Research Article

Characterization of Resistance Gene Analog Polymorphisms in sugarcane cultivars with varying levels of red rot resistance

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Abstract :
Resistance Gene Analog (RGA) strategy is being exploited perfectly for the identification, tagging and mapping of major genes or Quantitative Trait Loci for disease resistance. About 29 RGA primers designed from the conserved domains of resistance proteins, were used to analyse the genetic diversity among the 40 sugarcane cultivars that vary in their resistance to red rot disease. The genetic similarity values ranged from 58.4 - 90% with the mean genetic similarity of 74.2%. Cluster analysis resulted in a dendrogram with 3 major clusters and a clear distinction of resistant and susceptible varieties was observed. A total of 25 specific fragments amplified by 14 primers were identified to be associated with resistance and 8 specific fragments amplified by 8 primers were associated with susceptibility. The primers RGA – 137, RGA 396, RGA-231 and RGA-118 amplified maximum number of resistant or susceptible specific fragments (3). Amplification of the red rot resistant variety Bo 91 and the red rot susceptible variety CoC 671 with the twenty nine RGA primers, followed by sequencing and homology analysis revealed significant homologies with the RGA's of rice, maize and sugarcane. These RGA's that were found to be associated with red rot resistant/ susceptible varieties are a valuable source of markers that can be tested for screening red rot resistance in breeding programs.

Key words: Sugar cane, RGA, red rot resistance

Introduction

Sugarcane is the second most important agro industrial crop in India, next only to cotton. This commercial crop is cultivated in more than 90 countries all over the world, the largest areas being in Brazil and India. Cultivated sugarcane is an interspecific hybrid between *Saccharum officinarum* and *Saccharum spontaneum*, characterized by high ploidy levels with chromosome numbers of the cultivars ranging from approximately 100 to 130 and are both aneuploid and polyploid. The success of a sugarcane variety usually requires a balance between sugar yield and its related traits as well as stress and disease tolerance.

Biotic stresses represent a major challenge to the farmers and can greatly impact seasonal yields. In India, the estimated loss in crop production due to fungal diseases is about 18-31%. Of the common diseases of sugarcane, red rot, caused by the fungus *Colletotrichum falcatum* Went causes severe loss in yield and quality of the susceptible cultivars in the Indian sub-continent (Singh & Waraitch 1977; Alexander & Viswanathan 1996). The disease is best managed by growing resistant varieties, since till recently no chemical measures have been found to be successful. Agronomic measures are partially effective in managing the disease but need very systematic implementation. The use of resistant varieties is the most effective method of prevention and control.

Breeding for resistant varieties requires precise screening methodologies and selection for resistant plants, which are often laborious and require extensive knowledge on plant-pathogen interactions. Molecular markers tightly linked to the genes conferring resistance will be of advantage in such situations. Resistant Gene Analog Polymorphism (RGAP) technique (Chen *et al.*1998) has proven to be efficient in identification of molecular markers for disease resistance. Isolation of disease resistance gene analogs (RGAs) using the conserved motifs of the resistance genes has attracted considerable attention since it was first reported more than a decade ago (Bozkurt *et al*. 2007). In sugarcane genes associated with cold tolerance, oxidative stress, insect resistance and disease resistance were identified from the sugarcane EST databases (SUCEST) (Rossi *et al*. 2001). Approximately 130 different RGAs have been identified in sugarcane both from SUCEST databases and from cDNA libraries (Rossi *et al*. 2003; McIntyre *et al*. 2005a).

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The present paper reports the Resistance Gene Analog Polymorphisms (RGAP) in sugarcane cultivars varying in their resistance levels to red rot disease and and identification of markers that are associated with red rot resistance and susceptibility.

Materials and methods

Plant material and screening for red rot disease

The plant materials used for the study included 40 sugarcane cultivars (Table1) which have been characterized periodically at the Institute for their reaction to red rot fungus. The varieties comprise 4 resistant varieties, 15 moderately resistant varieties, 13 susceptible and 8 highly susceptible varieties to red rot disease. The varieties were subjected to confirmatory screening for this study. They were planted in the pathology screening plot and inoculated with the red rot fungus cf 671 and evaluated on a 0 - 8 scale. The clones were rated for resistance using the following scale. 0-2.0: Resistant, 2.1-4.0: moderately resistant, 4.1-6.0: moderately susceptible, 6.1-8.0: susceptible, 8 and above: highly susceptible.

Genomic DNA extraction and PCR amplification of RGAs

The genomic DNA to be used as template for PCR analysis was isolated from 40 sugarcane cultivars using the method developed by Walbot (1988) and checked for its integrity by resolving on 0.8% agarose gels. About twenty nine RGA primers were designed based on the conserved domains of cloned plant resistance genes and synthesized. Fifteen nanogram of the sugarcane DNA was amplified in a 15µl reaction mixture containing 10 mM Tris HCl (pH 9.0) with 1.5 mM $MgCl_2$, 100µM dNTPs, 50ng of each primer and 1 Unit of Taq Polymerase. The samples were denatured at 94ºC for 3 min, followed by 44 cycles of denaturation at 94ºC for 1 min, annealing at 50-56ºC for 1 min and extension at 72ºC for 2 min followed by a final extension at 72ºC for 7 min and storing at 4ºC. The PCR amplifications were performed in a thermocycler (MJ Research Inc. model: PTC-100). Amplification products were analysed on a 6% polyacrylamide gel and visualized by silverstaining.

Data analysis

For each genotype, the data was scored as "1" for "presence" and "0" for “absence”. The data thus obtained was analysed using the software DARwin 5.0 (Perrier *et al* .2003). The phenetic analysis was performed by calculating the genetic dissimilarities between cultivars *i* and *j* as d_{ij} by the Dice’s index d_{ij} = (b+c)/2a + (b+c) (where d_{ij} is the dissimilarity between units i and j ; a, b and c the number of variables). The matrix of pair wise d_{ij} values was used to construct a phenogram with the Neighbor joining method (Saitou and Nei. 1987). The clustering was validated by bootstrap analysis. One thousand bootstrap replicates were computed and was used in the construction of dendrogram.

Results and discussion

DNA Polymorphisms

Twenty nine primers that showed clear amplifications were screened on the 40 sugarcane cultivars consisting of 4 resistant, 15 moderately resistant, 13 susceptible and 8 highly susceptible varieties. Twenty-nine primers generated 529 distinct fragments, of which 458 were polymorphic and 71 were monomorphic (Table 3). A single primer amplified an average of 18 fragments. The percentage polymorphism ranged from 30 to 100%. The size of the amplification products ranged from 26bp to 1500bp. Primer RGA-183 (40) produced the most polymorphic fragments and RGA – 152 (3) the lowest with an average of 15.79 polymorphic fragments per primer.

In a study done by Chen *et al* (1998), 30 to > 130 bands were detectable among the rice, barley and wheat genotypes using Poly Acrylamide Gel Electrophoresis. The highest polymorphisms detected with a primer pair in rice, barley and wheat were 47, 48 and 39% respectively, and the mean polymorphisms were 31% in rice, 38% in barley and 21% in wheat. The results confirmed the observation that highly heterogeneous products are produced by PCR amplification with RGA primers and showed that, compared to agarose-gel electrophoresis, the high resolution of poly-acrylamide gel electrophoresis greatly increases the ability to detect polymorphism (Welsh & Mc Cleland. 1990, 1991).

Genetic diversity and cluster analysis

The genetic dissimilarity matrix was generated using the data generated on the individual varieties. The mean genetic similarity among the varieties was 74.2%. The highest similarity (100 minus dissimilarity value) was observed between Co 89006 and Co 91004 (90%) both of which are susceptible to red rot. The lowest similarity was observed between 88A162 and Co 99004 (58.4%) which are resistant and moderately resistant respectively (Table 4). The cluster analysis based on the data set of 29 markers resulted in a dendrogram with 3 major clusters. A clear grouping of the resistant and susceptible varieties was observed. The moderately resistant variety Co 99004 is found to be grouped along with the highly susceptible variety Co 95024. Co 99004 though classed under moderately resistant variety, it has shown susceptible reactions during recent screening. Previous studies using AFLP’s and RAPD’s and maize microsatellite markers on a set of 28 commercial sugarcane cultivars revealed 62%, 70% and 63.9% genetic similarities respectively (Nair *et al*. 2002; Selvi *et al*. 2003, 2006).

Markers associated with red rot resistance

Screening of 40 individual varieties with twenty-nine polymorphic RGA primers revealed a number of markers that are associated with red rot resistance and susceptibility. A total of 25 specific fragments amplified by14 primers were identified to be associated with resistance and 8 specific fragments amplified by 8 primers were associated with susceptibility. The primers RGA – 137, RGA 396, RGA- 231 and RGA-118 amplified maximum number of resistant or susceptible specific fragments (3). The primers RGA 137, RGA - 231 and RGA - 118 amplified 3 resistance-specific fragments each, with the sizes ranging from 145bp to 1337 bp. Minimum number of markers associated with resistance (1) was amplified by the primers RGA – 012, RGA – 183, RGA – 533, RGA- 267 and RGA-258 and the size of the markers was 1111 bp, 691 bp, 419bp, 506bp and 228bp respectively. RGA's viz., RGA – 088, RGA – 169, RGA – 019, RGA -396, RGA 184, RGA 162, RGA-275 and RGA-542 amplified one marker each that was specific to susceptible varieties. The details of the markers associated with resistance and susceptibility are given in Table 4.

In a study by McIntyre *et al*. (2006), fifty –five RGAs were identified within the sugarcane EST database with homology to typical disease *R*-genes. The 55 RGAs generated 272 polymorphic markers of which 177 segregated as single-dose makers in the R570 selfed progeny population. Of the 177 markers, 148 markers, corresponding to 50 different RGAs, were incorporated into the map of R570 (Rossi *et al.* 2003) with the remainder unlinked. Sequence analysis of markers obtained in this study revealed strong homology to disease resistance proteins like RPM1 of rice and to the disease resistance sequences of rice, Zea mays, Sorghum bicolor, Glycine max etc., containing various classes of disease resistance domains like NB-ARC, NBS-LRR,LRR, S/T kinase, protein kinase etc. This study provides a new way to develop molecular markers for assessing the genetic diversity of sugarcane cultivars that are resistant and susceptible to red rot and a more direct way to develop markers that are associated with resistance and susceptibility using potential candidate resistance genes that would be more reliable for screening breeding populations.

References

Alexander, K.C. and Viswanathan, R. 1996. Major diseases affecting sugarcane production in India and recent experiences in quarantine. (In) *Sugarcane germplasm conservation and exchange*, Croft,B.J., Piggin, C.M., Wallis,E.S. and Hogarth,D.M., (*Eds*). Proceedings 67, Australia. pp. 46-48.

Bozkurt O, Hakki E.E., Akkaya MS. 2007. Isolation and sequence analysis of wheat NBS-LRR type disease resistance gene analogs using degenerate PCR primers. *Biochemical Genetics*, **45** : 469-486

Chen X M, Line R F, Leung H. 1998. Genome scanning for resistance-gene analogs in rice, barley and wheat by high resolution electrophoresis. *Theor Appl Genet* **9**: 345-355.

McIntyre C,L., Casu, R.E., Drenth J, Knight D, Whan V.A., Croft V.J, Jordan D R., and Manners J.M., 2005. Resistant gene analogues in sugarcane and sorghum and their association woth Quantitative trait loci for rust resistance. *Genome* **48**: 391-400.

McIntyre CL, Jackson M, Cordeiro G, Amouyal O, Hermann S, Aitken KS, eliott F, Henry RJ, Casu RE, Bonnett GD .2006. The identification and characterization of alleles of sucrose phosphate synthase gene family III in sugarcane. *Molecular Breeding,* in press.

Nair NV , Selvi A, Sreenivasan TV, Pushpalatha KN .2002. Molecular diversity in Indian Sugarcane cultivars as revealed by Randomly Amplified DNA Polymorphisms. *Euphytica* **127**: 219-225.

Perrier, X., Flori, A. , Bonnot, F.2003. Data analysis methods. In: Hamon, P.,Seguin, M., Perrier, X. ,Glaszmann, J. C. Ed., Genetic diversity of cultivated tropical plants. Enfield, Science Publishers. Montpellier. pp 43 - 76.

Rossi, M., P.G. Araujo and M.A.van Sluys. 2001. Survey of transposable elements in sugarcane expressed tags (ESTs). *Genet. Mol. Biol*. **24**:147-154.

Rossi, M.M, Araujo, P.G., Paulet, F., Garsmeur, O., Dias, V.M., Chen, H., Van Sluys, M.-A., and D'Hont, A. 2003. Genomic distribution and characterization of EST-derived resistance gene analogues (RGAs) in sugarcane. *Mol, Genet. Genomics*, **269:**406-419.

Saitou, N., and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. *4: 406-425.*

Selvi, A., Nair, N. V., Balasundaram, and Mohapatra,T. 2003. Evaluation of maize microsatellite markers for genetic diversity analysis and fingerprinting in sugarcane. *Genome* **46**: 394-403.

Selvi, A., Nair, N. V., Noyer, J.L., Singh, N.K., Balasundaram, N., Bansal, K.C., Koundal, K.R. and Mohapatra,T. 2006. AFLP analysis of the phenetic organization and genetic diversity in the sugarcane complex, *Saccharum* and *Erianthus*. *Genetic Resour. Crop Evol.* **53**: 831-842.

Singh, O., and Waraitch, K.S.1977. Metabolic changes induced by *Colletotrichum falcatum* Went in sugarcane. *Sugarcane Pathol. Newsl*., **19**: 7-9.

Walbot .1988. Preparation of DNA from single rice seedlings. *Rice Genet Newsl* **5:** 149-151.

Welsh, J., R.J. Honeycutt, M.McClelland and B.W.S.Sobral .1991. Parentage determination in maize hybrids using arbitrarily primed Polymerase Chain Reaction (AP-PCR). *Theor. Appl. Genet.,* **82**: 473-476.

Welsh, J., and M.McClelland .1990. Finger printing genomes using PCR with arbitrary primers. *Nucl. Acids Res.,* **18**: 7213-7218.

Table 1 Cultivars used in the study and their reaction to red rot disease

S.No	Resistant cultivars	Reaction to red rot	S.no	Susceptible cultivars	Reaction to red rot
1	Co 86037	R	20	Co 95024	HS
2	Co 87263	R	21	Co 1148	S
3	Co 86249	MR	22	Co 6304	S
4	Co 93009	MR	23	Co J 94192	HS
5	Co 94008	MR	24	CoC 671	HS
6	Bo 91	R	25	CoC 85061	HS
7	Co 87268	MR	26	CoC 92061	HS
8	Co 7314	MR	27	CoC 90063	HS
9	88A162	R	28	Co 8371	S
10	Co 86250	MR	29	Co 85002	S
11	Co 87270	MR	30	Co 7204	S
12	Co 87271	MR	31	Co 7704	S
13	Co 87272	MR	32	Co 87023	HS
14	Co 88021	MR	33	Co 89006	S
15	Co 89010	MR	34	Co 91004	S
16	Co 89014	MR	35	Co 91010	S
17	Co 90014	MR	36	Co 798	S
18	Co 93027	MR	37	64A30	HS
19	Co 99004	MR	38	Co 92013	S
			39	Co 97009	S
			40	Co 92020	S

Note: R- resistant; MR- Moderately resistant; S –susceptible; HS- highly susceptible

Table 2 Primers used in the study

S.No	Primer	Annealing temp.
1	RGA-137	56°C
2	RGA-088	56°C
3	RGA-169	52°C
4	RGA-145	56°C
5	RGA-019	54°C
6	RGA-231	54°C
7	RGA-275	52°C
8	RGA-012	50°C
9	RGA-183	52°C
10	RGA-184	52°C
11	RGA-125	50°C
12	RGA-057	54°C
13	RGA-251	50°C
14	RGA-533	50°C
15	RGA-396	52°C
16	RGA-087	50°C
17	RGA-118	52°C
18	RGA-152	52°C
19	RGA-162	52°C
20	RGA-185	52°C
21	RGA-267	54°C
22	RGA-281	52°C
23	RGA-326	54°C
24	RGA-327	52ºC
25	RGA129	52ºC
26	RGA258	52ºC
27	RGA16	52ºC
28	RGA173	52ºC
29	RGA542	50ºC

Table 3 Resistance Gene Analog polymorphisms among the sugarcane cultivars with varying levels of red rot resistance

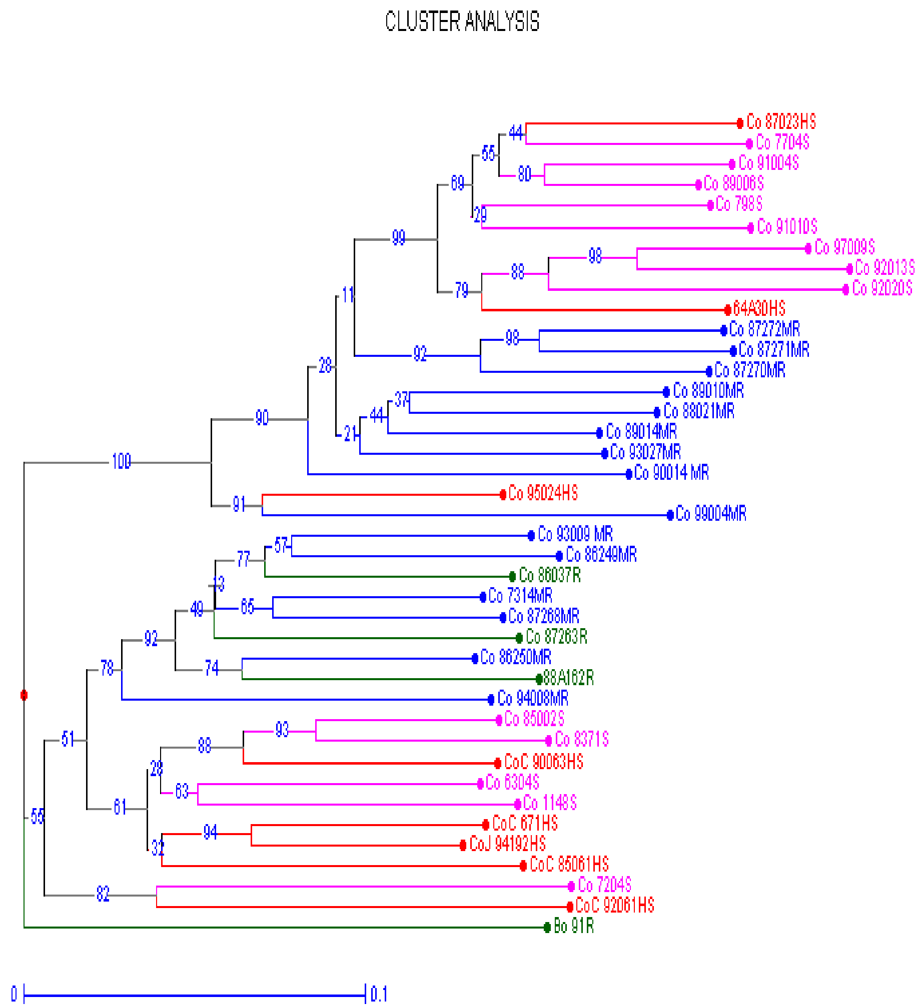
S.No.	Primer	TOTAL NO. OF BANDS	No. OF MONOMORPHIC BANDS	No. OF POLYMORPHIC BANDS	M%	P%
1	RGA 137	10	3	7	30	70
2	RGA 088	12	3	9	25	75
3	RGA 169	12	1	11	8	92
4	RGA 145	18	3	15	17	83
5	RGA 019	19	2	17	11	89
6	RGA 185	13	7	6	54	46
7	RGA 012	16	3	13	19	81
8	RGA 184	7	3	4	43	57
9	RGA 125	13	2	11	15	85
10	RGA 251	20	3	17	15	85
11	RGA 533	9	5	4	56	44
12	RGA 152	10	7	3	70	30
13	RGA 162	13	3	10	23	77
14	RGA 183	42	2	40	5	95
15	RGA 396	33	9	24	27	73
16	RGA 281	20	2	18	10	90
17	RGA 231	21	1	20	4.8	95.2
18	RGA 118	30	1	29	3.3	96.7
19	RGA 327	17	0	17	0	100
20	RGA 057	14	1	13	7.1	92.9
21	RGA 267	32	1	31	3.1	96.9
22	RGA 129	14	1	13	7.1	92.9
23	RGA 326	19	1	18	5.3	94.7
24	RGA 087	20	1	19	5	95
25	RGA 258	17	0	17	0	100
26	RGA 16	26	3	23	11.5	88.5
27	RGA 275	16	1	15	6.3	93.7
28	RGA 173	16	1	15	6.3	93.7
29	RGA 542	20	1	19	5	95
	Total	529	71	458	13.42	86.58

M% - Percentage Monomorphism   P% - Percentage Polymorphism

Table 4 Markers associated with red rot resistance and susceptibility

Primer code	Total no.of specific bands	R-Specific bands*	S-Specific bands*
RGA-137	3	3 (1337, 605, 396)	0
RGA-088	1	0	1 (114)
RGA-169	1	0	1 (348)
RGA-145	0	0	0
RGA-019	1	0	1 (482)
RGA-012	1	1 (1111)	0
RGA-183	1	1 (691)	0
RGA-184	1	0	1 (130)
RGA-125	0	0	0
RGA-251	2	2 (330, 198)	0
RGA-533	1	1 (419)	0
RGA-396	3	2 (56, 42)	1 (684)
RGA-152	0	0	0
RGA-162	1	0	1 (750)
RGA-185	2	2 (554, 550)	0
RGA 281	2	2 (154, 303)	0
RGA 231	3	3 (606,145,188)	0
RGA 118	3	3 (295, 325, 220)	0
RGA 327	0	0	0
RGA 057	1	1(318)	0
RGA 267	1	1 (506)	0
RGA 129	2	2 (540, 956)	0
RGA 326	0	0	0
RGA 087	0	0	0
RGA 258	1	1 (228)	0
RGA 16	0	0	0
RGA 275	1	0	1 (264)
RGA 173	0	0	0
RGA 542	1	0	1 (124)
TOTAL	33	25	8

* Specific bands along with their molecular weight in bp.


CLUSTER ANALYSIS
Co 87023HS
Co 7704S
Co 91004S
Co 89006S
Co 798S
Co 91010S
Co 97009S
Co 92013S
Co 92020S
64A30HS
Co 87272MR
Co 87271MR
Co 87270MR
Co 89010MR
Co 88021MR
Co 89014MR
Co 93027MR
Co 90014 MR
Co 95024HS
Co 99004MR
Co 93009 MR
Co 86249MR
Co 86037R
Co 7314MR
Co 87268MR
Co 87263R
Co 86250MR
88A162R
Co 94008MR
Co 85002S
Co 8371S
CoC 90063HS
Co 6304S
Co 1148S
CoC 671HS
CoJ 94192HS
CoC 85061HS
Co 7204S
CoC 92061HS
Bo 91R
0
0.1