Ife Journal of Agriculture, 2016, Volume 28, Number 1

RECOGNITION SITES OF RESTRICTION ENDONUCLEASES ON THE GENOMIC DNA OF TWO STARCH BRANCHING ENZYMES FROM CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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ABSTRACT

This study investigated the distribution, frequency and properties of restriction enzymes on genomic DNA of SBE I and II to facilitate production of modified starch in cassava. Genomic DNA of starch branching enzyme I and II were amplified, cloned, sequenced and restriction enzyme analyses conducted with bioinformatics tools. Twenty-two restriction enzymes' sites of 17 restriction enzymes were evenly distributed on the circular restriction map of SBE I gene genomic DNA. Three restriction enzymes produced blunt DNA ends, six enzymes produced 5' overhang while seven enzymes left 3' overhang at the end of their DNA cutting activity. Seven enzymes were sensitive to DNA methylation by N6-methyladenosine while 88.2% of the restriction enzymes that cut SBE I DNA with lambda DNA as their substrate. Similarly, the genomic DNA of SBE II had 30 restriction sites cut by 22 restriction enzymes. Seven enzymes produced blunt DNA end, 12 left 5' DNA end and three produced 3' DNA end. About 36.4%, 13.6%, 2% and 9.1% of the restriction enzymes were sensitive to N6-methyladenosine, N6-methylcytosine, N4-methylcytosine and N5-methylcytosine DNA methylation, respectively. The study concluded that cassava SBE I and II genomic DNAs contain adequate restriction enzymes' sites with appropriate properties relevant for manipulation of starch biosynthesis.

***Keywords**: Cassava; genomic DNA; Restriction enzyme; Starch branching enzyme*

INTRODUCTION

Cassava is a root crop and constitutes an important source of energy in the diet of 600 million people in tropical and subtropical countries (Defloor *et al*., 1998; Bull *et al*., 2011). Cassava native starch has limited food and industrial uses because of its physicochemical properties. Currently, there is huge market in food and industrial sectors for modified starch which is estimated to be US$10 million (Blennow *et al*., 2003). Consequently, there is considerable interest in generating cassava plants that can produce modified starches suitable for both food and industrial applications. Conventional breeding of cassava is challenging due to the highly heterozygous

nature of the crop (Ceballos *et al*., 2004). In the field, cassava is typically propagated clonally by stem cuttings. This propagation strategy is ideal for improvement through crop bio-engineering as gene segregation through outcrossing is limited (Taylor *et al*., 2004).

Modification and bio-engineering of starch properties required molecular alteration of biosynthesis genes such as starch branching enzyme I and II (Baguma *et al*., 2003). This process includes cutting and stitching of the gene DNA to obtain desired modified starch in the bio-engineered plants (Zeeman *et al*., 2000). The presence of natural restriction sites on DNA facilitates their cutting by restriction enzymes (Williams, 2003). In cassava, natural restriction enzyme sites have been documented in SBE I and II cDNAs (Salehuzzaman *et al*., 1992; Baguma *et al*., 2003). However, evolutionary, structural and functional information derived from cDNA sequences of the genes is limited because the origin of such DNA sequence is RNA which is an expressed part of the gene. The knowledge of the restriction sites had been employed to achieve effective cloning and sequencing of SBE I and II cDNAs and for various molecular work on their DNA. Expression patterns of the two genes have been made possible in cassava and sweet potato by the utilization of restriction sites (Salehuzzaman *et al*. 1992; Baguma *et al*., 2003; Hamada *et al*., 2006). Recently, restriction sites were utilized in the production of amylose-free cassava plants, virus-resistance cassava transgenic plants and high-starch producing cassava plants (Ihemere *et al*., 2006). The frequency, position and properties of restriction enzyme sites on genomic DNA of cassava SBEI and II genes have not been documented. Such information is essential for planning molecular cloning studies on the genes and provides insights on the degree to which the starch genes can be modified to achieve desired goals of starch modification (Oyelakin *et al*., 2015). The objective of this study was to describe the distribution, frequency and properties of natural restriction enzymes that have cutting sites on cassava genomic DNA of SBE I and II.

Materials and methods

The experiment was conducted at the Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan.

Plant materials and DNA extraction

DNA was extracted from young leaves (0.5–1.0 g) of field-grown cassava genotype TMS 4(2)1425. Detail DNA extraction procedure is as described by Opabode et al. (2013b). DNA was resuspended at a concentration of 500 ng/µl. The quality of the DNA was verified by running 2 µl of the DNA alongside a molecular weight marker λPst I on 0.8% agarose gel in 1 x TAE (Tris Acetate EDTA) buffer at 500 volts for one hour.

Primer composition, PCR amplification and cloning

Details of primer design and composition for this study can be find in Opabode et al. (2012). Amplification of SBE I and SBE II were carried out in a 50-µl reaction volume independently, which composed of 5µl of

10x buffer, 2.5 µl of $MgCl_2$ (25 mM), 5 µl each of primer F and R (1µM), 2.5 µl of dNTPs (2.5 mM), 1 µl template DNA (500 ng), 28 µl of H_2O and 1 µl of Taq DNA polymerase (5 U) (Bioline Inc., USA). Detail of the PCR amplification profile can be find in Opabode et al. (2012)

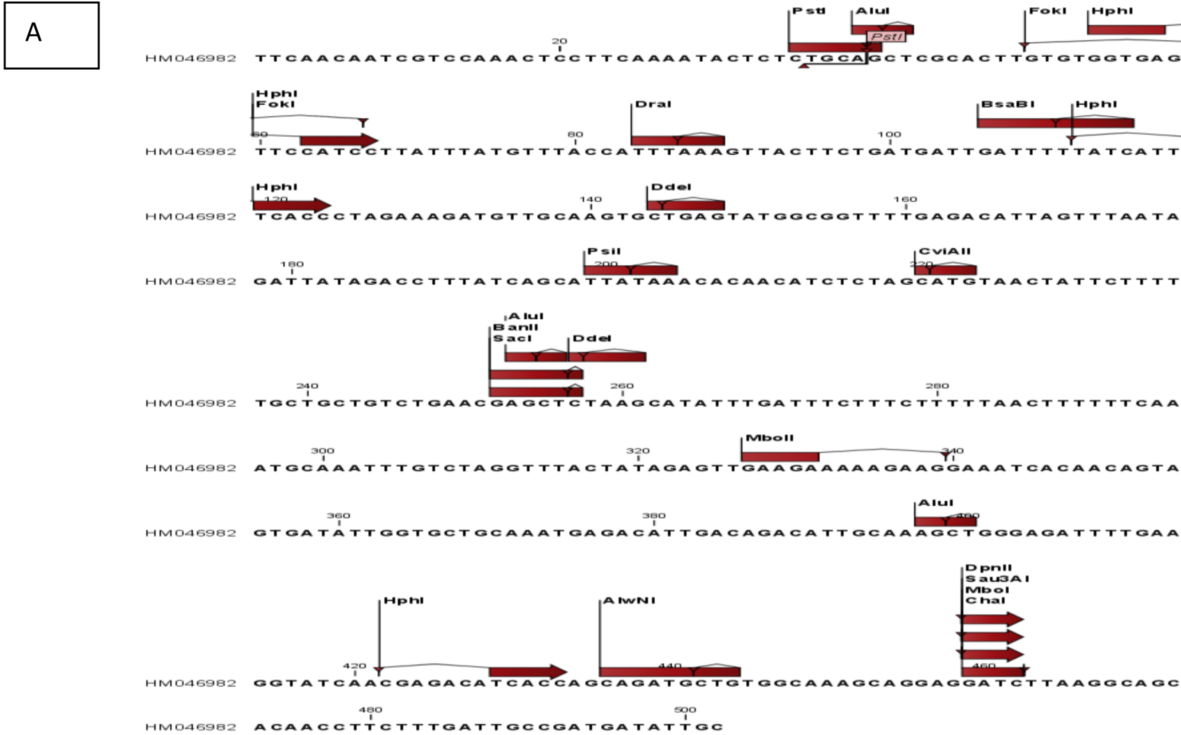
Bioinformatic analysis

Genomic DNAs of SBE I and II were submitted to CLC DNA WORKBENCH version 6.1 software for restriction enzymes analyses. Restriction map with restriction enzymes' sites were drawn using MAPVIEW programme of the software. Recognition frequencies, cutting frequency, cutting position, incubation temperature, palindrome recognition ability and popularity rating were obtained by computational method using the software. Restriction enzyme popularity scoring of Burge and Karlin (1998) was used for the study where 1= not very popular, 2= not popular, 3= popular, 4= very popular and 5= widely popular.

Results

Restriction maps of SBE I and II genomic DNAs

The linear restriction map of SBE I genomic DNA (HM046982) showed that the first site of restriction enzymes occurred after nucleotide 20. The first restriction enzyme to cut SBE I DNA was *AluI* and the last was *Sau3A* (Fig.1A). Twenty-two sites of 17 restriction enzymes were evenly distributed on 502 base pair genomic DNA of SBE I (Fig. 1A). Similarly, 30 restriction enzymes' sites of 22 restriction enzymes were distributed evenly on the circular restriction map of SBE II (Fig. 1B). Sixteen restriction sites were located on the right hemisphere of the map while 14 sites were located on the left hemisphere of the restriction map. Clockwisely, the first restriction site was *TaqI* and the last was *BspMI*.



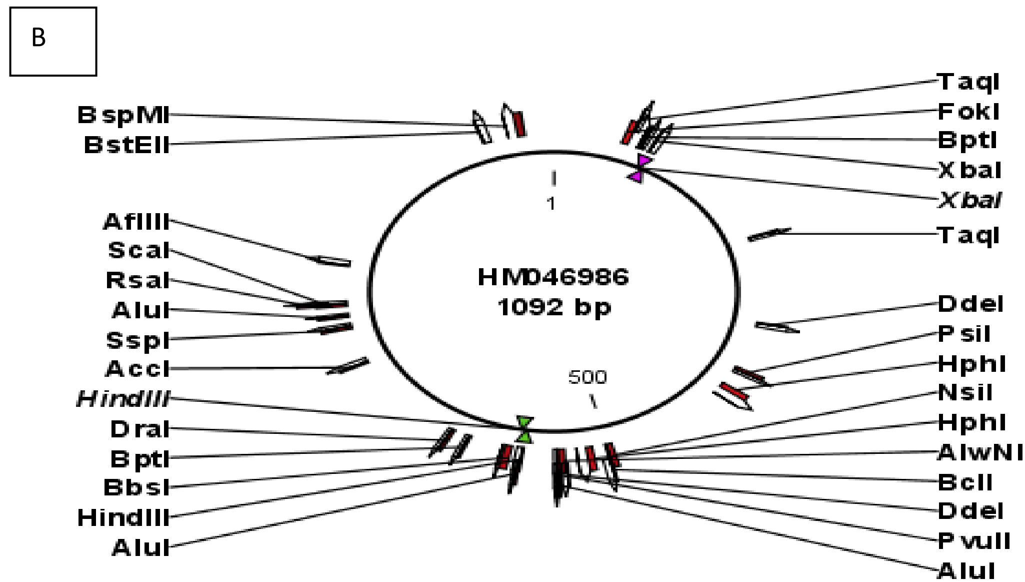


Fig. 1: Distribution of restriction sites on maps of SBE I (above) and SBE II (below) genomic DNA

Molecular characterization of restriction enzymes on genomic DNA of SBE I

All the recognition sequences of 17 restriction enzymes with 22 cutting sites were not less than four base pairs (Table 1). Only three restriction enzymes (*AluI, DraI, PsiI*) produce blunt DNA ends, six enzymes produced 5' overhang while seven enzymes left 3' overhang at the end of their DNA cutting activity. Restriction enzyme *Alu I and HphI* had the highest cutting sites (3) on SBE I DNA. Only *MboI II* was inhibited by N4-methylcytosine DNA methylation, 7 enzymes are sensitive to N6-methyladenosine while the activity of six enzymes are inhibited by N5-methylcytosine. Restriction enzymes *AlwNI, BsaBI, ChaI and Psi I* are not sensitive to DNA methylation of any type. About 88.2% of the restriction enzymes that cut SBE I DNA used lambda DNA as their substrate (data not shown). Only two enzymes did not utilized lambda DNA as their substrate; *CviAII* used pBR322 DNA while *Mbo I* recognized SV40 DNA as substrate. Incubation temperature of 37^0C was the optimum for 94.7% of the enzymes; only one enzyme *(Dpn II*) had 65^0C incubation temperature. Furthermore, about 82.5% of the restriction enzymes recognized palindromic sequences on SBE I DNA while three enzymes (*FokI, MboII ,HphI*) did not possess the ability to recognize palindromic sequences. One of the enzymes (pstI) was widely popular, another one very popular, 47.1% popular and 41.2% not popular in molecular experiments (Table 1).

Table 1: Recognition sequence, overhang, cutting site and position of restriction enzymes on genomic DNA of SBE I in cassava.

Enzyme	Recognition sequence	DNA ends	Cutting frequency	Cutting position	Methylation sensitivity	Popularity
*Alu*I	AGCT	Blunt	3	40, 254,398	5-MC	3
AlwNI	CAGNNNCTG	3'NNN	1	441	NL	2
BanII	GRGCYC	3'RGCYC	1	256	5-MC	2
BsaBI	GATNNNNATC	Blunt	1	110	NL	2
*Cha*I	GATC	3'-GATC	1	462	NL	3
CviAll	CATG	5'-AT	1	220	N6-MA	3
*Dde*I	CTNAG	5'-TNA	2	144, 257	5-MC	3
*Dra*1	TTTAAA	Blunt	1	86	N6-MA	3
DpnII	GATC	5'-GATC	1	458	N6-MA	2
Fok1	GGATG	5'-NA	1	49	N6-MA	3
HphI	GGTGA	3'-NA	3	66,111,421	N6-MA	2
MboI	GATC	5'-GATC	1	458	N6-MA	3
MboII	GAAGA	3'-NA	1	339	N4-MC	2
Psil	TTATAA	Blunt	1	201	NL	2
PstI	CTGCAG	3'-TGCA	1	39	N6-MA	5
SacI	GAGCTC	3'-AGCT	1	258	5-MC	4
Sau3AI	GATC	5'-GATC	1	458	5-MC	3

5-MC-5-methylcytosine N6-MA- N6-methyladenosine N4-MC- N4-methylcytosine NL-nil

Molecular characterization of restriction enzymes on genomic DNA of SBE II

A total of 30 cutting sites of 22 restriction enzymes were identified on SBE II genomic DNA (Table 2). Seven enzymes produced blunt DNA end, 12 5' DNA end and three 3' DNA ends. *AluI* had the highest (3) cutting frequency. About 36.4%, 13.6%, 2% and 9.1% of the restriction enzymes are sensitive toN6-methyladenosine, N6-methylcytosine, N4-methylcytosine and N5-methylcytosine, respectively. In the same vein, 22.7% of the enzymes was not sensitive to DNA methylation of any type. About 86.4% of the enzymes used lambda DNA as substrate; *BciI, NsiI* and *TaqI* utilized pBR322 DNA, SV40 DNA and θx-174 DNA, respectively as their substrates (data not shown). All restriction enzymes performed optimally at 37^0C incubation temperature except *TaqI* which had optimal incubation temperature of 65^0C. About 81.8% of enzymes that cut SBE II genomic DNA recognized palindromic sequences. Restriction enzymes *BclI, BspMI, FokI* and *NsiI* did not recognize palindromic sequences. About 50% of the enzymes that cut SBE II genomic DNA were popular for cloning work. According to popularity scoring, *XbaI* and *TaqI* are widely popular among laboratories involved in molecular cloning (Table 2).

Table 2: Recognition sequence, overhang, cutting site and position of restriction enzymes on genomic DNA of SBE II in cassava.

Enzyme	Recognition sequence	Overhang sequence	Cutting frequency	Cutting position	Methylation sensitivity	Popularity
AccI	GTMKAC	5'-MK	1	740	N6-MC	3
AflIII	ACRYGT	5'-CRYG	1	848	N4-MC	2
AluI	AGCT	Blunt	3	543,574,793	5-MC	3
AlwNI	CAGNNNCTG	3'-NNN	1	543	NL	2
BbsI	GAAGAC	5'-NA	1	589	NL	2
BclI	TGATCA	5'-GATC	1	503	N6-MC	3
BptI	CCWGG	5'-W	2	75, 619	NIL	2
BspMI	ACCTGC	5'-NA	1	1054	5-MC	2
BstEII	GGTNACC	5'-GTNAC	1	1033	MC	3
*Dde*I	CTNAG	5'-TNA	2	308,536	5-MC	3
*Dra*1	TTTAAA	Blunt	1	635	N6-MA	3
Fok1	GGATG	5'-NA	1	72	N6-MA	3
HindIII	AAGCTT	5'-AGCT	1	572	N6-MA	3
HphI	GGTGA	3'-NA	2	393,527	N6-MA	5
NsiI	ATGCAT	3'-TGCA	1	502	N6-MA	2
PsiI	TTATAA	Blunt	1	363	NIL	2
PvuII	CAGCTG	Blunt	1	543	N4-MC	4
RsaI	GTAC	Blunt	1	806	N4-MC	3
ScaI	AGTAC	Blunt	1	806	N4-MC	3
SspI	AATATT	Blunt	1	781	N6-MC	3
TaqI	TCGA	5'-CG	2	64,213	N6-MA	5
XbaI	TCTAGA	5'-CTAG	1	83	N6-MA	5

5-MC-5-methylcytosine N6-MA- N6-methyladenosine N4-MC- N4-methylcytosine NL-nil
N6-MC –N6 methylcytosine

Discussion

In this study, 17 and 22 cutting sites were identified on SBE I and SBE II genomic DNAs, respectively. These positions represent modification opportunities on the molecular structure of the genes and thus, on the activities of the genes. Restriction enzymes *SacI, AluI, FokI* and *MboI* have been reported to have cutting sites on cassava SBE I cDNA (Salehuzamman *et al.,* 1992). Similarly all the enzymes identified to have cutting sites on cassava genomic DNA of SBE I have been documented to have cutting sites on cassava cDNA of SBE II by Baguma *et al*. (2003). The number of restriction enzymes that cut SBE I and SBE II DNAs that produce overhang DNA ends were more than those with blunt DNA ends. This will have a positive impact on the manipulation of the genes of the two starch biosynthesis enzymes as both 5’ and 3’ DNA ends are desirable in molecular cloning and gene manipulation works while blunt DNA ends are not desirable. Similar pattern of DNA ends by restriction enzymes attached to SBE in maize and sweet potato have been reported (Yao *et al*., 2004; Hamada *et al*., 2006).

Only nine out of 39 restriction enzymes reported in this communication were not DNA methylation sensitive. Again, this situation brighten the prospect of generating information on methylation of cassava genome as large number of the restriction enzymes are sensitive to methylation to detect locations of DNA methylation in the genome. DNA methylation is a biochemical process that is important for normal development in higher organisms. Lambda DNA is a common and cheap DNA available for most molecular works. The prospect for future molecular manipulation of SBE I and II DNA for production of modified starch in cassava is further enhanced as most restriction enzymes that cut the DNA utilized lambda DNA as their substrate. Most of the enzymes that cut SBE I and II genome in cassava has 37^0C optimal incubation temperature. Similar observation have been made on restriction enzymes of potato and sweet potato SBE I and GBSS II genomic DNA and cDNA (Hofvander *et al*., 2004; Hamada *et al*. 2006). Most enzymes that cut SBE DNA recognize palindromic nucleotide sequences. This will allow a massive identification of both inverted-repeats and mirror-like palindromes which will be greatly exploited for starch modification.

It is noteworthy that both cassava SBE I and SBE II genomic DNA reported in this study have restriction sites of *Sac I, XbaI* and *Dra I* which have been used in molecular studies in cassava and other crops. For instance, Baguma *et al*. (2003) utilised *XbaI* site for effective screening of cDNA library for isolation of cassava of SBE II gene. To develop potato plants capable of producing modified starch by Hofvander *et al*. (2004), restriction enzymes’ sites of potato cDNA clones of SBE 1 and SBE II branching enzymes were used as a basis for constructing antisense fragments to inhibit the corresponding genes in potato. Some restriction enzymes (e.g. *PstI, SacI, TaqI* and *XbaI*) on the genomic DNA of cassava SBE I and II reported here have found use in Southern and Northern hybridizations to determine the copy number of genes and

gene expression pattern in cassava, potato, sweet potato, maize, barley and sorghum (Salehuzzaman *et al*., 1992; Baguma *et al*., 2003; 2003;Yao *et al*., 2004; Hamada *et al*., 2006, Regina *et al*., 2010).

In conclusion, the study identified 17 restriction enzymes with 22 cutting sites on 502 base pair- genomic DNA of SBE I and 30 cutting sites of 22 restriction enzymes on SBE II genomic DNA that was 1092 base pair long. The study established that the restriction sites were adequate and relevant for future manipulation and functional characteristics of the genes for production of modified starch in cassava.

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