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Molecular Markers for Smut Resistance in Mutated Population of Sugarcane Clone GT-54-9 through Amplified Fragment Length Polymorphism (AFLP)

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Abstract

Eight AFLP selective primer combinations were used to detect smut resistant markers in different sugarcane resistant and susceptible sugarcane mutants generated from sugarcane variety GT 54-9 using Gamma radiation. AFLP amplification using the eight primer pairs resulted in a total 390 scorable bands with an average of 48.75 bands per primer combination. The selective primer combination E-ACC/*Tru*-CTG was the only one able to distinguish between the resistant and susceptible mutants by the amplification of two bands ≈ 110-120 bp bands in resistant sugarcane mutants.

Keywords: *Molecular markers; AFLP; Sugarcane; Smut resistance; Ustilago scitaminea*

Introduction

Smut disease is an important economic disease of sugarcane in all sugarcane producing countries. The causal organism, *Ustilago scitaminea* Syd.; (*Sporisorium scitamineum* (Syd.) M. Piepenbr., M. toll & Oberw.) was first reported in Natal in 1877 in South Africa and now occurs in most of the sugarcane producing areas of the world [1]. The disease aggressively and dramatically affects plant growth and cane yield [2].

Molecular markers are powerful tools but may differ with respect to essential features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost effectiveness [3]. The developments in molecular technology in the last two decades of the 20th century led to the Innovation of a powerful molecular markers such as restriction fragment length polymorphisms (RFLPs) [4], randomly amplified polymorphic DNAs (RAPDs) [5]; microsatellites or simple sequence repeats (SSRs) [6,7]; amplified fragment length polymorphisms (AFLPs) [8] and multilocus sequence typing (MLST) [9].

The pedigree records and phenotypic traits are the only methods use in the assessment of the genetic diversity in sugarcane germplasm. To optimize and improve the breeding programs a more accurate method such as molecular markers need to be used in screening and evaluating the genetic diversity in sugarcane. Also, it will help to generate unique genetic fingerprints to different sugarcane varieties and increase the information about its genetic relationships on the molecular levels.

The AFLP technique developed by Pieter *et al.* [8] is used to visualize numerous amplified DNA restriction fragments simultaneously. AFLP technique has a number of advantages:

i. Minimal amounts of gDNA are required,
ii. No prior genome sequence data are necessary for primer construction,
iii. Markers are randomly distributed throughout the genome, and
iv. Many bands are generated that potentially provide a large number of polymorphisms [10,11].

The use of AFLP markers for determining genetic diversity and mapping in sugarcane has been assessed by [12,13]. Besse *et al.* [12] have used these markers as a to ol to provide a preliminary characterization of the diversity levels in wild collections of members of the *Saccharum* complex. Progress has been made in studies of the molecular basis of sugarcane smut resistance [14,15]. Thokoane and Rutherford [14] used cDNA-AFLP technique to detect differential gene action in smut resistant and susceptible sugarcane genotypes. Sequence homology analysis showed that a putative chitin receptor kinase, a Pto ser/thr protein kinase interactor, and an active gypsy-type LTR retrotranspon expressed in the resistant variety in response to challenge by the smut organism. In the current study, we are trying to measure AFLP sensitivity in detecting a smut resistant marker in different sugarcane resistant and susceptible sugarcane mutants generated from sugarcane variety GT 54-9 using Gamma radiation.

Materials and Methods

Plant material

Ten smut resistant sugarcane clones were obtained from Sugar Crops Research Institute, Giza, Egypt (GT 54-9, C9/0.5Kr-11, C9/0.5Kr-32, C9/0.5Kr-42, C9/0.5Kr-46, C9/1Kr-3, C9/2Kr-1, C9/3Kr-63, C9/3Kr-69 and C9/3Kr-8) and 10 susceptible clones (C9/2Kr-19, C9/2Kr-4, C9/3Kr-38, C9/3Kr-45, C9/3Kr-47, C9/3Kr-52, C9/3Kr-56, C9/3Kr-70, C9/1Kr-3 and C9/3Kr-79). All clones were selected from a former mutation induced clones by gamma radiation to the moderately resistant sugarcane cultivar GT 54-9.

Encore Adaptor	*Tru* 9I Adaptor
(*Eco*RI-1) 5'-CTCGTAGACTGCGTACC-'3	(*Tru*9I-1) 5'-GACGATGAGTCCTGAG-'3
(*Eco*RI-2) 5'-AATTGGTACGCAGTCTAC-'3	(*Tru*9I -2)5'-TACTCAGGACTCAT-'3

Table 1: *Structure of EcoRI and Tru9I adaptors.*

The oligonucleotides of each adaptor were mixed separately with buffer mixture as in Table 2. The final volume for both adaptor mixtures should be approximately 200 µl. Aliquot (50 µl) of the adaptor mixtures was transferred to 200 µl thin wall PCR tubes and placed in a thermocycler MJ-PTC-200 and run the following program. 95°C for 2min then 95°C decreasing to 25°C by 1°C per 1 min intervals. After the end of the thermocycler program, the adaptor mixtures were stored in -40°C.

Eco RI adaptors (5 µM (conc))	*Tru* 9I adaptors (50 µM (conc))
10 µl 100 µM RI. sequence 1	100 µl 100 µM *Tru* 9I. sequence 1
10 µl 100 µM RI. sequence2	100 µl 100 µM *Tru* 9I. sequence 2
2 µl 1 M Tris-HCl (pH 8.0)	2 µl 1 M Tris-HCl (pH 8.0)
2 µl 5 M NaCl	2 µl 5 M NaCl
0.4 µl 0.5 M EDTA	0.4 µl 0.5 M EDTA
175.6 µl ddH_2O	

Table 2: *Mixtures of preparing RI and Tru 9I adaptors.*

AFLP reaction

Two methods were used for restriction/ligation reaction. In the first method digestion and ligation were done in three separate steps while, in the snd method, the restriction/ligation reaction was done in one step.

In the first method, 5 µl genomic DNA (100-250 ng) digested by 5 units of *Tru* 9I (SibEnzyme Ltd., cat.#E199) at 65°C for 2hrs. in 1x SE-buffer W in a total volume 20 µlthen 2.5 µlof 10x SE-*Eco* RI buffer and 2 µlof BSA 10 mg/ml and 5 units of *Eco* RI (SibEnzyme Ltd., cat.# E057) to make the total reaction 25µl. The mixture incubated at 37 °C for 2hrs, after the incubation the enzymes were killed by incubating the reactions at 70°C for 15 min. For ligation, 12.5 µl of restriction reaction were added to 2.5 µl 10X SE-T4 ligase buffer containing ATP (10 mM), 0.5 µl *Eco* RI adaptor (10 µM), 0.5 µl *Tru* 9I (10 µM), 1 unite T4 Ligase (SibEnzyme Ltd., cat.#E319) and sterile HPLC grade H_2O up to 25 µl. The reaction incubated at 37°C for 3hrs.

In the second method, 5 µl genomic DNA (100-250 ng) was subjected to restriction and ligation in one reaction. The restriction ligation reaction consisted of, 1 µl 10x modified Tango buffer (330 mM Tris acetate, 10 mM Magnesium acetate, 66 mM Potassium acetate, 0.1 mg/ml BSA and 50 mM dithiothreitol (DTT), pH 7.9), 5 units Tru 9I, 5 units *Eco* RI, 0.5 µl *Eco* RI adaptor (10 µM), 0.5 µl *Tru* 9I (10 µM), ATP (10 mM) and 1 unit T4 Ligase, the reaction brought up to 25 µl using sterile HPLC grade H_2O). The reaction incubated for 3 hrs, at 37°C, and then killed by incubating the reaction at 70°C for 15 min.

Thermocycler MJ-PTC-200 with a hot lid was used in incubating the reactions in the two methods. Following the incubation, the digestion/ligation reactions were diluted (1:9 dilution using sterile HPLC grade H_2O) and vortexed then stored in -80°C.

Preselective PCR

Pre-selective PCR was performed using Preselective primers carrying a single 30-selective nucleotide. Core sequences of the *Eco* RI and *Tru* 9I primers (Table 3) are described in [8].

Primer Name	Sequence
Eco RI+A	5′-gactgcgtaccaattca-3′
Tru 9I+C	5′-gatgagtcctgagtaac-3′

Table 3: *AFLP Eco RI+A and Tru 9I +C preselective primers.*

PCR reactions were made in 25 µl reaction mixture consisted of 30 ng primer *Eco* RI+A, 30 ng primer *Tru* 9I+C, 1x PCR Buffer, 0.2 mM dNTPs, 1.5 mM Mg Cl_2, 2 units of BioReady rTaq DNA polymerase (BioFlux cat. BSA12M1) and 5 µl of the diluted ligation reaction mixture.

Amplification was performed in a MJ Research thermal cycler, the PCR program was 11 cycles of 94°C for 30s, 65°C (-0.7 °C/cycle) for 30s, 72°C for 1 min. then 22 cycles of 94°C for 30s, 56°C for 30s 72°C for, 1 min. and finally one cycle at 60°C for 30 min. After the PCR reaction a 5 µl aliquot from each reaction were mixed with 6x loading buffer and run in 1.2% agarose gel (containing ethidium bromide) in TEA buffer at 100 volts for 30 minutes to check the amplification of the restricted fragments. The pre-amplification PCR products were diluted 1:5 with sterile HPLC grade H_2O and stored in -40°C.

Selective PCR

Selective PCR was carried out using selective primers (Table 4) carrying three 3′-selective nucleotides. Primer combinations used (Table 5) were previously identified as being suitable combinations for sugarcane [12].

PCR reactions were conducted in 200 µl PCR tubes in a total reaction volume 20 µl. PCR master mix contained the tested selective primer combination were prepared according to Table 6 then aliquot of 15 µl of master mix, 5 µl of diluted pre-selective PCR reaction were added to the reaction mixture.

Eco RI primers	Sequence	*Tru* 9I primer	Sequence
Eco-ACA:	5′-gactgcgtaccaattcaca-3′	*Tru* 9I -CTG	5′-gatgagtcctgagtaactg-3′
Eco-ACC:	5′-gactgcgtaccaattcacc-3′	*Tru* 9I -CTA	5′-gatgagtcctgagtaacta-3′
Eco-ACG:	5′-gactgcgtaccaattcacg-3′	*Tru* 9I -CTC	5′-gatgagtcctgagtaactc-3′
		Tru 9I -CTT	5′ -gatgagtcctgagtaactt-3′

Table 4: *EcoRI and Tru9 three nucleotides selective primers and its sequences.*

	Eco RI-ACG	*Eco* RI-ACC	*Eco* RI-ACA
Tru 9I -CTG		x	
Tru 9I -CTA	x		x
Tru 9I -CTC	x	x	x
Tru 9I -CTT		x	x

Table 5: *AFLP primers combinations used in the present work.*

Reagents	µl/one rxn	Final conc.
Water	8.1µl	-
10X PCR buffer (w/15 mM $MgCl_2$)	2.0µl	1X (1.5 mM $MgCl_2$)
5 mM dNTPs	0.4µl	200 µM each
Eco RI I -A## primer (1µM)	1.0µl	0.046 µM
Tru 9I -C## primer (5µM)	1.0µl	0.275 µM
DNA polymerase (5 U/µl)	0.4µl	2 U/rxn
Total	15.0µl	

Table 6: *Composition of PCR master mix for the selective AFLP reaction.*

PCR tubes were transferred to thermocycler MJ-PTC-100 which adjusted to the program consisted of 12 cycle 94°C 30 sec; 65°C 30 sec decrease by 0.7°C/cycle and 72°C 1min then 30 cycles of 94°C 30 sec 56°C 30 sec 72°C 1 min. A final extension at 60°C 30 min. after the PCR the reactions a 5 µl aliquot from each reaction were mixed with 2 µl of 6x loading buffer and run in 1.2% agarose gel (containing ethidium bromide) in TEA buffer at 100 volts for 30 min to check the amplification of the restricted fragments before polyacrylamide electrophoresis. All the PCR reaction kept on -20°C.

Gel migration

The reaction was stopped by adding an equal volume of loading dye (98% formamide, 10 mM EDTA pH 8.0, bromophenol blue 0.25%, xylene cyanol 0.25%). The mixture was denatured for 3min at 90°C and stored on ice. Subsequently, 8 µl per sample was loaded on a 5% acrylamide/bis acrylamide (19:1) and 7.5 M urea gel, in 100 mM Tris, 100 mM boric acid, 2 mM EDTA, pre-run for 10 min at 140 W. The gel runs for 2 h at 140 W. The 46 samples were run on the same gel, with one gel run per primer combination.

Silver Staining

An improved photochemical derived methods for protein silver staining proposed by [17] with slight modification. All procedures were performed at room temperature with a constant agitation of the bath while all the staining steps were performed. After the completion of staining procedure, the gel was rinsed twice in water over 10 min and left to dry vertically overnight.

The dried gel was preserved at room temperature; the gel scanned using the Adobe Photoshop™ program (Adobe Systems, Mountain View, Ca, USA). The Adobe Photoshop program was used as needed to increase the contrast between bands and background staining.

Results and Discussion

The second method used for digestion ligation reaction gave a better result than the first method where the DNA digested in 2 stages with each restriction enzyme then the ligation reaction performed (Figure 1).

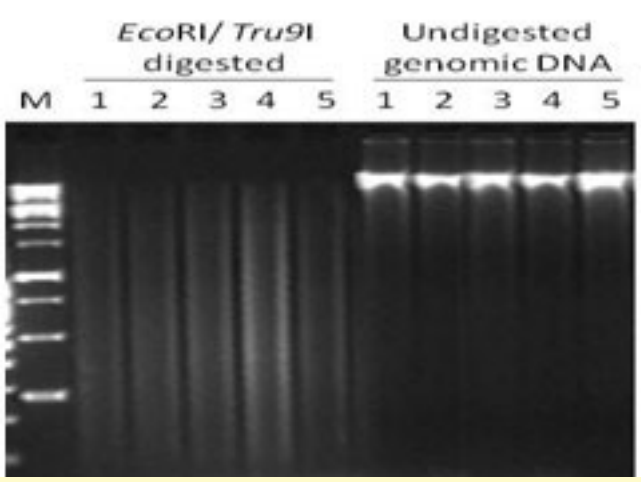

Figure 1: *Restriction digestion reaction of resistant and susceptible mutants using restriction enzymes Eco RI and Tru 9I M:1kb.*

After the restriction ligation reaction samples were amplified using the pre-selective primers to amplify those DNA fragments which ligated to the 2 enzyme adaptors. The preselective amplification reaction showed a good amplified band when separated on 1.2% agarose gel (Figure 2).

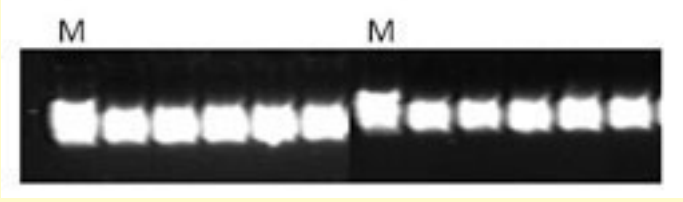

Figure 2: *Preselective AFLP reaction of digested resistant and susceptible DNA mutants. M = Molecular weight marker.*

AFLP amplification using the eight primer pairs resulted in a total 390 scorable bands (Table 7) with an average of 48.75 bands per primer combination.

Among the tested 8 selective primer combinations, the selective primer combination E-ACC/*Tru*-CTG was the only one able to distinguish between the resistant and susceptible mutants by the amplification of 2 bands ≈ 110-120 bp bands in resistant varieties (Figure 3).

Primer combination (*Eco* RI/*Tru* 9I)	Number of amplified bands
ACC/CTT	60
ACC/CTG	51
ACC/CTC	42
ACA/CTA	43
ACA/CTC	43
ACA/CTG	56
ACG/CTA	50
ACG/CTC	46
Total	390

Table 7: *Amplified bands obtained using eight selective AFLP primer pairs.*

The dendrogram generated from AFLP profile of the primer pairs E-ACC/*Tru*-CTG is shown in Figure 3 divided the ten resistant and susceptible sugarcane mutants into 2 major groups. Three of the sensitive mutants grouped under the first group while, the second group resolved in 2 distinct subgroups the first subgroup only included one susceptible mutant and the second one divided in 2 sub-sub groups the first one contained only one susceptible mutant while all the resistant mutants grouped under the second one in different levels.

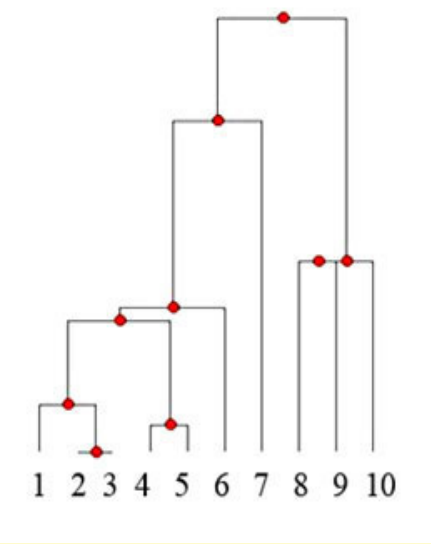

Figure 3: *AFLP profile and dendrogram showing genetic relationship among the smut resistant (lane 1-5) and susceptible (lane 6-10) sugarcane mutants based on AFLP primer combination E-ACC/Tru-CTG.*

This study revealed a high similarity among the tested sugarcane mutants; this is likely because all the mutants generated from the same sugarcane variety.

Amplified fragment length polymorphism relies on the selective amplification of a subset of DNA fragments from a more complex template pool that is created by ligation of adapters to restriction fragments [8]. The advantages of AFLP are: high reproducibility, high PCR multiplex ratio, amenability to any genome complexity, the possibility of generating a virtually infinite number of markers and the fact that no prior sequence information is required [18].

A few recent studies have identified several AFLP markers linked to resistance through QTL mapping [19,20]. Molecular markers can facilitate the development of smut-resistant lines via marker-assisted selection in sugarcane breeding. Recently, a number of AFLP markers associated with smut resistance were identified in an association mapping study of 154 sugarcane lines. These markers combined were found to account for up to 59% phenotypic variation for smut, and individually accounted for greater than 7% of the variance in disease resistance ratings [21]. Yu-hui *et al.* [22] conducted AFLP analysis for the mutants and reported that there were polymorphisms between mutants and the donor and the numbers of loci for the polymorphism were different among the mutants and many mutants had more than one mutation loci.

Conclusion

The present study suggests that AFLP can detect markers for disease resistance. Because of the high complexity of sugarcane genome it is critical to design and use different AFLP systems using different restriction enzymes to increase the chance of identifying selective resistant markers for smut disease. On the other hand, the use of more sensitive approaches such as cDNA differential display methods with AFLP and RAPD will be very helpful to identify resistance genes and markers.

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