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Development of sequence characterized amplified region (SCAR) marker for identifying drought tolerant sugarcane genotypes

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Abstract

A sequence characterized amplified region (SCAR) marker was developed for screening drought tolerant genotypes in sugarcane. Of 150 RAPD decamer primers, used to identify polymorphism amongst five sugarcane genotypes (3 drought tolerant and 2 drought sensitive), 12 were found to be polymorphic. A 724 bp DNA fragment was amplified through PCR with primer OPAK 12 in drought tolerant genotypes ROC22 and GT 18, while it was absent in both sensitive genotypes. The DNA fragment was purified, cloned and sequenced. The DNA sequence of the RAPD marker OPAK 12_{724} was used to generate SCAR marker, which was used for screening 23 genotypes, of which 13 were tolerant and 10 were susceptible to drought stress. SCAR amplification in 12 tolerant sugarcane genotypes, including one tolerant wild species, showed 92% specificity of marker and confirmed its location at the same loci as that of RAPD marker OPAK 12_{724}. The amplified fragment showed 91% homology with *Saccharum officinarum* cDNA sequences identified in SUCEST project. However, no other known gene sequence in GenBank was found to show 100% similarity with this sequence. Possibly, it might be related to some conserved sequences of sugarcane genome, still unidentified for their genetic function. High effectiveness in the detection of drought tolerant genotypes, as well as wild species of sugarcane, showed that this marker is appropriate in assisting selection of genotypes with drought tolerance.

Keywords: Drought; RAPD; SCAR; Sugarcane; Susceptible; Tolerant.
Abbreviations: RAPD_Randomly Amplified Polymorphic DNA; SCAR_Sequence Characterized Amplified Region; SUCEST_Sugarcane Expressed Sequence Tags Project.

Introduction

Currently, water stress (drought) is one of the most important environmental stresses affecting crop productivity worldwide and, largely, its magnitude and intensity are on the rising trend. It influences all stages of sugarcane growth and development and affects yield and sugar content (Shrivastava and Srivastava, 2006). Thus, it becomes imperative to view mitigation of drought induced damage in an integrated way to sustain productivity. Breeding drought tolerant sugarcane genotypes with an increased ability to survive and grow under drought conditions is an important component of this strategy. Generally, plant tolerance is a measure of the plant's ability to survive and even to grow proactively in the presence of stress. Efforts have been made to develop drought tolerant genotypes which, after being integrated into appropriate crop management programs, sustain sugarcane productivity. Normally, drought tolerant sugarcane genotypes are selected depending upon certain well defined morphological and physio-biochemical characteristics related to tolerance under natural field conditions (Zhou et al., 2011). Hybrid genotypes or clones possessing tolerance to drought at a specific level are used in hybridization to develop tolerant varieties. Screening of genotypes for a particular trait by traditional methods is a time-consuming and relatively laborious process. The use of molecular markers in breeding programs, during recent years, has been proved as an efficient tool in this regard. With the development of the random amplified polymorphic DNA (RAPD) method (Williams et al., 1990), random segments of a genome can be sampled and, thereby, genetic traits which differ between different genotypes can be compared. However, the RAPD procedure has several disadvantages, the main one being sensitivity to reaction condition that reduces reproducibility of the results obtained by different laboratories. Notwithstanding, the results can be used to identify polymorphism, and once a set of amplified regions have been secured, sequence characterized amplified region (SCAR) markers can be developed to produce reliable unique PCR-based results from the polymorphic RAPD markers (Paran and Michelmore, 1993). This methodology has been extensively utilized in the identification of various plant species (Choi et al, 2008; Devaiah and Venkatasubramanian, 2008a and b; Dhanya et al., 2009; Ladhalakshmi et al., 2009; Ray and Roy, 2009), pathotypes (Chen et al., 2010; Sudisha et al., 2009), and sex identification (Kim et al., 2008; Liu et al., 2009). In general, SCAR markers not only help in selecting the particular traits, such as seedlessness in grapes (Li et al., 2010), polyembryony (Kang et al., 2008) and detection of off-types and dwarf plants in banana among

micropropagated plantlets, but they also help in a breeding program for selection of male fertility (Ashutosh et al., 2007) and male sterility genes (He et al., 2009; Lee et al., 2010). The utility of SCAR markers in identifying the resistance to biotic stresses, such as bacterial wilt in egg plant (Cao et al., 2009), club-rot disease in cabbage (Hayashida et al., 2008), rust in Asparagus (Li et al., 2007), rust in common bean (de Souza et al., 2007), Gall Midge in rice (Sardesai et al., 2002), root-knot nematode in pepper (Wang et al., 2009), topovirus in tomato (Dnyaneshwar et al., 2006), covered smut in barley (Ardiel et al., 2002), etc., have been well exploited in breeding disease tolerant genotypes. In sugarcane also, a SCAR marker linked to smut resistance gene has been identified and utilized in the breeding program (Que et al., 2008). However, until now, the applicability and authenticity of SCAR markers have been found to be limited in the case of biotic stresses or for the identification of a particular trait corresponding to a single gene. The difficulties in selection procedures and long duration of the sugarcane crop necessitate the development of a technique to identify stress tolerant genotypes. SCAR markers, by being both accurate and cost efficient, offer the most practical method for screening numerous samples in a time and labor-saving manner (Kasai et al., 2000). Because of multi-species origin of sugarcane, it is thought to have one of the most complex plant genomes carrying variable chromosome numbers with a commensurately large DNA content. This complexity complicates the application of conventional genetics and breeding techniques. The present study is an attempt to develop a reliable and reproducible molecular marker which can be used as a selection tool to facilitate the sugarcane varietal breeding program for drought tolerance.

Results

Screening of RAPD primers

Out of 150 decamer RAPD primers screened, 12 primers produced polymorphic and reproducible amplification profiles for all five genotypes. Primer OPAK12 was selected, which consistently showed polymorphic bands in all the genotypes. In drought tolerant genotypes ROC 22 and GT 18, it also primed the amplification of a distinct DNA fragment of 724 bp which was absent in susceptible genotypes.

Development of SCAR Marker

The 724 bp DNA fragment was excised and amplified. The DNA was extracted from excised band, purified, cloned and confirmed by the bacteria solution PCR. The obtained sequence was 724 bp in length with 46% GC content (A-185; T-206; C-195; G-138), and the first ten nucleotides, of the sequence obtained, matched completely with the sequence of OPAK12 (Fig 1). Based on this sequence, a pair of primers (SCAR marker) was designed, namely, SCOPAK12F (5'-AGTGTAGCCCC ACACCA AGAC-3) and SCOPAK12R (3'-TTGACCTACCCA CCCGATGTGA-5). These primers were made to sit astride the RAPD amplicons corresponding to the RAPD primers and the internal sequence of each RAPD fragment. The annealing temperature of these primers was tested using DNA from different genotypes, and the best results were obtained at 60^{o}C.

Screening of sugarcane genotypes

The screening of 23 sugarcane genotypes using SCAR marker showed amplification of specific 724bp DNA fragment in 12 tolerant sugarcane genotypes including one tolerant wild species (Fig 2) which confirmed that SCAR marker SCOPAK12 is present at the same loci as that of RAPD marker OPAK 12_{724} and is probably linked to the unknown drought tolerance gene. The amplified SCAR marker from different drought tolerant genotypes were purified, cloned and sequenced again. These sequences were checked against the initial RAPD sequences and were found to be identical to them. Sequence of the SCAR amplified amplicon was submitted to GenBank (Accession number HQ529598).

Discussion

Since Epstein and Rains (1987) have outlined the steps for a general program for developing stress tolerant plants using biotechnological approaches, a great deal of work has been done, exploiting the genes from sugarcane or different organisms. The work on use of marker assisted selection in sugarcane is scarce and needs to be practised more in order to save time and breeding efficiency. Our studies resulted in the development of a marker SCOPK12, for the first time in case of drought tolerance, which may be useful in breeding programs tailored to breed drought tolerant sugarcane genotypes. Amplification of this specific marker in wild sugarcane species *Saccharum robustum*, as well as in high yielding drought tolerant commercial genotypes, indicated that it has not been affected by the environmental stress during the long course of exploitation. Practically, if the heritability of a selected trait is higher than the heritability of productivity, then the trait may be less influenced by the environment and could be used as a selection criterion (Quizenberry, 1982). We have identified a RAPD marker OPAK 12_{724} (AGTGTAGCCC) and the corresponding SCAR marker which might be closely linked to a drought tolerance gene in sugarcane commercial genotypes and wild species. Drought tolerant sugarcane genotype ROC22, GT 18 and other tolerant genotypes showed a single specific band on PCR amplification using SCAR marker. Using BLAST program, it was found that this marker had 91% homology with *S. officinarum* cDNA sequences (CA265131) identified in the SUCEST program. But, no other GenBank sequences showed similarity with this sequence, which may relate to some conserved unexpressed sequences in the sugarcane genome, still unidentified for its genetic function.

Unexpectedly, the SCAR marker was found in one drought sensitive genotype Gannan 95-108, but was absent in one drought tolerant genotype GT 21. The possible reasons for this are, primarily, the fact that most cultivated sugarcane are *Saccharum* spp. hybrids, selected at distant geographical regions and containing a wide range of germplasm, and secondly, the presence of newly developed SCAR marker near a gene which has been separated during recombination or on a gene which has been mutated during the long course of cultivation. However, the SCAR marker developed here showed 92% specificity. The genotypes tested by the SCAR marker have previously been identified for drought tolerance or susceptibility by various workers (Table 1). Generally speaking, the SCAR markers have been found accurate and efficient in case of biotic stresses or in screening of the gene specific traits. The plant's responses to abiotic stresses are temporary and controlled by overexpression or suppression of the genes. There may be some possibilities of this fragment for being a part of some specific gene that imparts tolerance to drought stress, for which the studies are under progress.

Table 1. Sugarcane commercial and wild genotypes used to screen with the SCAR marker.

SN	Genotype	Tolerant (T)/ Sensitive (S) Reference	SN	Genotype	Tolerant (T)/ Sensitive (S) Reference
1	ROC1	T (Chen et al., 2007)	13	GT97-69	T[a]
2	GT96-44	T (Chen et al., 2007)	14	ROC16	S (Liao et al., 2005)
3	MT92-505	S (Chen et al., 2007)	15	GT21	T (Huang et al., 2006)
4	MT88-103	S (Chen et al., 2007)	16	Gannan 95-108	S (Chen et al., 2007)
5	F172	S (Huang and Mo, 1995)	17	Co 421	T[a]
6	Yacheng 82-96	T (Chen et al., 2007)	18	Ya 71-374	S (Chen et al., 2007)
7	Funong 81-745	T (Chen et al., 2007)	19	Gannan 92-12	T[a]
8	Guifu 96-25	T[a]	20	*Saccharum sinense*	S (Shrivastava and Srivastava, 2006)
9	Yacheng 90-33	T (Chen et al., 2007)	21	*S. barberi*	S (Shrivastava and Srivastava, 2006)
10	Yacheng 64-389	S[a]	22	*S. robustum*	T (Shrivastava and Srivastava, 2006)
11	Ke 5	S (Chen et al., 2007)	23	ROC22	T (He, 2009)
12	GT28	T (Zhou et al., 2008)			

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1 AGTGTAGCCCCACACCAAGACAACACACCTGAGTCAACTAACATGAAACACTGGACTCAC

61 AAGCC CATGCGAATTTCTCAATGAACA CAGAT TATTAGGAT GCTCTGG ATC GTCACTTCA

121 CT GGCTT TGAT TATATAA TTAAA CTGA TGGATTA TTGATATGA CTA GTG TATTGG CTCCT

181 CCACT TCTGTTTC AGACACCGAG GCTGTGGAGACCACTTCCTCAGTGACCTCATCAGCAT

241 CTTCTT TGTCAGAT AGAGAGGCTGTT GAGACCTC CTCCTCGT TAGTGATCTC ATCCTCCA

301 CTTCACTA TCGTACAG CGAGG CTGCT GAGACC TCCTC GTCGGTTACCTCATTCTCCTCTT

361 CTCCGC CAGAT AGTGA ATTAGC TGCTGAGAC CTCGTC CTCTGT CATCTCATCCTCCTCTT

421 CTGTAT CGTACAAT GAGGCT TCTGAGAC ATCCTCGT CAGTGAC CTCATCCTCATCCTCTG

481 CCTGG AATTAAC ATGTATTGT TATAT ATTGTTC AAAAAAA GAGAGCAGAAACCAAATACC

541 CTTCTATA TAAA TGAGAAC CTTAAAG CAGAGT GCTGTTCTCAGTTCCTCCAGCATTGCAG

601 CCAGCC GTGGATA TTCAGCC GTAAGTA TCTGGAGCG CCCGTTCCAAATCCTCAATATCCT

661 CGGTCT GTTGCC AGAAGCAT GCAGAGTACTTCCGTTAAATACAACTGGATGGGTGGGCTA

721 CACT

Fig 1. Nucleotide **s**equence of the specific fragment amplified from sugarcane genotype ROC 22 using RAPD primer OPAK12

Materials and methods

Experimental location

The experiment was conducted at Guangxi Academy of Agricultural Sciences (GXAAS), Nanning, Guangxi, P.R. China.

Materials for screening RAPD Primers

Single bud sets of five genotypes, of which three were drought tolerant (GT 21, ROC 22 and GT 18) and two drought sensitive (ROC 16 and GY 9), were planted using standard culture techniques under soil-pot culture system in an intelligent greenhouse. Upon germination, normal crop cultural practices were followed. At the elongation stage of plants, samples of last transverse marked (LTM) leaves were taken in three replicates and frozen in liquid nitrogen for DNA extraction. The DNA isolated from these leaves was used to screen RAPD primers.

Materials for screening SCAR marker

In the latter part of the experiment, leaves from 20 commercial genotypes and three *Saccharum* species differing in drought tolerance (Table 1) were collected from Sugarcane Germplasm Center, Guangxi Sugarcane Research Institute, GXAAS (Nanning, China) and screened for drought tolerance using a SCAR marker developed in this study.

DNA Extraction

Total DNA was extracted from sugarcane leaves by the method described by Srivastava and Gupta (2001).

RAPD-PCR

150 random decamer primers (Shanghai Sangon Co. Ltd., Beijing) were used for amplification. The reaction mixture (20 µL) contained 1x PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM primer, 0.7 U Taq DNA polymerase (Shanghai Sangon BE Technology and Services Co. Ltd., Shanghai, China) and 40 ng of template DNA. Amplifications were performed in a ThermoCycler (Biometra, Germany) using denaturing at 94^{o}C for 4 min, and 10 cycles of 94^{o}C for 1 min, 37^{o}C for 1 min, 72^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 1 min, 37^{o}C for 20 sec, 72^{o}C for 90 sec, and a final extension step at 72^{o}C for 10 min. PCR products were visualized on 2% agarose gel with ethidium bromide staining. The presence of polymorphic bands was confirmed by repeating PCR amplifications three times under the same conditions.

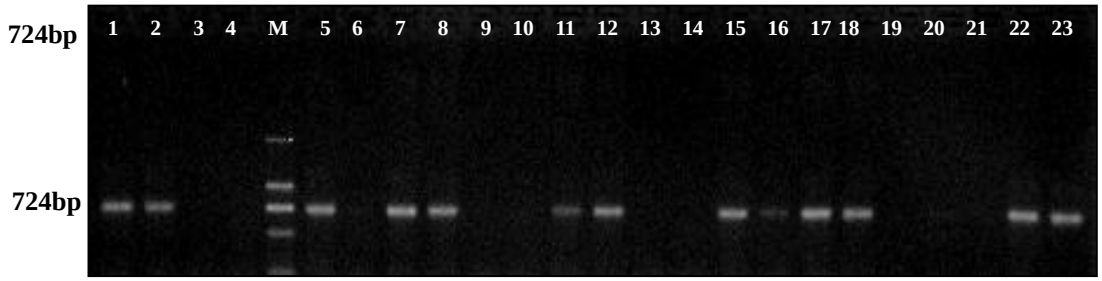

Fig 2. Screening of 23 drought tolerant and sensitive sugarcane genotypes with the SCAR marker (Forward: 5'-AGTGTAGCCCCACACCAAGAC-3'; reverse: 3'-TTGACCTACCCACCCGATGTGA-5') [1. ROC22(T), 2. *Saccharum robustum*(T), 3. *S.barberi* (S), 4. *S. sinense* (S), 5. Gannan 92-12 (T), 6. Yacheng 71-374 (S), 7. Co421 (T), 8. Gannan 95-108 (S), 9. GT21 (T), 10. ROC16 (S), 11. GT97-69 (T), 12. GT28 (T), 13. Ke 5 (S), 14. Yacheng 64-389 (S), 15. Yacheng 90-3 (T), 16. Guifu 96-25 (T), 17. Funong 81-745 (T), 18. Yacheng 82-96 (T), 19. F172 (S), 20. Mintang 88-103 (S), 21. Mintang 92-505 (S), 22.GT96-44 (T), 23.ROC1 (T)] [T-Tolerant; S-Susceptible]

SCAR marker development

The selected RAPD polymorphic bands were excised from agarose gel and purified using Biospin Gel Extraction Kit (Bioflux; Cat# BSC02M1; Beijing Biomars-Technology Co. Ltd., China) following the procedure mentioned in the user's manual. Purified DNA fragment was ligated into pUCm-T vector (Shanghai Sangon Co. Ltd., Beijing, China; Cat # SK 2211) following the procedure mentioned in the user's manual. The ligated vector was introduced into competent *Escherichia coli* cells (JM109; Shanghai Sangon Co. Ltd., Beijing, China) following protocol for transformation as described by Sambrook and Russell (2001). Eight white colonies were picked up with sterilized tooth pick and were cultured separately in the LB liquid medium (containing 100mg/L Ampicillin) at 37^{o}C for about 8 h. After culturing the cells, bacteria solution-PCR was performed to confirm the presence of inserted DNA fragments in clones. After confirmation, the bacteria solution was used for sequencing (Shanghai Sangon Co. Ltd., Beijing, China). Based on the DNA sequence of each RAPD amplicon, two oligonucleotides were designed as SCAR marker. Marker was designed in order to have (i) an experimental Tm equal or higher to 50^{o}C, (ii) more than 19 base pair length and (iii) a 3' end rich in GC bases. Where possible, SCAR marker was selected to sit astride the RAPD amplicons corresponding to the RAPD primers and the internal sequence of each RAPD fragment. The selected marker was synthesized by Shanghai Sangon Co. Ltd., Beijing, China. The SCAR amplifications were performed in 20 µL of reaction mixture containing 20 ng of template DNA, 1x PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM primer, 0.7 U Taq DNA polymerase (Shanghai Sangon BE Technology and Services Co. Ltd., Shanghai, China). Thermal cycling conditions were 94°C for 3 min, followed by 32 cycles at 94°C for 1 min, annealing temperature for 45 sec and 72°C for 90 sec; and a final extension at 72°C for 10 min. For each primer pair, the annealing temperatures were tested using DNA samples and highest temperatures were selected. SCAR amplifications were analyzed by electrophoresis of 10 µL of PCR products on 2% (w/v) agarose gel stained with ethidium bromide. The specificity of SCAR markers was tested using DNA from 23 sugarcane genotypes and species differing in sensitivity to drought.

Conclusions

Generally, the breeding for drought tolerant sugarcane genotypes involves a long procedure of identification and selection of morpho-physiological traits, *vis-à-vis*, yield and sucrose contents under field conditions. The present study is a successful demonstration of screening several commercial genotypes as well as wild species of sugarcane from different origins around the world for drought tolerance using SCAR marker. This suggests that the newly developed SCAR marker achieved its aim for the targeted objective. This study, for the first time, reports for the identification of SCAR marker for the abiotic stress, specifically, the drought. The SCAR marker developed in this study represents a new tool, easy to use by sugarcane breeders, in attempting to breed for drought tolerance in sugarcane, and will reduce the time needed to develop a drought tolerant sugarcane genotype.

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