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Mutation Identification in Sugarcane Somaclones Using Simple Sequence Repeat Markers (SSR)

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Abstract

There have been several reports suggesting that the tissue culture technique produced genetic variations of somaclones from a wild type plant. In this study, SSR markers was used to detect the genetic variation in regenerated plants from leaves and shoots via callus culture of sugarcane variety K84-200. DNA from 58 somaclones and the wild type plant K84-200 were isolated. Ten primers of simple sequence repeat (SSR) markers were used to determine mutation of somaclones. Among all ten amplified SSRs, 3 primers MCSA205C07, SMC226CG and SMC319CG showed different polymorphisms from the wild type plant. The clustering analysis separated 58 somaclones and the wild type plant into 2 groups. The wild type is in group A while group B was different from the wild type in regards to DNA pattern and sequence variations including insertions, deletions, and substitutions in somaclones. This result showed that the somaclonal variation technique can induce genetic variation from the wild type. SSR markers and sequencing of SSR polymorphic bands are a useful tool for detection of genetic variations in somaclones.

Introduction

Sugarcane (*Saccharum* sp. hybrids) is one of the most important industrial crops in tropical and subtropical regions of the world and is the principal raw material for the sugar industry. Breeding for improved cultivars of sugarcane is difficult because of the complexity of the genome and the long duration required for breeding. Tissue culture techniques are widely used in sugarcane improvement programs (Eldessoky et al., 2011). The phenotypic change in the regenerated plants is called somaclonal variation (Larkin & Scowcroft, 1981; Oono et al., 1984). In sugarcane, variations can be created through tissue culture. An understanding of the molecular nature and mechanisms of somaclonal variation is important to evaluate the background

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mutations in plants and to utilize somaclonal variation efficiently to obtain new lines for crop breeding. Among different classes of molecular markers, simple sequence repeat (SSR) markers have a variety of applications in plant breeding and genetics because of their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage (Powell et al., 1996). In sugarcane, SSR markers have been frequently used in sugarcane improvement for many purposes, including genome mapping, gene tagging, association mapping, genetic diversity analysis, varietal identification and purity testing (Cordeiro et al., 1999; Cordeiro et al., 2001; Cordeiro et al., 2003; Pan et al., 2003; Rossi et al., 2003; Aitken et al., 2005; Govindaraj et al., 2005; Wei et al., 2006; Singh et al., 2007; Liu & Pan, 2011; Singh et al., 2011a,b). Several studies have reported the use of SSRs to assess somaclonal variation in plants (Chowdari et al., 1998; Rahman & Rajora, 2001; Rodríguez López et al., 2004; Ryu et al., 2007; Schellenbaum et al., 2008; Gao et al., 2009; Marum et al., 2009). Nonetheless, few studies have addressed the molecular basis or nature of somaclonal variation (Al-Zahim et al., 1999; Yang et al., 1999). In sugarcane, most of the research on somaclonal variation has focused on detection of genetic variability, but no studies have been conducted to assess somaclonal variation at the nucleotide sequence level. This research was aimed to determine if genetic change occurs in the new sugarcane somaclones at the nucleotide sequence level by using SSR and nucleotide sequence analysis.

Materials and methods

1. Plant materials

Sugarcane variety of K84-200, which is widely grown in many parts of Thailand, was used for this study. The shoots and young leaves of K84-200 were cut in small pieces and the surface was sterilized in 5% (v/v) and 10%(v/v) sodium hypochlorite with 0.1%(v/v) Tween-20 for 15 min, followed by three rinses with sterile distilled water for 5 min each. The shoots and the young leaves were cut to a length of approximately 0.5 cm x 0.5 cm and were cultured on MS medium (Murashige & Skoog, 1962) containing 3 $mg.L^{-1}$ 2,4-D for callus induction. The callus was cut in small pieces and subcultured in the same medium for 3 week periods until the callus had grown enough. The callus was transferred to culture on the MS medium without 2,4-D for shoot regeneration. Each first shoot was named and propagated. The shoots were transferred on root formation medium (MS medium add 0.4 $mg.L^{-1}$ IBA) for new complete somaclone plants. These somaclones were collected in the bottles prior to use for DNA extraction.

2. DNA extraction and polymerase chain reaction amplification

Young leaves from 58 somaclones and the wild type plant K84-200 were collected (Table 1) and ground in liquid nitrogen. Total DNA was extracted following a modification of the procedure of Dellaporta et al. (1983). A total of 10 SSR markers (Cordeiro et al., 2000, 2001) were used in this study. The primers' names and their sequences are listed in Table 2. The PCR reactions were carried out in 25 µL volumes in a mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP (Promega), 50 pmole each of forward and reverse primers, 75 ng of genomic DNA, and 1 unit of Taq DNA polymerase from Invitrogen (Brazil). The PCR amplification was performed in a MJ Mini Thermal Cycler PTC-1148 (Bio-Rad). For each amplification process, a preheating denaturation of DNA at 94ºC for 2 min was followed by 30 cycles consisting of 30 sec at 94ºC, 1 min at 55ºC (annealing), and 1 min at 72ºC (extension). A final incubation for 5 min at 72ºC was performed. Amplified DNA fragments were separated by 4% MetaPhor agarose electrophoresis and visualized by UV. φx-174/Hae III was used as the size standard DNA marker.

Table 1 The sugarcane somaclones used in the present study

No.	Somaclone Name	No.	Somaclone Name	No.	Somaclone Name
1.	K84-200*	21.	27L5N	41.	67CS5
2.	99S5M	22.	65L5N	42.	1Sh10
3.	10S5M	23.	77L5M	43.	52Sh10
4.	20S5M	24.	4L5M	44.	54Sh10
5.	9S5M	25.	83L5M	45.	57Sh10
6.	15S5M	26.	9L5N	46.	59Sh10
7.	58S5M	27.	33L5N-T	47.	92Sh10
8.	27S5N	28.	17L5M-T	48.	93S5NK
9.	69S5M	29.	54L5M-T	49.	30S10M
10.	75S5M-T	30.	93L5M-T	50.	12
11.	80S5M-T	31.	59L5M-T	51.	22
12.	93S5M	32.	10L10M	52.	27
13.	6L5M	33.	31L5M	53.	55
14.	2L5M	34.	93L5M	54.	AL5M
15.	9L5M	35.	CL5M	55.	63S5M
16.	30L5N	36.	36L5	56.	87S10M
17.	21L5M	37.	4S5	57.	31S10M
18.	57L5M	38.	29S5	58.	99S5N
19.	90L5M	39.	6CS5	59.	34S5M
20.	38L5M	40.	33CS5		

Remark: * The wild type plant

Table 2 List of SSR primers and their properties for studying in sugarcane somaclones

Marker code	Primers (5′-3′)	T_m (°C)	Type of repeat
MCSA068G08	F 5′CTAATGGCATGCCCCAGAGG3′ R 5′GCTGGTGATGTCGCCCATCT3′	57.2	$(CAG)_6$
MCSA077C02	F 5′CAACCACCTCGCTCGATTCG3′ R 5′TGATGAGCCAGCAATCCTCCT3′	59.1	$(AGG)_8$
MCSA175A08	F 5′CAGGGGCAATGCTCGATGGA3′ R 5′TGACACGCATGGGGTTGTCG3′	60.1	$(CGG)_6$
MCSA180E02	F 5′CAACGACGCAGGATCGAACC3′ R 5′AGCAGGCACGACTTCCCCAC3′	59.5	$(CCG)_5$
MCSA205C07	F 5′GCTACCAGCTCTCGGTGCTTC3′ R 5′GCACGGGCTAGAACCTAGAAGG3′	59.4	$(CGA)_5$
SMC226CG	F 5′GAGGCTCAGAAGCTGGCAT3′ R 5′ACCCTCTATTTCCGAGTTGGT3′	50	$(CA)_{10}$
SMC319CG	F 5′CCTTTCATCCACAGAGGACAG3′ R 5′GGTTCACCGAAGCAAGAGAAC3′	50	$(CA)_{17}$
SMC477CG	F 5′CCAACAACGAATTGTGCATGT3′ R 5′CCTGGTTGGCTACCTGTCTTCA3′	55	$(CA)_{31}$
SMC863CG	F 5′CGGTCGCTGTTGCATTGTAG3′ R 5′TGGATCACTCAATCTCACTTCG3′	55	$(TC)_9$
SMC1039CG	F 5′AGGTGAGAGTTCCTGGCTTTCCA3′ R 5′TGTGCTGGCAAGCCCCTACTT3′	55	$(TG)_{17}$

Remark: Cordeiro et al. (2000, 2001)

3. Data analysis

Amplified DNA bands detected after electrophoresis separation in each accession were scored using the binary number, "1" for the presence of an SSR product band and "0" of the absence for a product band of similar length. Only distinct bands were considered for analysis; faints bands were omitted. The resulting matrices of molecular data for all primers were submitted for analysis. The binary data contained in an Excel file was imported into the NT Edit module of NTSYS-pc, version 2.01 (Rohlf, 2000). The resultant similarity matrix was employed to construct a dendrogram using sequential agglomerative hierarchical nesting (SAHN) based on the unweighted pair group method with arithmetic means (UPGMA) (Sneath & Sokal, 1973) to infer their genetic relationships.

4. DNA cloning and sequencing

The SSR polymorphic fragments were purified by PureLink Quick Gel Extraction Kit (Invitrogen, Germany). The DNA fragments were ligated into a vector pGEM-T Easy using a pGEM-T kit (Promega), essentially as recommended by the manufacturer. *Escherichia coli (E. coli)* strain JM109 was used as the host for the plasmid. The clones were selected for sequencing. GenBank nucleotides databases were searched for sequences having homology sequences using BLASTN program (NCBI). Comparisons of all nucleotide sequences were performed using the CLUSTALW program.

Results and discussion

1. Somaclones regenerated via the callus induction

MS medium supplemented with 3 mg L^{-1} 2, 4-D could clearly induce calluses from small pieces of young leaf in 6 weeks (Fig.1A.). The grown calluses were transferred to shoot regeneration medium (MS medium without 2, 4-D) (Fig.1B). The first shoot regeneration was separated and named (Table1). These shoots were propagated before transferring to the root formation medium. There were 58 complete somaclone plants, which were collected in the bottles (Fig 1C).

2. Genetic diversity and phylogenetic Clustering analysis

Ten SSR primers were used for evaluation of somaclonal variation among the 58 somaclones. Eight of ten primers showed good amplifications, and two primers did not give any amplified products or gave poorly amplified products. Eight primers produced multiple fragments. The total number of scorable fragments (alleles) was 28, out of which 5 (17.85%) were

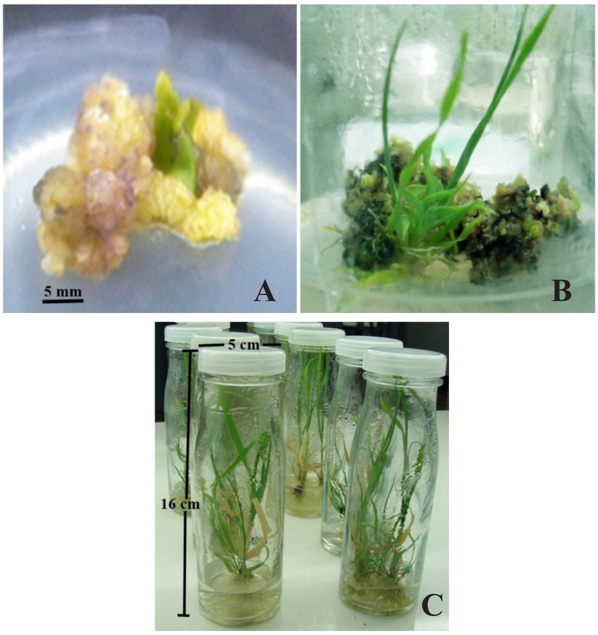

Fig. 1 (A) Callus induction from young leaf was cultured on MS medium with 3 mg L^{-1} 2,4-D (B) Shoot regeneration from callus culture on medium (MS without 2,4-D) (C) sugarcane complete somaclone plants.

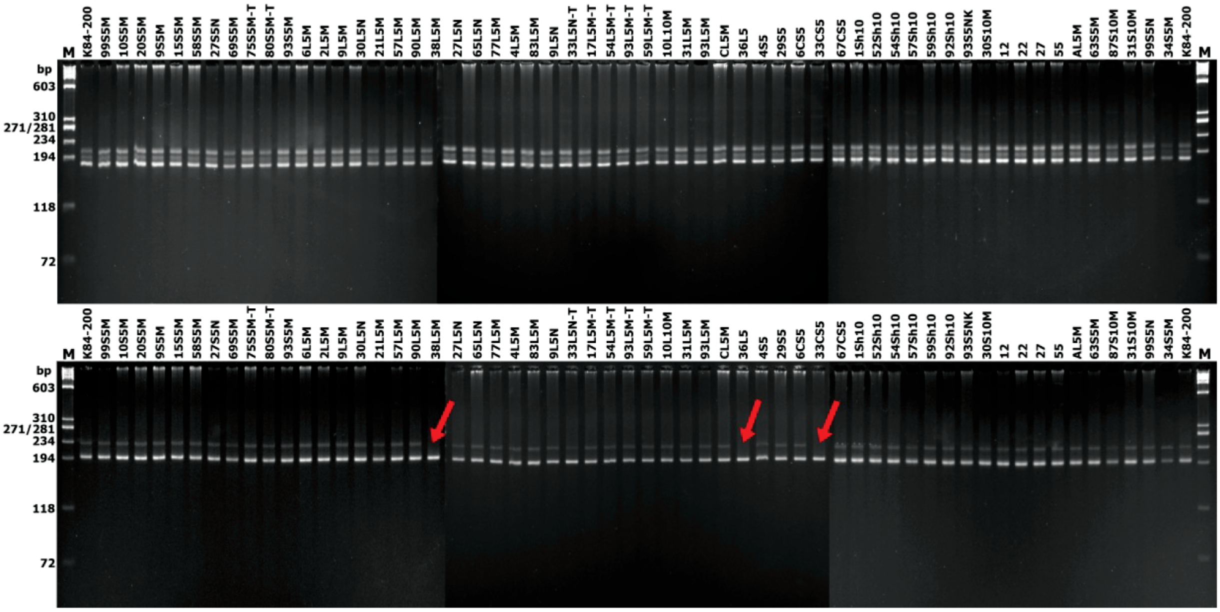

Fig. 2 Electrophoresis pattern of DNA amplified products obtained with primers (A) MCSA068G08 and (B) MCSA205C07. (Lane M=ϕx-174/*Hae* III, Lane). Fragments showing somaclonal variation are marked by arrows.

polymorphic and 23 (82.15%) were monomorphic. The number of fragments produced by various primers ranged from 2-7 (Table 3). The electrophoresis patterns of DNA amplified products obtained using primers MCSA068G08 (A) and MCSA205CO7 (B) are shown in Fig 2. The amplified products from primer MCSA068G08 showed that the DNA patterns of the 58 somaclones were the same as the wild type plant (A). The DNA pattern of the amplified products from primer MCSA205C07 (B) showed polymorphism in 3 somaclones, 38L5M, 36L5 and 33CS5. The polymorphic fragments were indicated by arrows (Fig. 2). Among all ten SSRs, 3 primers, MCSA205C07, SMC226CG and SMC319CG, showed polymorphisms of the somaclones from the wild type plant (Fig.4 A,B, and C), and seven of them were monomorphic. Cluster analysis of the somaclones was performed using similarity coefficient to generate a dendrogram. The overall genetic relationships among the somaclones and the wild type plant K84-200 were studied. The somaclones and the wild type plant K84-200 could be separated into 2 groups (Fig. 3). The genetic similarity values varied from 0.86 to 1.00. Group A contained 55 somaclones and the wild type plant K84-200, and group B contained 3 somaclones: 38L5M, 36L5 and 33CS5 (Fig. 4). Two major fragments were amplified using primer MCSA205C07 (Fig. 4A, bands 1A and 2A). The wild type plant contained all the two bands. Band 2A was shared by all 3 somaclones (38L5M, 36L5 and 33CS5) and the wild type plant. On the other hand, there were 4 fragments amplified by primer SMC226CG (Fig. 4B, bands 1B, 2B, 3B and 4B). Bands 1B, 3B and 4B were amplified in all genotypes and band 2B was amplified only in the wild type plant. Primer SMC319CG produced five fragments (Fig. 4C, bands 1C, 2C, 3C, 4C and 5C). Band 1C and 4C were amplified in all genotypes. Band 2C and 5C were amplified only in the wild type plant and band 3C was absent only in the wild type plant. All the bands in Fig.4 were cloned and sequenced.

Table 3 Numbers of polymorphic bands generated from 58 sugarcane somaclones and the wild type plant

Primer	Polymorphic	Monomorphic	Total no. of allele
MCSA068G08	0	3	3
MCSA077C02	0	0	0
MCSA175A08	0	2	2
MCSA180E02	0	7	7
MCSA205C07	1	1	2
SMC226CG	1	3	4
SMC319CG	3	2	5
SMC477CG	0	2	2
SMC863CG	0	3	3
SMC1039CG	0	0	0
Total	5 (17.85%)	23 (82.15%)	28

In most primers, polymorphic bands were absent in the DNA pattern of the variable regenerated plants when compared to that one of the wild type plant. Amplification differences (i.e. absence or presence of a band) can be the result of base substitution, base deletion or base insertion, and result in a situation suitable for detecting possible mutation sites.

The SSR technique has been used for identification of somaclonal variation and their relationship in sugarcane (Cordeiro et. al., 2003; Thumjamras et al., 2011), olive (Noormohammadi et al., 2014), oil palm (Inpuay et al., 2012), cotton (Jin et al., 2008) and rice (Gao et al., 2009). The results show that the SSR marker could be clearly identified. Similar results have also been reported with SSR markers that have been used to assess somaclonal variation in many plants.

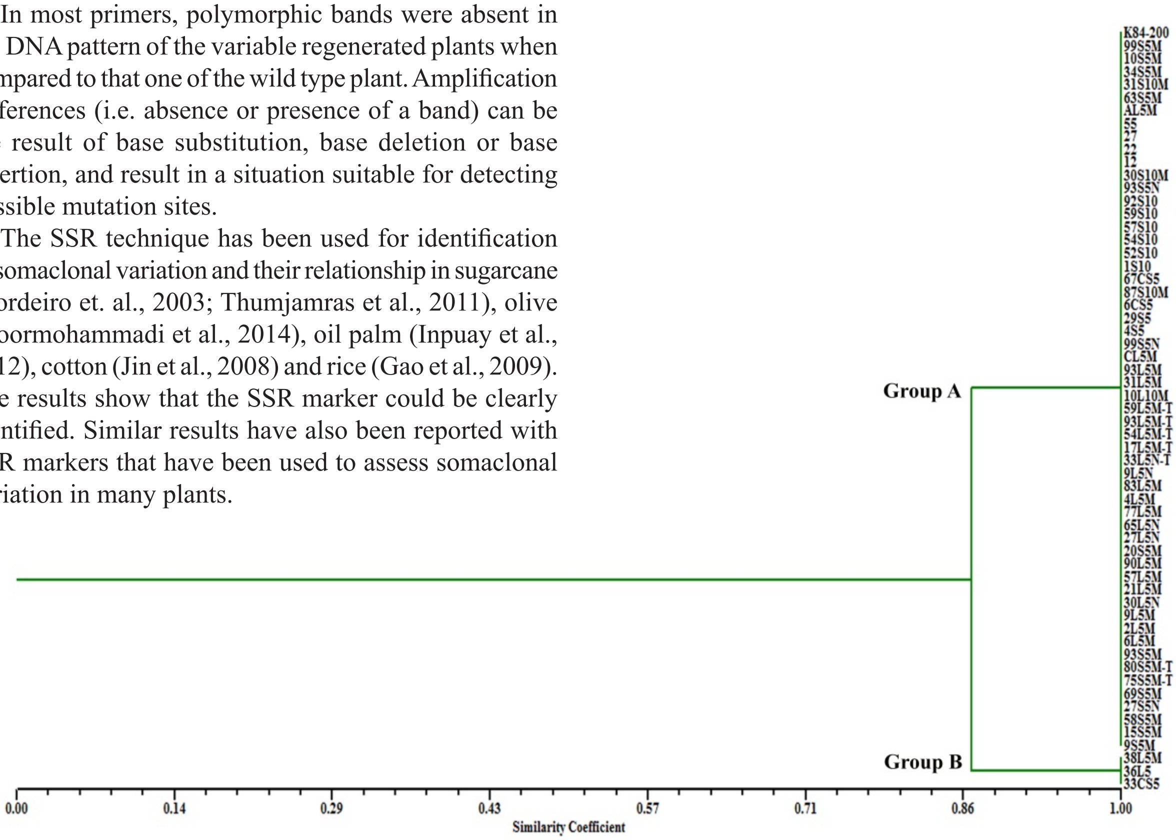

Fig. 3 Dendrogram based on the estimation of genetic similarity coefficients and UPGMA clustering presenting the relationships between 58 plants regenerated and wild type plant K84-200, as revealed by SSR markers.

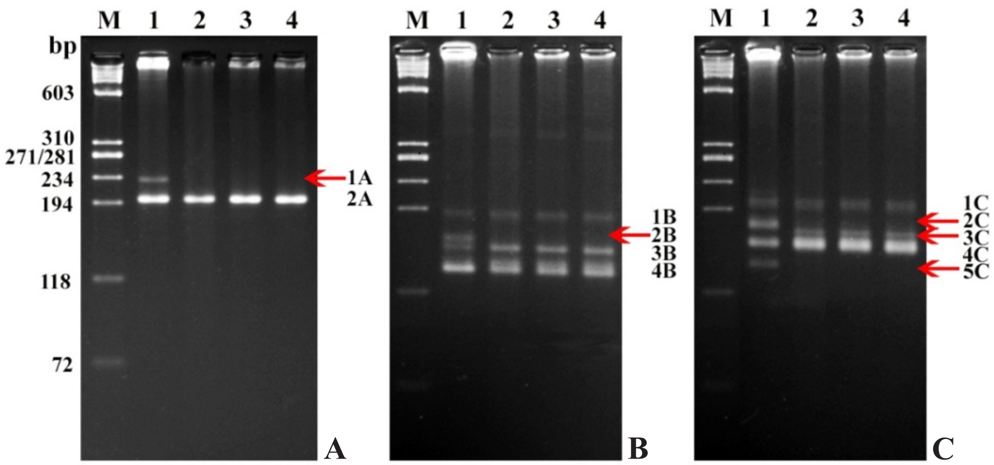

Fig. 4 Electrophoresis pattern of DNA amplified products obtained with primers (A) MCSA205C07, (B) SMC226CG and (C) SMC319CG. (Lane M=ϕx-174/Hae III, Lane 1= wild type plant (K84-200), Lane 2=38L5M, Lane 3=36L5 and Lane 4=33CS5). Fragments showing somaclonal variation are marked by arrows.

Table 4 Nucleotide sequence comparison at the primary sequence level and predicted homology of the three somaclones and wild type plant from SSR fragments primer MCSA205C07, MCSA226CG and MCSA319CG

Clone	Changes in nucleotide sequence (′5-3′)	Analytical results					
		Size (bp)	DNA fragment	Single-base-pair substitutions			
				Transition	Transversion	Insertion	Deletion
MCSA205C07-1A							
K84-200	5′GCACGGGCTAGAACCTAGAAGGCCGCACA TGCACGGCATGGCAGCTGGCTGCTGGTCTG CTCGTCGTCGTCGTCGTCGTCGATGAAAGC GCCGGCAAGCAGCAAACGGCGGCGACGCC TGCCTGGTCATCCGGATCCGGGACTGCATC TCCTCACCGGACACTTCTGCAGCGCGATGA AGATGGAAGAAGCACCGAGAGCTGGTAGC 3′	207					
38L5M	No fragment		absence	0	0	0	0
36L5	No fragment		absence	0	0	0	0
33CS5	No fragment		absence	0	0	0	0
MCSA205C07-2A	1 53 82 103 105 138 150 187 190						
K84-200	5′ - A G A C T T G T 3′	200					
38L5M	5′ G G A T T A G G - 3′	200	-	3	3	1	1
36L5	5′ G G G T T A G A T 3′	201	-	3	3	1	0
33CS5	5′ G A A T T A G G T 3′	201	-	2	3	1	0
SMC226CG -1B	29 85 86 87 88 89 90 127 165 166						
K84-200	5′ A C A C A C A - T - 3′	165	-				
38L5M	5′ A C A C A C A - T - 3	165	-	0	0	0	0
36L5	5′ A C A C A C A - G T 3′	166	-	0	1	1	0
33CS5	5′ T - - - - - - A G T 3′	161	-	0	1	2	6
SMC226CG -2B							
K84-200	5′GAGGCTCAGAAGCTGGCATGCATCGTGAAA 145 ATACGACAGCTTTGTGCATAACAGCCTAAGCA CACACACACACACACACACACACACACATCA GAGAGCAGGGCAATTACACGACACATACTGA AACCAACTCGGAAATAGAGGT3′						
38L5M	No fragment		absence	0	0	0	0
36L5	No fragment		absence	0	0	0	0
33CS5	No fragment		absence	0	0	0	0
SMC226CG -3B	30 85 86 87 88 89 90 99 100 101 102 116						
K84-200	5′ A C A C A C A G A - G - 3′	146	-				
38L5M	5′ A C A C A C A G A - G - 3′	146	-	0	0	0	0
36L5	5′ T - - - - - - - - - A A 3′	139	-	0	0	1	8
33CS5	5′ T - - - - - - C A G G - 3′	141	-	0	0	2	6

Remark: - = single base deletion

Clone	Changes in nucleotide sequence (′5-3′)	Analytical results					
		Size (bp)	DNA fragment	Single-base-pair substitutions			
				Transition	Transversion	Insertion	Deletion
SMC226CG -4B							
	7 27 28 29 101 102 103						
K84-200	5′ - T - - T G C 3′	136	-				
38L5M	5′ - T - - T A C 3′	136	-	1	0	0	0
36L5	5′ - T - - T G C 3′	136	-	0	0	0	0
33CS5	5′ T G A G G G G 3′	139	-	0	3	3	0
SMC319CG -1C							
	29 46 47 48 49 50 74 98 118 124 128 139 141 144 160 163 178 179						
K84-200	5′A T T A G T A G T C T C T T T T A T 3′	179	-				
38L5M	5′A T T A G T A G T C T C T T T T A T 3′	179	-	0	0	0	0
36L5	5′A A G T G T G T G G A G G T G - G - 3′	177	-	2	10	0	2
33CS5	5′G - - T T A G T G G A G G C G - G - 3′	175	-	4	10	0	4
SMC319CG -2C							
K84-200	5′GGTTCACCGAAGCAAGAGAACTGATTGAAA 179 TCTTAGGCAAAGACCTTAGTGTGTGTGTGTG TATTAATTTGCAATCCTATGGATGATTGCAGT CCTAGGGATAATTGCAATAGAAATTGCTTACG TTTACATTCATTTCTTCTTCTGTCCTCTGTGG ATTAATCTATATCTCTTTAGAT3′						
38L5M	No fragment	absence	0	0	0	0	
36L5	No fragment	absence	0	0	0	0	
33CS5	No fragment	absence	0	0	0	0	
SMC319CG -3C							
	46 47 48 74						
K84-200	- - - - No fragment						
38L5M	5′ A G T G 3′	165	presence	-	-	-	-
36L5	5′ T T A A 3′	165	presence	-	-	-	-
33CS5	5′ T T A A 3′	165	presence	-	-	-	-
SMC319CG - 4C							
	29 46 47 48 73 111 128 144						
K84-200	5′ A T T A A T A T 3′	165	-				
38L5M	5′ A A G T G T A T 3′	165	-	1	3	0	0
36L5	5′ G T T A G C T T 3′	165	-	3	1	0	0
33CS5	5′ G T T A G T A C 3′	145	-	3	0	0	0
SMC319CG -5C							
K84-200	5′GGTTCACCGAAGCAAGAGAACTGATTGAG145 ATCTTAGGCAAAGACCTTAGTGTGTGTGTG TATTAATTTGCAGTCCTATGGATAATTGCAAT AGAAATGGCTTAGGTTAACATTCATTTGTGC TCCTGTCCTCTGTGGATGAAAGG3′						
38L5M	No fragment	absence	0	0	0	0	
36L5	No fragment	absence	0	0	0	0	
33CS5	No fragment	absence	0	0	0	0	

Remark: - = single base deletion

3. Sequence variation of polymorphic SSR markers

The 31 DNA sequences of difference fragments in somaclones and wild type plant were blasted and aligned using the program BLASTN, BLASTX and CLUSTALW, respectively. Comparisons of all nucleotide sequences were performed using the CLUSTALW program. The nucleotide comparison of clones MCSA205C07-1A, SMC226CG-2B, SMC319CG-2C and SMC319CG-5C showed that certain fragments were present only in the wild type plant (K84-200) and contained 207, 146, 179 and 145 bps respectively. These fragments were absent in all three somaclones (38L5M, 36L5 and 33CS5). The 165 bp fragment of clone SMC319CG-3C was absent only in the wild type plant but was present in all three somaclones (38L5M, 36L5 and 33CS5). There were nucleotide insertions in somaclones. Clone MCSA205C07-2A of three somaclones (38L5M, 36L5 and 33CS5) showed base substitution (transition & transversion), base deletion and base insertion from the nucleotide sequence of the wild type plant. Clone SMC226CG-1B of 38L5M, showed the same nucleotide sequence of the wild type plant. Clone SMC226CG-1B of 36L5 and 33CS5 showed base substitution (transversion), base insertion and base deletion from the nucleotide sequence of the wild type plant. Clone SMC226CG-3B of 38L5M, showed the same nucleotide sequence of the wild type plant. Clone SMC226CG-1B of 36L5 and 33CS5 showed base insertion and base deletion from the nucleotide sequence of the wild type plant. Clone SMC226CG-4B of 36L5, showed the same nucleotide sequence of the wild type plant. Clone SMC226CG-4B of 38L5M showed base substitution (transition) and clone SMC226CG-4B of 33CS5 showed base substitution (transversion) and base insertion from the nucleotide sequence of the wild type plant. Clone SMC139CG-1C of 38L5M, showed the same nucleotide sequence of the wild type plant. Clone SMC139CG-1C of 36L5 and 33CS5 showed base substitution (transition & transversion) and base deletion from the nucleotide sequence of the wild type plant. Clone SMC139CG-4C of three somaclones (38L5M, 36L5 and 33CS5) showed base substitution (transition & transversion) from the nucleotide sequence of the wild type plant (Table 4). The main type of variation at the nucleotide sequence level was nucleotide substitution (Table 4). DNA pattern and DNA sequencing analysis of the SSR polymorphic fragments of these somaclones showed DNA deletion, DNA insertion and single base-pair substitutions, as shown in Table 4. Similar results were also reported by Ngezahayo et al. (2007) in rice. These results showed that the somaclonal variation technique can induce genetic variation from the wild type plant. In our study, at the nucleotide sequence level, single base-pair substitutions occurred were mostly transversions (Table 4). In contrast to this, Ngezahayo et al. (2007) reported that single base-pair substitutions occurred were mostly transitions, while transversions were rare in rice. The nucleotide sequences of transition and transversion, including DNA deletion, DNA insertion and single base-pair substitutions are referred to as point mutations. Point mutations are small changes in the sequence of DNA bases within a gene. In general, all point mutants that are important in plants may or may not affect protein synthesis. (Mba, 2013).

At the primary nucleotide sequence level, nucleotide sequence of clones MCSA205C07-1A, MCSA205C07-2A, SMC226CG-1B, SMC226CG-2B, SMC226CG-3B, SMC226CG-4B, SMC139CG-1C, SMC139CG-2C, SMC139CG-3C, SMC139CG-4C and SMC139CG-5C of somaclones 38L5M, 36L5, 33CS5 and wild type plant K84-200, the cloned sequences queried to the GenBank databases showed non-sequence homology in GenBank nucleotides databases (data not shown). DNA sequences of SSR have distribution on the whole genome (Powell et al., 1996). However, fragmentation is not always even, depending on the type of organism. The SSR are mostly distributed into the non-coding regions, as opposed to coding regions (Paniego et al., 2002).

Conclusion

Somaclonal variation could be found in callus culture. SSR pattern and sequencing analysis of these somaclones show deletion, insertion and substitution. The main type of variation at the nucleotide sequence level was nucleotide substitution. These results show that the somaclonal variation technique can induce genetic variation from the wild type plant. More primers should be included to increase the efficacy of somaclone identification. SSR markers proved to be a useful tool for identifying the genetic variation of somaclones regenerated from callus. This information will enrich the ongoing breeding program by somaclonal variation.

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