DISTRIBUTION, ABUNDANCE AND PROPERTIES OF RESTRICTION ENZYMES ON GENOMIC DNA OF GRANULE-BOUND STARCH SYNTHASE I AND II IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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ABSTRACT

There is high demand for modified starch globally for both food and industrial uses because of its ability to withstand processing conditions such as extreme temperature, diverse pH, high shear stress and freeze-thaw variations. The study described the distribution, abundance and properties of restriction enzymes on genomic DNA of granule-bound starch synthase (GBSS) I and II with a view to manipulate the genes for production of modified starch in cassava. Thirty-one sites of 16 restriction enzymes were evenly distributed on 721 base-pair granule-bound starch synthase I (GBSS I) genomic DNA. About 63% of the restriction enzymes on GBSS I produced overhang DNA end, 88% were methylation sensitive and 69% utilized lambda DNA as substrate. Most of the enzymes (94%) have optimum incubation temperature of 37^{0}C while 95% of the enzymes recognized palindromic sequences. Similarly, 1690 base-pair long genomic DNA of GBSS II had 20 restriction sites cut by 16 restriction enzymes. About 67% of the restriction enzymes on GBSS II produced overhang DNA end, 88% were methylated sensitive, 93% use lambda substrate as substrate and all the enzymes has optimum incubation temperature of 37^{0}C. About 88% of the enzymes recognized palindromic sequences. Restriction enzymes were evenly distributed on GBSS I and II and *EcoRI* is the cheapest enzyme on both DNAs. The study concluded that cassava GBSS I and GBSS II genomic DNAs contain sites for suitable and abundant restriction enzymes with desirable properties for manipulation of the genes for production of modified starch.

Key words: Amylose synthesis, Cassava, Endonuclease, Genomic DNA.

INTRODUCTION

Starch synthase (EC 2.4.1.21) is an important enzyme responsible for the biosynthesis of starch in plant tissues. The two major forms of this enzyme are granule-bound starch synthase (GBSS) and soluble starch synthase (SSS) (Wang *et al.,* 1999; Zeeman *et al.*, 2010). GBSS, which is also known as the WAXY protein, is associated with starch granules, while SSS can be found in the soluble fraction of amyloplasts or the stroma of chloroplasts. Biochemically, GBSS is involved in the synthesis of amylose through the transference of glucose units from adenosine 5'-diphosphate (ADP)-glucose or uridine 5'-diphosphate (UDP)-glucose to non-reducing ends of -1, 4 glucose polymers, although the rate of transfer from ADP-glucose is higher than from UDP-glucose (Wang *et al.*,1999). The GBSS can be classified into two major types based on molecular mass and localization: GBSS I and GBSS II. GBSS I with a molecular mass of 5860 kDa is tightly bound to starch granules and offers the largest proportion of total GBSS activity. It has been established that GBSS I is responsible for amylose synthesis in plants. GBSS II with a higher molecular mass of 7779 kDa is present in starch granules as well as in the soluble fraction of plastids. Also, GBSS II plays an important role for determining amylopectin structure and starch granule morphology (Craig *et al.,* 1998).

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). Cassava native starch has limited food and industrial uses because of its poor pasting and retrograding properties. Currently, there is huge market in food and

industrial sectors for modified starch such as low-amylose starch which is estimated to be US$10 million (Blennow, 2003). Consequently, there is considerable interest in generating cassava plants that can produce modified starches suitable for both food and industrial applications through different breeding techniques. The success from conventional breeding is limited due to the highly heterozygous nature of the crop which prevents backcrossing and poor flowering with limited seed set of many varieties (Ceballos *et al.,* 2004). To date, only two starch mutants have been reported in cassava: a natural mutation in a *gbss* gene resulting in production of amylose-free starch and a gamma irradiation-induced mutation in an isoamylase gene resulting in high-amylose starch (Ceballos *et al.,* 2007; Ceballos *et al.*, 2008). It has been observed that genes that are involved in starch modifications through natural mutation are recessive. Thus, identification of natural mutants in cassava that produce modified starch through conventional breeding becomes an ardous task. In the field, cassava is typically propagated clonally by stem cuttings and 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 require molecular alteration of biosynthesis genes including GBSS I and II. This process includes cutting and stitching of the gene DNA to obtain desired modified starch in the bio-engineered plants. The presence of natural restriction sites on DNA facilitates their cutting by restriction enzymes. Restriction enzymes are enzymes that cut DNA at specific recognition nucleotide sequences known as restriction sites (Roberts, 1978). Basically, restriction enzymes are used to assist in insertion of genes into plasmid vectors during gene cloning and genetic engineering experiments. Furthermore, restriction enzymes are used to manipulate DNA at restriction sites for different biotechnological applications such as genomic and cDNA libraries construction and screening, Southern and Northern hybridizations, gene construct design and gene and QTLs mapping (Roberts, 1980; Williams, 2003). In cassava, natural restriction enzyme sites have been documented in GBSS I and II cDNAs (Salehuzzaman *et al.*, 1993; Munyikwa *et al.*, 1997). The knowledge of the restriction sites had been employed to achieve effective cloning and sequencing of GBSS I and II cDNAs and for various molecular work on their DNA. Expression patterns of the two genes have been made possible by the utilization of restriction sites (Munyikwa *et al.*, 1997; Salehuzzaman *et al.*, 1999). Recently, the use of restriction sites was utilized in the production amylose-free cassava plants (Raemakers *et al.*, 2005) and high-starch producing cassava plants (Ihemere *et al.*, 2006). 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. To close this information gap, we have cloned, sequenced and described the genomic arrangement of GBSS I and II in cassava (Opabode et al., 2011; Opabode et al., 2013). Still, the frequency, positions and properties of restriction enzyme sites on genomic DNA of cassava GBSS I and II genes have not been documented. Such information is essential for planning molecular cloning of the genes and provides insights into the degree to which the starch genes can be modified to achieve desired goals of starch modification. In addition, the information is necessary for planning for procurement, storage and handling of the restriction enzymes. The objective of this study was to describe the distribution, abundance and properties of natural restriction enzymes that have cutting sites on cassava genomic DNA of granule-bound starch synthase I and II.

MATERIALS AND METHODS

Location of the Study

The study was conducted at the Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan between 2006 and 2010.

Plant Materials and DNA Extraction

DNA was extracted from young leaves (0.51.0 g) of field-grown cassava genotype TMS 4(2) 1425 as described by Dellaporta *et al.* (1983). About 800 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 1% PVP) and 20 µl 0.7% beta mercarpto-ethanol, which

have been preheated at 65 ^{0}C was added to the tubes. These were mixed with a pipette tip until all tissue became dispersed in the buffer. About 100 µl of 20% sodium diodecyl sulphate (SDS) was added and mixed thoroughly for one minute. The mixture was then removed from 65^{0}C condition and cooled to room temperature for two minutes. About 300 µl of ice-cold 5M Potassium acetate was added to the mixture followed by gentle inversion six times. Thereafter, the mixture was incubated on ice for 20 minutes and centrifuged at 3,000 g in microfuge tube for ten minutes. The supernatant was carefully transferred to two new 1.5 ml eppendorf tubes.

One volume of ice-cold iso-propanol (approx. 700 µl) was added and mixed by inverting gently 8-10 times. This was followed by incubation at -80^{0}C for one hour and centrifugation at 3,000 g for one minute. The supernatant was tipped off and the last drop of iso-propanol was removed by placing tubes face down on paper towel. Pellets were suspended in 250 µl of high salt TE and 4 µl RNase was added. This was followed by incubation at 37^{0}C for one hour. Iso-propanol (500 µl) was added and mixed by gentle inversion 8-10 times. Then, the mixture was kept at -80^{0}C for one hour and centrifuged at 3,000 g for 10 minutes. Supernatant was decanted and the last drop of iso-propanol was removed by placing tubes face down on paper towels. The pellets were washed twice with cold 70% ethanol. Pellets were allowed to dry by leaving it on paper towel for one hour. Depending on the size of the pellet, about 100-200 µl sterile distilled water was added. Tubes were stored at 4^{0}C overnight to dissolve the DNA pellet. The supernatant was transferred to eppendorf tube and stored at -20^{0}C for further use. DNA was resuspended at a concentration of 500 ng/ l. The quality of the DNA was verified by running 2 l of the DNA alongside a molecular weight marker Pst I on 0.8% agarose gel in 1 x TAE (Tris Acetate EDTA) buffer at 500 volts for one hour.

Primer Design and Synthesis for GBSS I and II Gene Amplification

A cassava granule-bound starch synthase I gene-specific primer pair was designed from cDNA sequences (accession X14760) earlier deposited in GenBank database from nucleotide +99 to +530. The downloaded sequences were used as template to design primer specific for the genes using *Lasergene* sequence analysis software (DNASTAR Inc, Madison, USA). The composition of the GBSS I primer pairs designed and used for the study were as follows: Forward 5'-TGGACCCAAACTATCACTC -3' and Reverse 5'-AGTTTTGCCCCATACCTTC -3.' Similarly, a cassava granule-bound starch synthase II gene-specific primer pair was designed from cDNA sequences (accession AF173900) earlier deposited in *GenBank* database from nucleotide +3 to +369. The downloaded sequences were used as template to design primer specific for the gene using *Lasergene* sequence analysis software (DNASTAR Inc, Madison, USA). The composition of the GBSS II primer pairs designed and used for the study were as follows: Forward 5'-GGCATTTATAGGATCACTTCC-3' and Reverse5'-GAGTTTTCCCTGTTCATGAG-3'. Synthesis of the primers was done by Integrated DNA Technologies Incorporation (Iowa, USA).

PCR Amplification and Cloning

Amplification of GBSS I and GBSS 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). The PCR amplification profile consisted of initial denaturation at 94^{0}C for one minute and 30 cycles of amplification (94 ^{0}C for 30 seconds, 50^{0}C for 30 seconds, 72^{0}C for 45 seconds) with a final cycle of 5 minutes at 72^{0}C. All PCR amplifications were carried out in a Peltier thermal cycler (PTC 2000, MJ Research, India). The PCR fragments were purified and cloned into pDRIVE vector (QIAGEN, CA, USA). The presence of the insert in the recombinant plasmid was confirmed by restriction digestion.

DNA Sequencing and Analyses

For each PCR reaction, two to three independent clones were sequenced using automated sequencer. Both strands of the DNA inserts were sequenced and any sequence ambiguities were resolved by re-sequencing. The sequences were manually edited and vector sequences removed.

DNA sequencing was performed by Iowa State University, USA. BLAST searches were conducted at NCBI's website using blastn algorithm. Gene prediction analysis of the genomic DNA was conducted at GENSCAN web server (Burge and Karlin, 1998). The sequences of cassava GBSS I and II have been submitted to *GenBank* and published under the accession numbers HM038439 and HM038440, respectively.

Bioinformatic Analyses of Restriction Sites

Genomic DNAs of GBSS 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. To determine the average cost in US dollar per unit of restriction enzymes, information on the cost of restriction enzymes in 14 leading biotech companies was obtained from their websites. 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

Distribution, Recognition Sequence, Dna End, Cutting Site and Position

Thirty-one sites of 16 restriction enzymes were evenly distributed on the circular restriction map of GBSS I genomic DNA (*GenBank* accession HM038439) (Fig. 1). Clockwisely, the first enzyme's site on the DNA was that of *SimI* while the last was that of *HinfI*. Sixteen restriction enzyme's sites were located on the right wing of the map while 15 sites occupied the left wing. Similarly, a total of 43 restriction enzyme's sites of 16 restriction enzymes were distributed on GBSS II genomic DNA (*GenBank* accession HM038440) (Fig. 2). Clockwisely, two *SimI* sites were first located on the genomic map and the last were two sites of *BstEII*. Twenty restriction enzymes' sites were situated at the right wing of the map while 23 sites were on the left wing.

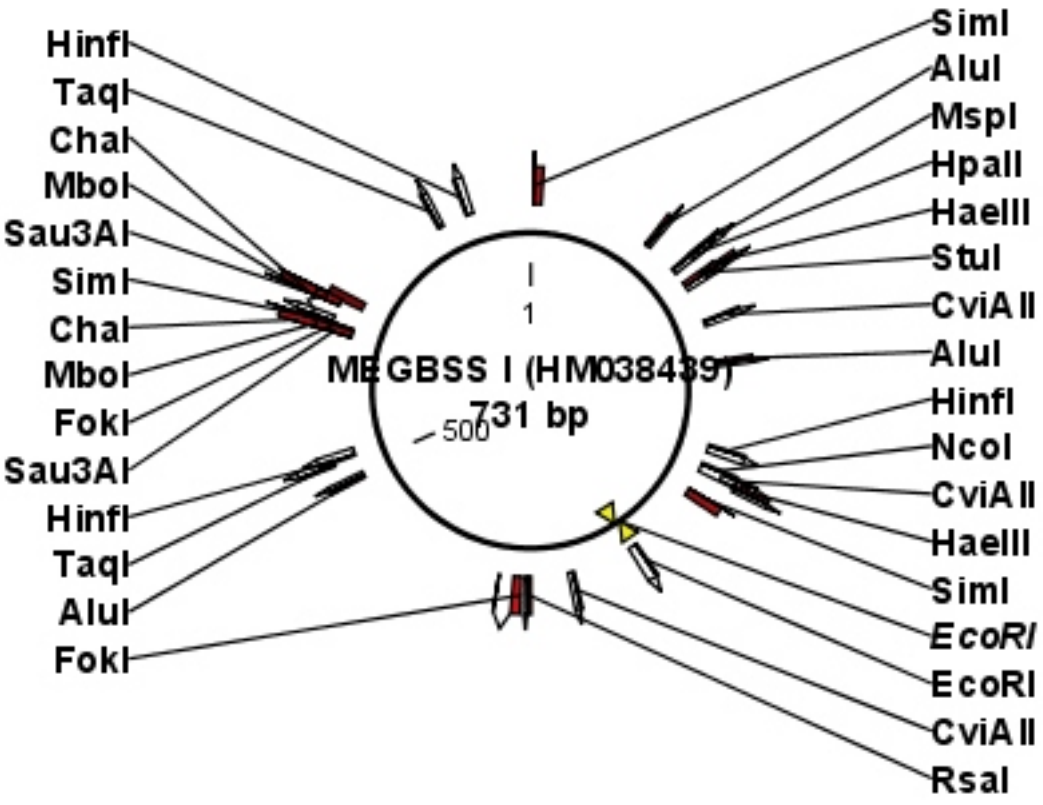

Fig. 1: Restriction map of cassava GBSS I genomic DNA

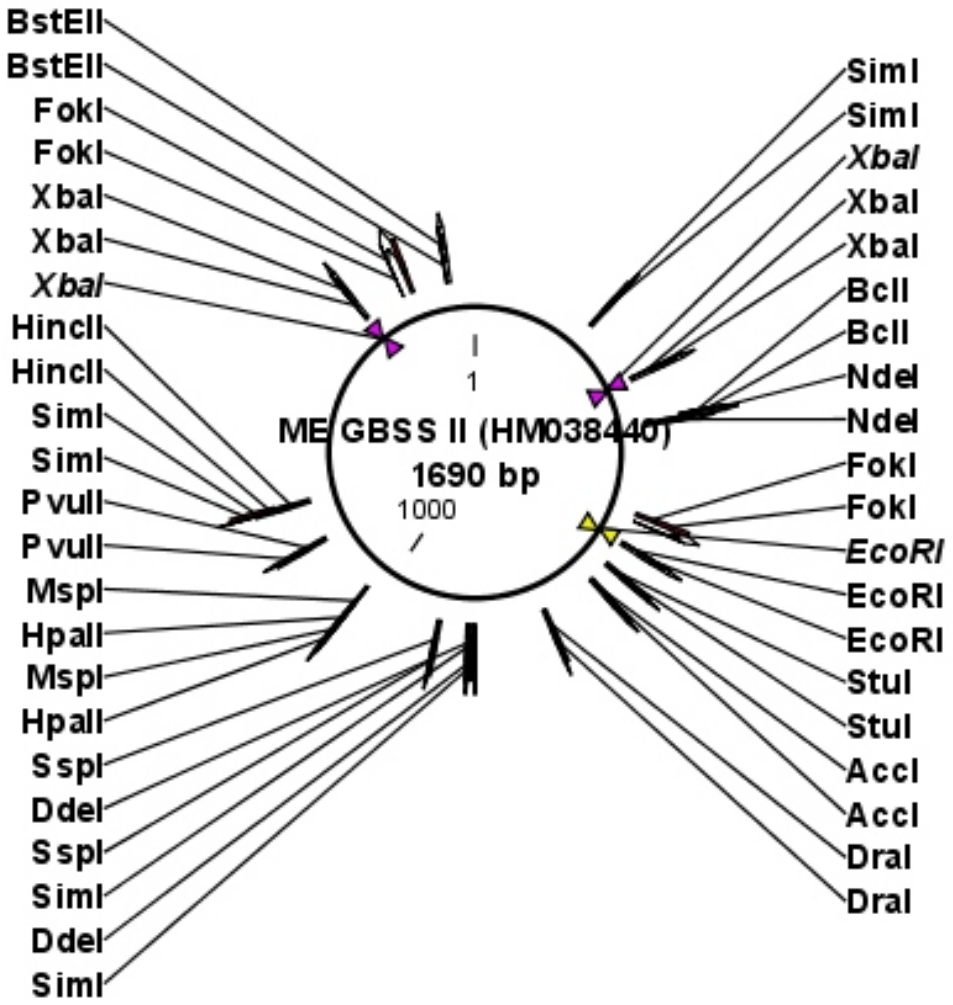

Fig.2: Restriction Map of Cassava GBSS II Genomic DNA

A total of 16 restriction enzymes with 31 sites were located on the isolated GBSS I sequence (Table 1). About 63% of the recognition sequence had four nucleotide bases in GBSS I whereas 60% of recognition sequence had six nucleotide bases in GBSS II. The nucleotide base of the recognition sequence of both GBSS I and II ranged from 4 and 7. Four of the restriction enzymes (*AluI*, *HaeIII*, *RsaI*, *StuI*) have a blunt DNA end, one (*CviAII*) has 3' overhanging DNA end while the rest of the enzymes produced 5' overhanging DNA end. On the GBSS I genome, six enzymes have one cutting site each, six have two sites each and four restriction enzymes have four sites each. About 46.2% of the enzymes are sensitive to N6-methyladenosine, 38.5% sensitive to 5-methylcytosine, 15.4% to N4-methylcytosine and two enzymes (*SimI*, *Stu1*) are not methylation sensitive (Table 1). Fifteen enzymes have restriction sites on GBSS II genomic sequence (*GenBank* accession HM038440) (Table 2). Only one enzyme (*SimI*) has three cutting sites on the genomic DNA, one enzyme (*FokI*) has two restriction sites, while the rest of the enzymes have one cutting site on the genomic DNA of GBSS II. About 33.3% of the enzymes have blunt overhang while the rest posses 5' overhang. Two of the enzymes exhibit no methylation sensitivity, 86.7% have N6-methyladenosine sensitivity, one of the enzymes each is sensitive to N5-methylcytosine and N6-methylcytosine.

Table 1: Recognition Sequence, DNA Ends, Cutting Site and Position of Restriction Enzymes on Genomic DNA of GBSS I in Cassava.

S/N	Enzyme	Recognition Sequence	DNA end Sequence	Cutting Frequency	Cutting Position	Methylation Sensitivity
1.	*Alu*I	AGCT	Blunt	3	79, 166,494	5-methylcytosine
2.	*Cha*I	GATC	3'-GATC	2	586,601	nil
3.	*CviAII*	CATG	5 -AT	3	139,231,339	N6-methyladenosine
4.	*Eco*RI	GAATTC	5 -ATT	1	296	N6-methyladenosine
5.	*Fok1*	GGATG	5 -NA	2	385,587	N6-methyladenosine
6.	*HaeIII*	GGCC	Blunt	2	114, 235	5-methylcytosine
7.	*HinfI*	GANTC	5 -ANT	3	218,508,691	N6-methyladenosine
8.	*HpaII*	CCGG	5 -CG	1	101	5-methylcytosine
9.	*MboI*	GATC	5 -GATC	2	582,597	N6-methyladenosine
10.	*MspI*	CCGG	5 -CG	1	101	5-methylcytosine
11.	*NcoI*	CCATGG	5 -CATG	1	230	N4-methylcytosine
12.	*RsaI*	GTAC	Blunt	1	367	N4-methylcytosine
13.	*Sau3AI*	GATC	5 -GATC	2	582,597	5-methylcytosine
14.	*SimI*	GGGTC	5 -GTC	3	2,247,588	nil
15.	*StuI*	AGGCCT	Blunt	1	114	nil
16.	*TaqI*	TCGA	5 -CG	2	506,672	N6-methyladenosine

Table 2: Recognition Sequence, DNA Ends, Cutting Site and Position of Restriction Enzymes on Genomic DNA of GBSS II in Cassava.

S/N	Enzyme	Recognition Sequence	DNA end Sequence	Cutting Frequency	Cutting Position	Methylation Sensitivity
1.	*Acc*I	GTMKAC	5 -MK	1	645	N6-methyladenosine
2.	*Bc*II	TGATCA	5 -GATC	1	372	N6-methyladenosine
3.	*BstE*II	GGTNACC	5 -GTNAC	1	1646	nil
4.	*Dde*I	CTNAG	5 -TNA	1	854	5-methylcytosine
5.	*Dra*1	TTTAAA	Blunt	1	734	N6-methyladenosine
5.	*EcoR1*	GAATTC	5 -AATT	1	570	N6-methyladenosine
6.	*Fok1*	GGATG	5 - NA	2	533,1575	N6-methyladenosine
7.	*HincII*	GTYRAC	Blunt	1	1191	N6-methyladenosine
8.	*HpaII*	CCGG	5 -CG	1	1026	N6-methylcytosine
9.	*MspI*	CCGG	5 -CG	1	1026	N6-methyladenosine
10.	*NdeI*	CATATG	5 -TA	1	377	N6-methyladenosine
11.	*PvuII*	CAGCTG	Blunt	1	1127	N6-methylcytosine
12.	*SimI*	GGGTC	5 -GTC	3	198,845, 1191	nil
13.	*SspI*	AATATT	Blunt	1	901	N6-methyladenosine
14.	*StuI*	AGGCCT	Blunt	1	612	N6-methyladenosine
15.	*XbaI*	TCTAGA	5 -CTAG	2	302, 1507	N6-methyladenosine

Table 3: Restriction Enzyme Substrate, Incubation Temperature, Palindrome Recognition, Popularity and Cost of Restriction Enzymes on Genomic DNA of GBSS I in Cassava.

S/N	Enzyme	Enzyme Substrate	Incubation Temperature (^{0}C)	Palindrome Recognition	Popularity	Cost (unit per US$)
1.	*AluI*	Lambda DNA	37	yes	3	16
2.	*ChaI*	Lambda DNA	37	yes	3	17
3.	*CviAll*	pBR322 DNA	37	yes	3	2
4.	*EcoRI*	Lambda DNA	37	yes	5	191
5.	*Fok1*	Lambda DNA	37	no	3	16
6.	*HaeIII*	Lambda DNA	37	yes	4	63
7.	*HinfI*	Lambda DNA	37	yes	3	89
8.	*HpaII*	Lambda DNA	37	yes	3	34
9.	*MboI*	SV40 DNA	37	yes	3	7
10.	*MspI*	Lambda DNA	37	yes	3	100
11.	*NcoI*	Lambda DNA	37	yes	4	5
12.	*RsaI*	Lambda DNA	37	yes	3	19
13.	*Sau3AI*	Lambda DNA	37	yes	3	4
14.	*SimI*			yes	3	35
15.	*StuI*	Unmethylated lambda DNA	37	yes	3	18
16.	*TaqI*	x-174 DNA	65	yes	4	71

Table 4: Restriction Enzyme Substrate, Incubation Temperature, Palindrome Recognition, Popularity and Cost of Restriction Enzymes on Genomic DNA of GBSS II in Cassava.

S/N	Enzyme	Enzyme Substrate	Incubation Temperature ^{O}C	Palindrome Recognition	Popularity	Cost (Unit Per Us$)
1.	*Acc*I	Lambda DNA	37	yes	3	15
2.	*Bc*II	Lambda DNA	37	yes	3	56
3.	*BstE*II	Lambda DNA	37	yes	3	36
4.	*DdeI*	Lambda DNA	37	yes	3	15
5.	*Dra*1	Lamda DNA	37	yes	3	39
6.	*Eco*R1	Lambda DNA	37	yes	5	191
7.	*Fok1*	Lambda DNA	37	no	3	16
8.	*HincII*	Lambda DNA	37	yes	3	21
9.	*HpaII*	Lambda DNA	37	yes	3	34
10.	*MspI*	Lambda DNA	37	yes	3	100
11.	*NdeI*	Lambda DNA	37	yes	4	68
12.	*PvuII*	Lambda DNA	37	yes	4	93
13.	*SimI*		37	no	3	35
14.	*SspI*	Lambda DNA	37	yes	3	16
15.	*StuI*	Unmethylated lambda DNA	37	yes	3	18
16.	*XbaI*	Adenovirus 2 DNA	37	yes	4	47

Restriction Enzyme Substrate, Incubation Temperature, Palindrome Recognition, Popularity and Cost

About 69% of restriction enzymes with recognition sites on cassava GBSS I genomic DNA used lambda DNA as their substrate (Table 3). Restriction enzymes *CviAII*, *MboI* and *TaqI* had p BR322 DNA, SV40 DNA and 174 DNA, respectively as substrate. The incubation temperature for the 94% of the enzymes was 37^{0}C. Only *TaqI* incubated optimally at 65^{0}C. All the recognition enzymes could recognize palindrome sequences except *FokI*. Out of 16 enzymes, one is widely popular, two very popular, and the rest enzymes popular in use for molecular studies. Based on the prevailing cost of the restriction enzymes from 15 leading Biotech firms in the United States of America and Britain, the cheapest enzyme with recognition site on cassava GBSS I DNA is *EcoRI* and the most expensive enzyme was *CviAII*. The four least expensive enzymes are *EcoRI*< *MspI*<*HinfI*<*HaeIII*. About 93% of the restriction enzymes with sites on GBSS II DNA had lambda DNA as their substrate, only *XbaI* enzyme used adenovirus 2 DNA as its substrate (Table 4). The optimum incubation temperature for all restriction enzymes with site on GBSS II DNA was 37^{0}C. All the recognition enzymes can recognize palindrome sequences except two which are *FokI* and *SimI* .

Out of 15 enzymes, only two are widely popular, another two very popular in use for molecular studies among restriction enzymes, the rest (80%) are popular in use for DNA work. From the prevailing market price for restriction enzymes, the most expensive enzyme on GBSS II DNA were *AccI* and *DdeI* and the cheapest was *EcoRI* (Table 4)

DISCUSSION

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts DNA at specific recognition nucleotide sequences known as restriction sites (Robert, 1980). Their discovery led to the development of recombinant DNA technology. Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA (Williams, 2003). In the present study, recognition sequences varied between 4 and 7 nucleotides. Generally, restriction enzymes are grouped into two: natural and artificial. Naturally occurring restriction endonucleases are further categorized into four groups (Types I, II III, and IV) based on their subunit composition, enzyme cofactor requirements, recognition sequence and cleavage position (Roberts, 1980, William, 2003). This study reports naturally occurring restriction enzymes that cut cassava GBSS genome. Out of 16 restriction enzymes that have cutting sites on cassava GBSS I genomic DNA, six (*HpaI, MboI, MspI, NcoI, RsaI, StuI, TaqI*) have been reported to have sites on cassava GBSS I cDNA (Salehuzzaman *et al.*, 1993). Restriction enzyme *HindIII* has many cutting sites on GBSS I cDNA but has no site on GBSS I genomic DNA. However, only five (*DdeI, FokI, SimI, SspI, XbaI*) enzymes reported in this study to have sites on cassava GBSS II genomic DNA has no sites on cassava GBSS I cDNA as reported by (Munyikwa *et al.*, 1997).

A high proportion of restriction enzymes reported in this work has more overhangs than blunt DNA ends. According to Williams (2003), the simplest DNA end of a double stranded molecule is called a *blunt end.* Non-blunt ends are created by various *overhangs.* An overhang is a stretch of unpaired nucleotides in the end of a DNA molecule. These unpaired nucleotides can be in either strand, creating either 3' or 5' overhangs. DNA end or sticky end refers to the properties of the end of a molecule of DNA or a recombinant DNA molecule (Williams, 2003). Similar pattern of DNA ends by restriction enzymes attached to GBSS I in sweet potato and potato have been reported (Salehuzzaman *et al.*, 1992; Wang *et al.*, 1999). According to Sambrook *et al.* (1989) the concept is important in molecular biology, especially in cloning or when subcloning inserts DNA into vector DNA. The sticky ends or cohesive ends form base pairs. Any two complementary cohesive ends can anneal, even those from two different organisms. This bondage is temporary however, and DNA ligase will eventually form a covalent bond between the sugar-phosphate residues of adjacent nucleotides to join the two molecules together. In a blunt-ended molecule both strands terminate in a base pair. According to Russel and Sambrook (2001) blunt ends are not always desired in biotechnology since when using a DNA ligase to join two molecules into one, the yield is significantly lower with blunt ends. When performing subcloning, it also has the disadvantage of potentially inserting the insert DNA in the opposite orientation desired.

Only four out of 16 enzymes that can cut cassava GBSS I and II DNA reported in this study are not sensitive to DNA metyhlation. DNA methylation is a biochemical process that is important for normal development in higher organisms. According to Binz *et al.* (1998), DNA methylation involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring (cytosine and adenine are two of the four bases of DNA). This modification can be inherited through cell division. Significant progress has been made in understanding DNA methylation in the model plant *Arabidopsis thaliana*. The principal *Arabidopsis* DNA methyltransferase enzymes, which transfer and covalently attach methyl groups onto DNA, are DRM2, MET1, and CMT3 (Binz *et al.*, 1998). As a large number of restriction enzymes that cut GBSS DNA are sensitive to DNA methylation, a unique opportunity is created to have an extensive detection of methylated DNA in cassava genome and enrich our understanding of cassava evolutionary development. Lambda DNA is a common and cheap DNA available for most molecular works.

The prospect for future molecular manipulation of GBSS I and II DNA for production of modified starch particularly low-amylose starch in cassava is bright as most restriction enzymes that cut the DNA utilized lambda DNA as their substrate.

Incubation temperature represents the temperature of optimal activity for restriction enzymes. Most of the enzymes that can cut GBSS I and II genome in cassava has 37^0C optimal incubation temperature. Similar observations have been made on restriction enzymes of potato, sweet potato and bean GBSS I and GBSS II genomic DNA and cDNA (Leij *et al.*,1991; Munyikwa *et al.*, 1997; Isono *et al.*, 2003). However incubation temperature of most restriction enzymes on rice and barley GBSS I and II genome are greater than 37^0C (Sato *et al.*, 2002; Radchuk *et al.*, 2009). A palindromic sequence is a nucleic acid sequence (DNA or RNA) that is the same whether read 5' (five-prime) to 3' (three prime) on one strand or 5' to 3' on the complementary strand with which it forms a double helix. Most enzymes (91%) that cut GBSS 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.

Isolated naturally occurring restriction enzymes are used to manipulate DNA for different scientific applications. It is noteworthy that both cassava GBSS I and GBSS II genomic DNA reported in this study have *EcoRI* restriction sites. The enzyme is the most popular and cheapest restriction enzyme in molecular studies in cassava and other crops. Other enzymes reported in this study have been used for several purposes. For instance, Zhang *et al.* (2005) constructed cassava storage-root cDNA by producing double-stranded cDNAs with *EcoRI* ends from storage root mRNA and ligated them to *EcoRI*-digested ZAPII arms and packaged into phage particles with Gigapack Gold packaging extracts. Earlier, subcloning of the products of a cassava cDNA library screening was conducted with the help of *EcoRI* sites on the product when a positive cDNA inserts were subcloned into the *EcoRI* site of pMTL-25 (Salehuzzaman *et al.*, 1993). Similarly, Salehuzzaman *et al.* (1993) used *EcoRI* sites on cassava GBSS I and II cDNA to produce gene constructs for genetic modification for production of modified starches. The *HindIII-EcoRI* sense and antisense gene inserts were ligated between the *HindIII* and *EcoRI* sites of plant transformation vector pBin resulting in the vectors pCS and pCAs. In the same vein, Zhang *et al.* (2005) utilized *EcoRI* and *NcoI* sites for construction of cassava promoter/GUS fusion for genetic modification of cassava and arabidopsis. To achieve this, CaMV35S promoter between *EcoRI* and *NcoI* of binary vector pCAMBIA1301 was replaced by *EcoRI-NcoI* fragments of pBP15GUS and pB54GUS to generate new binary vectors pCP15GUS and pCP54GUS, respectively. The binary vectors were introduced into *Agrobacterium tumefaciens* strain LBA 4404 via electroporation.

In a similar manner, restriction enzymes are used to digest genomic DNA for gene analysis by Southern blot. This technique allows researchers to identify how many copies (or paralogues) of a gene are present in the genome of one individual, or how many gene mutations (polymorphisms) have occurred within a population. The latter example is called restriction fragment length polymorphism (RFLP). To analyse the number of inserted transgene copies and GUS expression levels of wild-type and transgenic cassava plants, Southern and Northern hybridizations were performed by digesting the genomic DNA with *EcoRI*, which cuts only once inside the T-DNA of pCP15GUS and pCP54GUS and by hybdrising to the promoter probes and GUS probe (Zhang *et al.*, 2005). To further demonstrate the usefulness of other enzymes that cut GBSS DNA, Zhang *et al.* 2005 produce cassava plants resistant to mosaic virus by constructing three binary vectors, firstly the *EcoRI* fragment from pDH51, containing CaMV 35S promoter and terminator, was inserted into the *Eco*RI restriction site of the polylinker of pPZP100. The complete antisense (as) sequence of the AC1, AC2 or AC3 gene of ACMV-KE was generated by PCR and cloned between the *Xba*I and *Hin*dIII site of paRNA14 to give rise to vector pasAC1, pasAC2 or pasAC3.

CONCLUSION

With an even distribution of abundant restriction enzymes' sites on genomic DNA of both GBSS I

and II, prospect is bright for precise molecular manipulation of amylose synthesis to create cassava varieties that produce modified starches suitable for both food and industrial uses. Also, the high proportion of sites of restriction enzymes that could produce overhang DNA ends and sensitive to methylation would facilitate successful gene cloning experiments.

REFERENCES

Binz, T., D'Mello , N. and Horgen, P. A. 1998. A comparison of DNA methylation levels in selected isolates of higher fungi. *Mycol.* 90, 785-790.

Blennow, A. Engelsen, S. B., Nielsen, T. M., Baunsgaard, L. and Mikkelsen, R. 2003. Starch phosphorylation: a new front line in starch research. *Trends Plant Sci.* 7, 445-450.

Burge, C. B. and Karlin, S. 1998. Finding the genes in genomic DNA. *Curr. Opin. Struct. Biol.* 8, 346 - 354.

Ceballos, H., Iglesias, C. A., Perez, J. C., and Dixon, A. G. O. 2004. Cassava breeding: opportunities and challenges. *Plant Mol. Biol.* 56, 503-516.

Ceballos, H., Sanchez, T., Morante, N. ,Fregene, M. , Dufour, D., Smith, A. M., Denyer, K., Perez, J. C., Calle, F. and Mestres, C. 2007. Discovery of an amylose-free starch mutant in cassava (*Manihot esculenta* Crantz). *J. Agric. Food Chem.* 55, 7469-7476.

Ceballos, H., Sanchez, T., Tofino, A. P., Rosero, E. A., Denyer, K., Smith, A. M., Dufour, D., Morante, N., Perez, J. C. and Fahy, B. 2008. Induction and identification of a small-granule, high-amylose mutant in cassava (*Manihot esculenta* Crantz). *J. Agric. Food Chem.* 56, 7215-7222.

Craig, J., Lloyd, J.R., Tomlinson, K., Barber, L., Edwards, A.,Wang, T.L., Martin, C. Hedley, C.L. and Smith, A.M. 1989. Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. *Plant Cell* 10, 413-426.

Defloor, I., Dehing, I. and Delcour, J. A. 1998. Physico-chemical properties of cassava starch *Trop. Sci.* 31, 189-207.

Dellaporta, S. L, Wood, J., and Hicks, J. B. 1983. A plant DNA mini preparation: version II. *Plant Mol. Biol. Rep. 1*, 19-21.

Ihemere, U. Ariaz-Garzon, D. Lawrence, S. and Sayre, R. 2006. Genetic modification of cassava for enhanced starch production. *Plant Biotech. J.,* 4, 453-465.

Isono, N., Ito, H., Senoura, T., Yoshikawa. M., Nozaki, K. and Matsui, H. 2003. Molecular cloning of a cDNA encoding granule-bound starch synthase I from kidney bean seeds and expression in *Escherichia coli*. *J. Appl. Glycosci.* 50, 355-360

Leij, F. R., Visser, R. G., Ponstein, A. S., Jacobsen, E. and Feenstra,W. J. 1991. Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the amf allele. *Mol. Gen. Genet.* 228 , 240-248.

Munyikwa, T. R.I., Jacobsen, E. and Visser, R.G.F 1997. Developmental regulation of cassava granule bound starch synthase II. *African J. Root Tuber Crops* 2, 116-120

Opabode, J.T., Oyelakin, O.O.,Akinyemiju, O.A. and Ingelbrecht, I. L. 2011. Isolation of genomic clones encoding granule-bound starch synthase (GBSS I) in cassava (*Manihot esculenta* Crantz). *J. Plant Sci.* 6, 174-181

Opabode, J.T., Oyelakin, O.O.,Akinkunmi, J. A., Akinyemiju, O.A. and Ingelbrecht, I. L. 2013. Occurrence of two isoforms of granule-bound starch synthase II (GBSS II) gene in cassava (*Manihot esculenta* Crantz). *Annals Biol. Res.* 4,105-114

Radchuk,V.V., Borisjuk, L., Sreenivasulu, N., Merx, K. and Mock, H. P. 2009. Spatiotemporal profiling of starch biosynthesis and degradation in the developing barley grain *Plant Physiol.* 150, 190-204.

Raemakers, K., Schreuder, M., Suurs, L., Vincken, J-P. Jacobsen, E. and Visser, R.G.F 2005. Improved cassava starch by antisense inhibition of granule-bound starch synthase I. *Mol. Breed.* 16, 163-172.

Russell, D. W. and Sambrook, J. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory. ISBN 0-87969-576-5.

Roberts , R. J. 1980. Restriction and modification enzymes and their recognition sequences" *Nucleic Acids Res.* 8, 6380.

Salehuzzaman, N. I. M., Jacobsen, E. and Visser , R.G.F. 1992. Cloning, partial sequencing and expression pattern of a cDNA coding for branching enzyme in cassava. *Plant Mol. Biol.* 20, 809-819.

Salehuzzaman, N.I.M., Jacobsen, E., and Visser, R.G.F. 1993. Isolation and characterization of cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato. *Plant Mol. Biol.* 23, 947-962.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual,* 2nd ed, Cold Spring Harbour laboratory Press, Cold Spring Harbour, NewYork

Sato, H., Suzuki, Y., Sakai, M. and Imbe, T. 2002. Molecular Characterization of Wx-mq, a Novel Mutant Gene for Low-amylose Content in Endosperm of Rice (*Oryza sativa* L.). *Breeding Sci.* 17, 345-351

Taylor, N., Chavarriaga, P. Raemakers, K. Siritunga, D. and Zhang, P. 2004. Development and application of transgenic technologies in cassava. *Plant Mol. Biol.* 56, 671-688.

Wang, S. J.,Yeh, K.W., and Tsai, C. Y. 1999. Molecular characterization and expression of starch granule-bound starch synthase in the sink and source tissues of sweet potato. *Physiol. Plant.* 106, 253-261

Williams, R. J. 2003. Restriction endonucleases: classification, properties, and applications. *Mol. Biotech.* 23, 225-243.

Zeeman, S. C., Kossmann, J. and Smith, A.M. 2010. Starch: Its metabolism, evolution and biotechnological modification in plants. *Annual Rev. Plant Biology* 61, 209-234

Zhang, P., Vanderschuren, H., Futterer, J., Gruissem, W. 2005. Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. *Plant Biotech. J.* 3, 385-397.