximum number of polymorphic loci (128 and 102 respectively) with all 24 primers, while CSSG-676 gave a total of 93 bands and 67 polymorphic loci which were the least (Table 2). The degree of polymorphism also could not be linked to resistance or susceptibility against red rot in the sugarcane genotypes. The number of monomorphic loci for the resistant and susceptible genotypes was 29 and 52 respectively. However, none of the loci could be solely linked to either resistance or susceptibility of red-rot as they were also found to be present in some genotypes belonging to the other category as well.

A total of 10 bands produced by 7 primers (A-5, C-16, E-19, F-7, J-5 and K-11) were genotype specific (Table 3). Nine of these 10 bands were found in the resistant genotypes (CSSG-668, NSG-555, NSG-311, SPSG-79 and SPSG-26), while only one band of 300bp generated by the primer F-7 was specific to the susceptible genotype Coj-84.

The mean genetic similarity among the genotypes recorded was 74.37% which shows that a large part of the genome is similar. This may be due to the lack of parental diversity. The greatest similarity (83.25%) was recorded among the susceptible genotypes SPSG-114 and SPF-234 followed by the resistant genotypes CPF-236 and CPF-237 (78.86%). The resistant genotype NSG-555 had the least genetic similarity (67.15%) with the other sugarcane genotypes. The clustering pattern showed that except for the three genotypes NSG-555, CSSG-676 and NSG-39 the remaining genotypes appeared to form

Table 2: Total number of RAPD bands and polymorphic loci produced by each sugarcane genotype

Genotype	Total No. of Bands	Polymorphic loci	Genotype	Total No. of Bands	Polymorphic loci
SPSG-114	105	79	CSSG-676	93	67
SPF-234	99	73	NSG-555	125	99
HSF-242	107	81	NSG-39	105	79
Coj-84	107	81	NSG-311	128	102
Co-1148	97	71	SPSG-26	121	95
CSSG-668	119	93	SPF-213	106	80
SPSG-79	120	94	CPF-236	98	72
HSF-240	105	79	CP-77/400	116	90
CPF-237	102	76			

Table 3: List of Genotype Specific Bands

Primer	Sequence (5'-3')	Size of Band	Genotype
A-5	AGGGGTCTTG	300bp	CSSG-668
A-5	AGGGGTCTTG	1200bp	NSG-555
C-16	CACACTCCAG	600bp	NSG-311
E-19	ACGGCCTATG	400bp	SPSG-79
E-19	ACGGCCTATG	600bp	CSSG-668
F-7	CCGATATCCC	300bp	COJ-84
J-5	CTCCATGGGG	550bp	CSSG-668
J-12	GTCCCGTGGT	600bp	SPSG-26
K-11	AATGCCCCAG	1100bp	NSG-311
K-11	AATGCCCCAG	1300bp	NSG-311

a single cluster (Fig. 1). However, these three remaining genotypes could not be categorized as a separate cluster either. Parentage of the genotypes did not contribute significantly to the genetic similarity or have any significant effect on the clustering pattern. Most of the genotypes with same single or both parents e.g. SPSG-26, SPSG-79 and SPSG-114 with the common parent SP-70/1143 and, CSSG-676 and CSSG-668 with the common parent CP-74/2005 showed less similarity than some with totally different parents e.g. SPSG-114 and SPF- 234. Moreover, the susceptible genotype Co-1148 is the parent of Coj-84, but Coj-84 showed more homology with HSF-240 (77.24%).

Discussion

A number of sugarcane genotypes are released every year and tested for commercial cultivation by various commercial and research institutes in the country. As there are

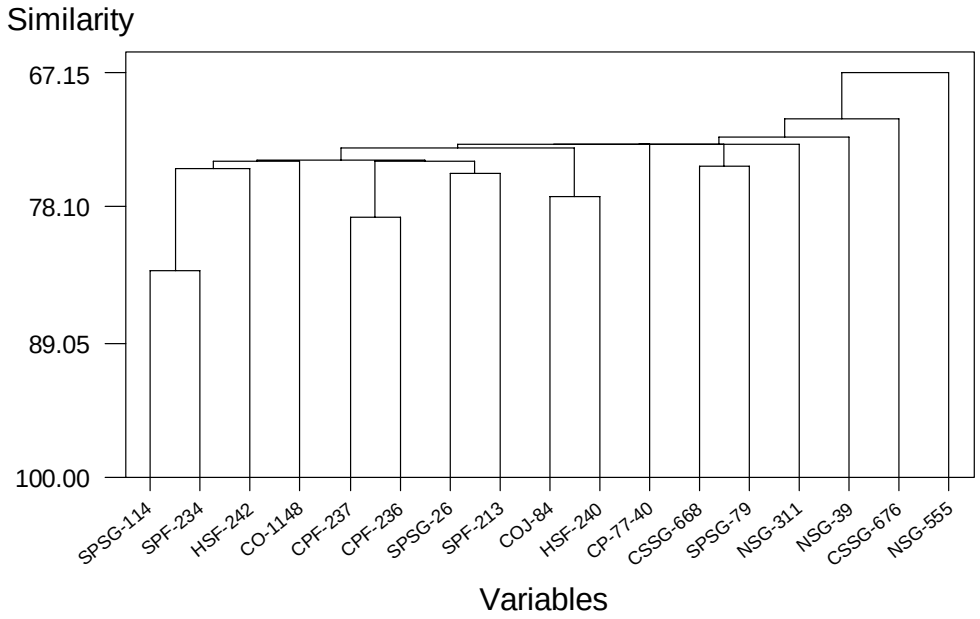

Fig. 1: Homology tree constructed showing the genetic similarity among sugarcane genotypes.

limited areas for sexual reproduction of sugarcane in Pakistan and most of the genotypes are produced *via* tissue culture and cloning techniques a conscious effort must be made to ensure a broad and diverse genetic base of sugarcane genotypes. The genetic similarity ranged from 67.15 to 83.25% among the sugarcane genotypes used. The high genetic similarity in the above results indicated that the genotypes studied were from a similar genetic parentage. This is almost the same range as given by Pan *et al.* (2004) for *Saccharum spontaneum* and elite accessions. Harvey & Botha (1996) reported similarities of 77-95% among 20 elite sugarcane varieties whereas, Harvey *et al*. (1994) reported nearly 80% genetic similarity among most of the 21 South African sugarcane varieties studied. They also found that a *S. spontaneum* clone and an elite variety were more divergent with almost 30% similarity and suggested hybridization of hybrid varieties with such clones and other species variants to develop new commercial varieties which are genetically more diverse.

Parentage of the genotypes did not contribute significantly to the genetic similarity or have any significant effect on the clustering pattern. This probably is due to the repeated use of a few set of varieties which are themselves related as parents (Nair *et al*. 2002).

The 10 genotype specific primers show that there is a potential of using RAPD-PCR markers for identifying *Saccharum spp.* hybrids and clones (Pan *et al.* (2004). The degree of polymorphism could not be linked to resistance or susceptibility against red rot in the sugarcane genotypes. Moreover, none of the monomorphic loci for the resistant or susceptible genotypes could be solely linked to either resistance or susceptibility of red-rot as they were also found to be present in some genotypes belonging to the other category as well. The reason for this may be that resistance or susceptibility to red rot in modern sugarcane hybrids is due to more than one genetic reason.

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