Red rot resistant gene characterization using RGAP markers among sugarcane cultivars resistant and susceptible to the red rot disease

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Abstract Sugarcane is the major source of sugar in Asia and Europe, grown primarily in the tropical and sub-tropical zones of the world. The main disease responsible for its low yield is red rot. Therefore, in the present study, characterization of red rot disease was performed among 55 different sugarcane cultivars varying in red rot resistance level. 18 fragments were found to be associated with red rot resistance and were identified as resistant specific markers. The resistant specific fragments were amplified by RGA169, RGA396, RGA129, RGA231, RGA251, RGA057, RGA118, RGA152, RGA327, RGA542, RGA012, RGA173, RGA184, RGA275, RGA019, RGA267, RGA281 and RGA533. 7 fragments were found to be associated with red rot susceptibility and were considered as susceptible specific markers amplified by RGA088, RGA162, RGA396, RGA231, RGA251, RGA087 and RGA275. Sequencing of five resistant fragments, viz., RGA169, RGA231, RGA251, RGA267 and RGA533 was performed and the data thus obtained showed 80–99% similarity when compared with other resistant gene sequences previously submitted in NCBI database.

Keywords *Saccharum* · RGAP · Sugarcane · Red rot · Resistance · Susceptibility

Introduction

Sugarcane is an important cash crop primarily used for the production of commercial sugar, all over the world, accounting for approximately two-third of total world sugar production (Menossi et al. 2008). India occupies the second position in terms of the production of sugarcane (FAOSTAT 2013). Although different biotic and abiotic factors cause the reduction in its yield, diseases are the major concern. Around 100 sugarcane diseases have been reported from all around the world (Rott et al. 2000). Globally, about 100 fungi, 10 viruses, 10 bacteria and 50 species of nematodes have been found as the pests of sugarcane (Singh and Waraitch 1981).

The approximate loss of crop production in India because of fungal diseases accounts for about 18–31% (Jayashree et al. 2010). Red rot disease is one of the oldest disease of sugarcane caused by fungus *Colletotrichum falcatum* (Ahmad 1988). It occurs in most of the sugarcane growing countries but is a major constraint for subtropical countries. It is the major threat for sugarcane cultivation and is responsible for the intense losses in the content of sucrose in most of the cane cultivated areas of the world (Hussnain and Afghan 2006). This disease causes the failure of some highly significant Indian commercial *Saccharum* varieties (Babu 2010).

This alarming situation warrants genetically improving the varieties not only by developing the disease resistant cultivars but also by improving their genetic base. Now a days, molecular methods based on polymerase chain

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reaction (PCR) are frequently being used to uncover the complex genetics of sugarcane and to assist breeders in genetic improvement of varieties.

Molecular markers that are linked tightly to the resistance genes have advantage over other molecular markers. Resistant gene analogs (RGAs) used the conserved motifs of the resistance genes across different plant species. In sugarcane, genes related to cold tolerance, disease resistance, insect resistance and oxidative stress were identified from the sugarcane EST databases (SUCEST) (Rossi et al. 2001). About 130 different sugarcane RGAs have been identified from SUCEST databases as well as from cDNA libraries (Rossi et al. 2003; McIntyre et al. 2005a). The present study was designed to characterize the genes related to red rot resistance in sugarcane.

Materials and methods

In the present study, the plant samples (leaves) collection, DNA extraction, PCR amplification and primer designing were done according to Sharma and Tamta (2016, communicated). Table 1 shows the sugarcane cultivars used in the study and their response to red rot disease whereas Table 2 gives the information about primers used in the study with their standardized annealing temperature (both Tables 1, 2 have been taken from Sharma and Tamta 2016, communicated).

Markers associated with red rot resistance and susceptibility

The PCR gel was analyzed for identifying the red rot resistant markers. The fragments that were associated specifically with red rot resistant cultivars were the markers associated with the red rot resistance. The same procedure was followed for identifying the markers associated with red rot susceptibility by analyzing the fragments associated with red rot susceptible cultivars specifically.

Data analysis of resistant fragments

The sequence data of the different plant resistant genes of Poaceae family were obtained from plant resistant gene database (PRGdb), which is a web accessible opensource (<http://www.prgdb.org>) database representing the first bioinformatics resource providing a comprehensive overview of resistance genes in plants (Sanseverino et al. 2010). Resistant markers identified in the present study were aligned with resistant genes procured from PRGdb and the comparative data thus obtained were constructed into phylogenetic tree by employing the CLC genomics

workbench 7.5 and the accuracy of the phylogenetic tree was also assessed by bootstrap analysis.

Sequencing of the amplified products

Five resistant specific fragments were selected as the representative markers for sequencing. Sequencing reaction of the selected markers was performed by Xcelris Labs Ltd, Ahmedabad, India, through outsourcing.

Prediction of chromosomal location of resistant fragments

The resistant fragments that were sequenced were compared with the resistant gene sequences from National Centre for Biotechnology Information (NCBI) database using BLASTn and the loci with which the RGAs were found to be most similar were predicted as the probable chromosomal location of RGAs in sugarcane as well.

Multiple sequence alignment and phylogenetic analysis

The sequence data of the resistant markers were compared to resistant gene sequences previously described in NCBI using BLASTn and BLASTx algorithm. The sequenced markers and the genes obtained from PRGdb were aligned using CLC genomics workbench 7.5 and a phylogenetic tree was constructed using Neighbor-Joining method as implemented in the CLC genomics workbench 7.5. Bootstrapping (100 replicates) was used to evaluate the degree of support for particular grouping patterns in the phylogenetic tree.

Results

Markers associated with red rot resistance

A number of red rot resistant and red rot susceptible markers were identified on screening of 55 different sugarcane cultivars using 28 different RGA primers. A total of 18 fragments were identified as resistant specific markers and were amplified by RGA169, RGA396, RGA129, RGA231, RGA251, RGA057, RGA118, RGA152, RGA327, RGA542, RGA012, RGA173, RGA184, RGA275, RGA019, RGA267, RGA281, RGA533 while 7 fragments were identified as susceptible specific markers amplified by RGA088, RGA162, RGA396, RGA231, RGA251, RGA087, RGA275 (Table 2).

Most of the markers amplified only a single specific band in the case of both red rot resistance and susceptibility except RGA396, RGA231, RGA251, RGA275 and

Table 1 Sugarcane cultivars used in the study and their response to red rot disease (Sharma and Tamta 2016 communicated)

S. no	Selected sugarcane cultivars	Response to red rot disease	Place of origin
1	CoPant97222	MR	Pantnagar
2	CoPant3220	MR	Pantnagar
3	CoS88230	MR	Shahjahanpur
4	Malani	MR	Coimbatore
5	CoPant90223	MR	Pantnagar
6	CoS97264	MR	Shahjahanpur
7	CoS96268	MR	Shahjahanpur
8	CoSe96436	MR	Seorahi
9	CoPant3219	MR	Pantnagar
10	CoS10231	MR	Shahjahanpur
11	CoH10261	MR	Hisar
12	6-80	MR	Unknown
13	6-225	MR	Unknown
14	CoH10262	MR	Hisar
15	Co90022	MR	Coimbatore
16	CoPant2218	MR	Pantnagar
17	Co10037	MR	Coimbatore
18	CoPant96269	MR	Pantnagar
19	CoPant10221	MR	Pantnagar
20	K14/05	MR	Unknown
21	CoLK9204	MR	Lucknow
22	CoPb10181	MR	Punjab
23	CoPb9214	MR	Punjab
24	CoLK9202	MR	Lucknow
25	CoH9264	MR	Hisar
26	CoS8432	MR	Shahjahanpur
27	CoPant5224	MR	Pantnagar
28	CoPant94211	MR	Pantnagar
29	CoS8233	MR	Shahjahanpur
30	CoPb8211	MR	Punjab
31	CoS98247	R	Shahjahanpur
32	Co09022	R	Coimbatore
33	CoPb10211	R	Punjab
34	UP9530	R	Uttar Pradesh
35	CoS96275	R	Shahjahanpur
36	CoS8436	MS	Shahjahanpur
37	Co0118	MS	Coimbatore
38	CoS767	MS	Shahjahanpur
39	CoPant84211	MS	Pantnagar
40	Co10036	MS	Coimbatore
41	Co10039	MS	Coimbatore
42	Co10035	MS	Coimbatore
43	Co0238	S	Coimbatore
44	CoJ88	S	Jalandhar
45	CoPant84212	S	Pantnagar
46	CoPant99214	S	Pantnagar
47	Co0239	S	Coimbatore

Table 1 continued

S. no	Selected sugarcane cultivars	Response to red rot disease	Place of origin
48	Co419	S	Coimbatore
49	CoPant84213	S	Pantnagar
50	UP0097	S	Uttar Pradesh
51	CoJ64	S	Jalandhar
52	CoPb10183	HS	Punjab
53	CoPb10182	HS	Punjab
54	CoH10263	HS	Hisar
55	Co1148	HS	Coimbatore

RGA267. RGA396 amplified 3 specific fragments of which 1 was resistant specific and 2 were susceptible specific. RGA231, RGA251 and RGA275 amplified 2 specific fragments of which 1 was found to be resistant specific and 1 was susceptible specific. RGA267 amplified 3 specific fragments all of which were found to be resistant specific.

Data analysis of resistant fragments

The markers that were identified as resistant were aligned with the resistant genes of Poaceae family obtained from PRGdb database. The comparative data thus obtained were used to construct the phylogenetic tree (Fig. 1). The phylogenetic tree was clearly differentiated into two major clusters and each cluster was further differentiated into different sub-clusters. RGA533 was found to be in close relationship with *Pi2*. RGA019 and RGA267 were found to be of same lineage. RGA173 was found to be closely related to *Pid2* gene. RGA129 was observed to be associated with *Pi5-1*. RGA281 and RGA118 were observed to be closely related with each other. RGA169 and gene *Pm3* were found to be closely associated. Similarly, RGA542 was in close relationship with *Pi36* gene. RGA152, RGA012, RGA184, RGA327 and RGA057 were associated with different resistant genes but were observed to be of different lineage.

Sequencing of the amplified products

PCR profiles of different primers used for sequencing are shown in Fig. 2. Sequencing of the amplicons showed length variation of nucleotide sequences among all the 5 RGAs. The length of the RGAs varied from 238 to 449 bp. The sizes of RGA169, RGA231, RGA251, RGA267 and RGA533 were found to be 292, 279, 238, 449, 300 bp, respectively.

The nucleotide sequences of RGA169, RGA231, RGA251, RGA267 and RGA533 were submitted in NCBI with accession number KT156964, KT156965, KT156966,

Table 2 Markers associated with red rot resistance and susceptibility

S. no	Primers	Primer sequence (5'–3')		Annealing temp (°C)	Total no. of specific bands	R-specific bands	S-specific bands
		Forward	Reverse				
1	RGA 088	GAGTGTCGGTGCACCTTTGA	TTTCCCCCATCAAATTGGTA	50	1	0	1
2	RGA 169	CCGCATCCTTGCTTATGAAT	ATGATATGAGGCTGCGCTTT	50	1	1	0
3	RGA 162	TTCAATGTTCCAAACAGCCA	GCACGAAGGAATAACAGGACA	51	1	0	1
4	RGA 396	ATGACTTTCCTGGCCTCCTT	CACTCCAAATGCAGAAAGCAA	51	3	1	2
5	RGA 129	CTTCTTGCTAGCTGGCCTC	TGGGATGCATGAACTTGTGT	52	1	1	0
6	RGA 231	CGAGAAACTTCCGACCTGAG	GATGCGAAGGTTCAGAGAAA	52	2	1	1
7	RGA 251	CGGGAAAACAACACTAGCAC	GTTTGAAGTCCACACGTCAT	52	2	1	1
8	RGA 326	CAGTGGGTGTGTAAGGATT	TTATCACTGCAGCCTGATCG	52	0	0	0
9	RGA 016	TAGCTCGCCGAGCAGATTA	GCTTGTGCTGCTGTGTTCAA	53	0	0	0
10	RGA 057	CACATCATGAGTGGCAGCTT	GACTGCTACCGGCTTATGGA	53	1	1	0
11	RGA 087	AGGCTCCACTGTCATTCTCG	AACACCTGAGAATCGATGGG	53	1	0	1
12	RGA 118	TCCATGTCTGCGAACTCTTG	TGTCCAGAGTGTGGAGGTTG	53	1	1	0
13	RGA 125	ACACTTTGAACAGCACCAGC	ATCTTCGGGGGTGGTTTGAAG	53	0	0	0
14	RGA 152	AGGCTCCACTGTCATTCTCG	AACACCTGAGAATCGATGGG	53	1	1	0
15	RGA 185	GCTACGGCACCTTCTAGCTG	ATTTGCACAATCAGCAGCAC	53	0	0	0
16	RGA 327	TTGACGACGATATGGCTGCT	CTCCCCGAAGCAATACACT	53	1	1	0
17	RGA 542	AAGTGCCCTCACCAGCTAAA	CCTTCTGCTACCCACCTTCT	53	1	1	0
18	RGA 012	GGGGTGACAAAGGGGTGAA	ATGCAGAGAACCCATGGTGG	54	1	1	0
19	RGA 137	GAGTGTCCCACTGAGGTGGT	TCAGCAACTTGTTTACAGGG	54	0	0	0
20	RGA 145	TACACATGGGGAAAGGGTGC	CAAGTTGAATGGCTGACGGC	54	0	0	0
21	RGA 173	CCGTCTCTCCTGGGTCAATG	TGCCTTGGATTGAGATGGCA	54	1	1	0
22	RGA 184	GGATGCTGCTCTCGGTTTCT	TAGACCAGCAACCTCTTGCC	54	1	1	0
23	RGA 258	TAGCACTTGATGCTGCCAGG	TAGCTGGCCATGGCTAAAGG	54	0	0	0
24	RGA 275	GTGCTGATGCGTGTACCAAC	ACACACCAAAAGTCCCAGG	54	2	1	1
25	RGA 019	GTTCTCCGTCCGTTCTCTG	CGGATCAACTGGCGATGGTA	55	1	1	0
26	RGA 267	CACCTCTGTTGGGGGTATGG	CGGGAGGGGCGCATTTATTA	55	3	3	0
27	RGA 281	AGCTACCTTACTGCCCTGGA	CAGCTAGAAGGTGCCGTAGC	55	1	1	0
28	RGA 533	AGTCTTGGATGGGGCCATTG	GCATCCTGTGGATACCCTCG	55	1	1	0

KT156967 and KT156968, respectively. The sequence data of the RGAs were compared to resistant gene sequences already submitted in NCBI database and showed 80–99% similarity. This further confirmed that RGAs identified in this analysis are resistant genes and have potential to provide resistance to sugarcane against red rot disease.

Predicted chromosomal location of resistant fragments

The sequenced resistant fragments were compared with the resistant gene sequences previously submitted in NCBI and the loci with which the RGAs were found to be most similar were predicted as the probable chromosomal location of RGAs in sugarcane as well.

RGA231 and RGA169 both were found to be present on the chromosome 3 of *Sorghum bicolor* while RGA267 was observed to be on the chromosome 1 of *S. bicolor*.

RGA533 was observed to be located on chromosome 2 of *Zea mays*. The location of RGA251 was not determined as no details related to the chromosomal location of the same were present in NCBI.

It was also observed that when the nucleotide BLAST was done for the sequences which were used to design the primers in the present study, the same chromosomal location was observed (data not shown) as it was depicted for the sequenced resistant fragments, so, the chromosomal location of the resistant fragments that were not sequenced could also be presumed by performing the nucleotide BLAST for the same.

Multiple sequence alignment and phylogenetic analysis

The sequence data of the resistant markers were aligned with the plant disease resistant genes deduced from PRGdb

Fig. 1 Phylogenetic tree showing the relationship between resistant fragments obtained from this study (*RGA169*, *RGA129*, *RGA057*, *RGA118*, *RGA152*, *RGA327*, *RGA542*, *RGA012*, *RGA173*, *RGA184*, *RGA019*, *RGA267*, *RGA281*, *RGA533*) and genes obtained from PRGdb

database and the data obtained were used to prepare a phylogenetic tree (Fig. 3). The phylogenetic tree clearly depicted that *RGA533* was in close relationship with *Pid2* gene. *RGA267* and *RGA169* were found to be of different lineage. *RGA231* was observed to be closely associated with *Pit* gene. *RGA251* as observed from phylogenetic tree was not much closely associated with any of the fungal resistance gene.

Discussion

Markers associated with red rot resistance

Genotype specific bands have been an important feature for genotypes identification based on a particular trait (Chandra et al. 2006). RGAs are widely spread in the flanking regions of a number of R genes all over the plant genome (Meyers et al. 1999). The tightly linked RGAP markers observed in resistant cultivars can be further used to select resistant cultivars obtained from the breeding of same progenitors or by crossing the same resistant female and other susceptible males. In addition, the mapping of the race specific resistance loci could be done for a better

understanding of the genetic basis of resistance against the different races of the causative fungi.

In the present study, 18 markers were identified as resistant specific markers and 7 markers were identified as susceptible specific markers. This may be due to either the very close association of specific markers to red rot resistant or susceptible genes or the closely related genetics of these varieties (Singh et al. 2011). In previous studies, RAPD markers were used for selecting sugarcane cultivars resistant or susceptible against various diseases, i.e. sugarcane mosaic virus (Zambrano et al. 2003); red rot (Alvi et al. 2008; Mumtaz et al. 2011) and rust (Ali et al. 2013; Muhammad et al. 2013). AFLP, RFLP, SSR and ISSR markers were also identified against different diseases in sugarcane (McIntyre et al. 2005b; Aljanabi et al. 2007; Virupakshi and Naik 2008). Few studies have also been done by taking RGAs against rust, mosaic and smut diseases in sugarcane (Rossi et al. 2003; Brune and Rutherford 2005; McIntyre et al. 2005a). Jayashree et al. (2010) identified 25 specific fragments linked to red rot resistance and 8 specific fragments linked to susceptibility in sugarcane genotypes with the same set of RGA primers as used in the present study.

Fig. 2 PCR amplification profile of primers used for sequencing. (Lane 1 and 7: 100 bp DNA ladder; Lane 2: RGA169; Lane 3: RGA231; Lane 4: RGA251; Lane 5: RGA267; Lane 6: RGA533)

This study revealed the relevance of RGAP markers in marking the resistance and susceptibility in sugarcane against red rot disease. These markers could act as a potential tool in identifying the genus *Saccharum* in addition to the identification of clones, genotypes and hybrids (Pan et al. 2004). The outcomes of this study will be helpful in selecting the parents for a breeding program to develop red rot resistant cultivars and the markers identified can be used selectively for further gene mapping applications.

Fig. 3 Phylogenetic tree showing the relationship among sequenced resistant fragments (RGA169, RGA231, RGA251, RGA267, RGA533) and resistant genes retrieved from PRGdb

Data analysis of resistant fragments

The alignment of resistant markers was done with the resistant genes to determine the similarity among them and using the alignment data, a phylogenetic tree was constructed to determine the phylogenetic relationship between them. In the present study, RGA533 was found to be similar to *Pi2* gene, a blast resistant gene in rice that provides resistant against *Magnaporthe grisea* (Zhou et al. 2006). RGA173 was found to be similar to *Pid2* gene that was identified by Shang et al. (2009) as a blast resistant gene in rice. RGA129 marker was closely associated with gene *Pi5-1* which was a resistant gene isolated from *O. sativa* that provides immunity against blast disease caused by *Magnaporthe oryzae* (Lee et al. 2009). RGA169 was observed to be closely associated with *Pm3* gene. *Pm3* gene encodes CC-NB-LRR proteins that confer resistance to *Blumeria graminis* f. sp. *tritici*, the wheat powdery mildew fungus (Yahiaoui et al. 2009). RGA542 was in close relationship with *Pi36* gene that provides resistance against blast fungus (Liu et al. 2007).

Sequencing of the amplified products

On sequencing of the fragments observed in the resistant cultivars, it was confirmed that these fragments could be served as resistant markers or RGAs against red rot disease in sugarcane. RGA169 marker was 292 bp in size and on performing protein BLAST, it was identified to belong to protein kinase like superfamily which is an important class of plant disease resistant proteins. RGA231 marker was found identical to kinase resistant protein of *Saccharum* hybrid cultivar LCP 85–384 with 95% similarity on performing protein BLAST and was 279 bp in size. RGA251 (238 bp) was found to be 95% similar to RGA-Q36 resistance gene like mRNA of *Saccharum* hybrid cultivar Q117 and 93% similar to RGA-Q9 resistance gene like mRNA of *Saccharum* hybrid cultivar Q117. On performing protein BLAST of RGA267 (449 bp), it was identified to be 91% similar to protein kinase superfamily. RGA533 (300 bp) was identified to be similar to *pk8-b* gene for putative protein kinase with 80% similarity (NCBI). The RGAs identified in this study were found to be similar to different plant disease resistance genes already identified by different researchers; hence, it could be inferred from this observation that these RGAs could be served as an efficient markers for providing resistance to sugarcane against dreadly red rot disease.

Predicted chromosomal location of resistance fragments

There is limited knowledge about the locations of resistance gene loci on the sugarcane maps constructed to date. A limited number of QTL studies have been done in sugarcane using these maps and out of these, only two are related to disease resistance which includes the identification of markers associated with brown rust resistance gene (Asnaghi et al. 2000; Rossi et al. 2003), the only monogenic trait identified in sugarcane so far and the identification of a RAPD marker associated with eyespot resistance (Mudge et al. 1996). Recently, 50 RGAs have been mapped into the sugarcane map by Rossi et al. (2003).

A considerable synteny has been revealed among the genomes of sugarcane, sorghum and maize (members of the Andropogoneae tribe) on performing comparative mapping (Hulbert et al. 1990; Grivet et al. 1994; Dufour et al. 1996; Guimaraes et al. 1997; Ming et al. 2002). Sorghum is a diploid species and has a small genome size (Arumuganathan and Earle 1991). The use of the small diploid genome of sorghum as a reference has greatly assisted the researchers in analyzing the sugarcane genome. In a consensus map constructed by Ming et al. (2002), 6 of the 8 basic chromosomes of *S. spontaneum* and 7 of the 10

basic chromosomes of *S. officinarum* could be identified and linked with sorghum linkage groups.

Sugarcane is a complex interspecific polyploidy. Its 100+ chromosomes can be categorized into 8 homology groups, each containing 12 chromosomes approximately of which 2 or 3 chromosomes are donated by *S. spontaneum* and 9 or 10 chromosomes are donated by *S. officinarum* (Hoarau et al. 2001; Rossi et al. 2003; Aitken et al. 2004). RGA genes with low level of sequence similarity may be members of the same RGA family but represent paralogous loci, whereas RGA genes with a high level of sequence similarity may be alleles at orthologous loci. There is a greater distance observed between paralogous loci as compared to that between orthologous loci (Feuillet et al. 2001; Rossi et al. 2003) in other plant species.

Recently, 55 sugarcane RGAs have been identified having a great homology with known resistance genes (Rossi et al. 2003). McIntyre et al. (2005a) identified 54 unique RGAs from stem, leaf and root RNA of sugarcane cultivar 'Q117', of which 31 RGAs were mapped in sugarcane and 31 in sorghum, of which 23 RGAs were common in both species.

In this study RGA 231, 169, 533 and 267 were observed on different chromosomes of *Sorghum* and *Zea mays* which clearly indicates a close homology of these RGAs with already known resistance genes in *Sorghum* and maize.

Multiple sequence alignment and phylogenetic analysis

The data of the sequencing of the resistant markers were aligned with the plant disease resistant genes and similarity was determined between them and the data thus obtained were used for constructing a phylogenetic tree. On analyzing the phylogenetic tree, it was observed that RGA533 was related to *Pid2* gene which is a resistant gene isolated from rice and is effective against blast disease of rice (Shang et al. 2009) while RGA231 was found to be in close relationship with *Pit* gene. *Pit* gene was also isolated from rice as a resistant gene against blast disease (Hayashi and Yoshida 2009). On the other hand, RGA267, RGA169 and RGA251 were found to be of different lineage. As the resistant markers identified in present study were determined to be closely associated with disease resistance genes, they could act as an effective and reliable source in selection of suitable parent generating the mapping population for tagging the red rot resistance gene(s) in sugarcane.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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