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Starch Branching Enzyme I from Cassava (*Manihot esculenta* Crantz): Genomic Organization, Intron Identification and Phylogenetic Analysis

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Abstract: Cassava (*Manihot esculenta*) is a major source of starch in tropical and subtropical countries. The genomic organization of the starch branching enzyme I (SBE I) in cassava was examined to increase starch biosynthesis knowledge and facilitate the production of modified starch in cassava. Three genomic clones encoding a fragment of SBE I gene were produced by PCR amplification of genomic DNA from a primer pair. Sequence analyses revealed that the insert in the clones had complete homology at both nucleotide and amino acid levels. One of the clones, pOYE303-6-1 (accession no. HM046982) was 502 base pair long. HM046982 encoded a polypeptide of 35 amino acids with molecular weight of 3.90 kDa and pI of 4.8. BLAST search retrieved *Manihot esculenta sbe* I cDNA (accession X77012) with 100% identity. HM046982 was rich in glutamic acid (14.3%), glycine (8.5%) and alanine (8.5%). Phylogenetic analysis showed that cassava SBE I belongs to dicot first subgroup and closely related to poplar (85%), lotus (83%), sweet potato (80%) and potato (80%). *In silico* identification of intron revealed that HM046982 had one intron that is 259 base pair long located between nucleotide 52 and 310 and consist of 41.7% T, 26.6% A and 16.6% C and 15.4% G. Application of the results of this study to starch modification in cassava by transgenic approach and other related DNA recombinant technologies was discussed.

Abreviations: cDNA complementary DNA. PCR polymerase chain reaction. SBE-starch branching enzyme. A adenine. T thymine. C cytosine. G guanine.

Key words: Cassava · Starch branching enzyme I · *Manihot esculenta* · Amylopectin · Starch

INTRODUCTION

Cassava native starch, irrespective of it source, cannot withstand processing conditions such as extreme temperature, diverse pH, high shear stress and freeze-thaw variations [1, 2]. As a result, cassava native starch is not suitable for many industrial and food applications such as food thickeners, binding agent and adhesive materials because of its physicochemical properties. The demand for modified starch has increased considerably, creating huge market globally that cannot be met by established sources of starch such as cereals and potato [3]. This is because modified starch has wide application as binders, fillers, emulsion stabilizer, consistent modifier and adhesives [1, 4]. In order to obtain modified starch from cassava, several modification methods are being used. These are conventional breeding, chemical method, physical treatment and biological method. There are some difficulties and weaknesses in these modification methods. For instance, conventional breeding of cassava varieties with modified starch properties is long and cumbersome; carrying out sexual crosses in cassava is difficult and many varieties rarely flower. Chemical modification of starch is long and laborious. High cost of chemicals makes the starch modification process not profitable. In addition, the method could pose threats to the environment if processing wastes are not properly disposed. Moreover, there are restrictions on the kinds of chemicals for treating starch granules as a result of consumers' health concern [3]. Physical treatment often

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disrupts starch structure, making it uneconomical by producing low quality starch. There are limitations on biological materials allowed for treating starch to protect consumer health [1]. Thus, there is an urgent need for alternative and sustainable means of modifying cassava starch to obtain novel qualities with expanded functionality.

Gene transfer approach offers a unique opportunity to develop cassava plants that are producing starch with specific characteristics such as low-amylose content [5, 6]. The method allows modification of starch directly on plant. Also, the molecular structure of starch can be precisely modified and tailored to certain uses. Once modification is achieved through transgenic method, it is permanent and cost-effective in the long run. In addition, the method is not environmentally risky and its products are safe for human consumption. It is prudent to use modern biotech tools to obtain novel cassava starch in the country since there is restriction on movement of modified plants and their products across international borders because of intellectual property rights.

Application of transgenic approach for starch modification requires molecular knowledge of key starch biosynthesis enzymes and genes. One of the enzymes involved in starch biosynthesis is starch branching enzyme (SBE) which is under the control of starch branching enzyme gene. Starch branching enzymes (SBEs) are involved in amylopectin synthesis, an important component of starch [7]. The prominent role of SBEs is to introduce branches to linear glucan chains of amylopectin and together with other starch biosynthesis enzymes (e.g. starch synthase, debranching enzymes and glucan water dikinases) govern the structure of amylopectin and in starch viscosity characteristics ([8, 9]. To date, two classes of SBEs have been identified: SBE I and SBE II. Starch branching enzyme I has been identified in maize, rice, pea, cassava and wheat [7]. According to Hovfander [10], SBE II has been identified in pea, maize, rice, barley, wheat, cassava and sorghum. Notably, SBE I and SBE II isoforms exhibit different substrate preferences. SBE II has lower affinity for amylose than SBE I, implying that SBE I uses longer glucan chains than SBE II. SBE I has greater branching activity and preferentially uses amylose as a subtrate [11], SBE isoforms are differentially and independently expressed during organ and tissue development and within the amyloplast. The genes encoding SBE I and II in cassava have been cloned and expression pattern determined [7, 12]. Study of expression patterns by northern hybridization showed that the gene is highly expressed in tubers. The transcript is detectable in stem and petiole, but not in leaves. In roots, the mRNA was hardly present. The expression levels at different stages of tuber growth are similar with exception of very young tubers in which it is relatively low. It was also shown that there is a difference in the level of branching enzyme expression between different cassava genotypes. Similarly, Baguma *et al*. [7] monitored the spatial and temporal expression patterns of the SBEII and SBEI genes, encoding starch branching enzyme II and I, respectively, in cassava (*Manihot esculenta* Crantz) at different phenological stages of the crop. As the cassava plant aged, the transcriptional activity of the *sbeII* and *sbeI* genes in the underground storage roots increased, whereas the activity in other organs remained the same or declined. At 180 days after planting (d.a.p.), levels of *sbeII* and *sbeI* transcripts in storage roots were very low, whereas at 360 d.a.p., the levels had increased dramatically.

Although complementary DNA (cDNA) encoding starch branching enzyme I in cassava has been isolated and cloned [7, 13], genomic organization of the enzyme in cassava has not been described. Similarly, the locations of introns in SBE I gene have not been identified. Also, limited phylogenetic analysis of SBE I was conducted with few available sequences, comprehensive phylogenetic relationship of cassava SBE I with other plants SBE I has not been investigated. The knowledge of genomic organization, intron sequences and phylogenetic relationship of SBE I are essential for manipulation of the gene to facilitate biosynthesis of starch suitable for desired food and industrial applications. Such information will provide insights into expression of the genes and the degree to which their activities can be altered to achieve desired goals of starch modification. For example, a well-characterize cassava granule-bound starch synthase I (GBSS I) gene has facilitated genetically modified and facilitate the production of cassava plants capable of producing amylose-free starch [2]. The objectives of this study were to (i) describe genomic DNA of 3' end of starch branching enzyme I (ii) identify the location and composition of intron sequences in the *sbe* I gene and (iii) conduct phylogenetic relationship of cassava SBE I with other plants.

MATERIALS AND METHODS

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 as described by Dellarporta *et al.* [14]. DNA was suspended 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 ëPst I on 1% agarose gel in 1 x TAE (Tris Acetate EDTA) buffer at 500 V for one hour.

Primer Design and Composition: The cDNA sequence of cassava SBE I (*GenBank* accession no X77012) earlier submitted at the international nucleotide sequence database of National Center for Biotechnology Information (NCBI), USA was downloaded. The downloaded sequence was used as a template to design three primer pairs specific for SBE I targeting 3' end (+2357 to +2500) of the gene using *primerselect* programme of Lassergen sequence analysis software (DNASTAR Inc, Madison, USA). The composition of the primer pairs, which were used for PCR amplification, were as follows:

- SBE1: Forward 5'-CAAAAGGGCTGTGGAAAGAAAC-3', Reverse 5'-AGCCCACTCACGATAGACAAT-3',
- SBE2:Forward 5'CGGCGCCTGATTTTGGGTCTT-3 Reverse 5'-GGTTTTGGAGGCCGGGGATAGTT-3'
- SBE3: Forward 5'-TTCAACAATCGTCCAAACTCCTT-3, Reverse 5'-GCAATATCATCGGCAATCAAA-3'.

Synthesis of the primer pairs was done at the laboratory of Integrated DNA Technologies Incorporation (Iowa, USA) according to our specification and diluted to 50µM per primer as stock primer in the laboratory before use.

PCR Amplification and Cloning: Amplifications of DNA were done by polymerase chain reaction as described by Sambrook *et al.* [15]. A preliminary PCR amplification was conducted to know the appropriate annealing temperature and primer pairs for amplification of SBE I gene. The PCR reactions were performed in a Peltier thermal cycler (PTC 2000, MJ Research, India). The preliminary PCR experiment consisted of the three primer pairs and three annealing temperatures (45, 54.3 and 60°C). The PCR reaction compositions for each of the primer pairs was: 1µl of 10x buffer, 0.5 µl of $MgCl_2$ (25 mM), 0.4 µl of primer F (1µM), 0.4 µl of primer R (1µM), 0.5 µl of dNTPs (2.5 mM), 1 µl template DNA (500 ng), 6.0 µl of H_2O and 0.2 U of Taq DNA polymerase. The reaction volume was 10 µl. The three PCR programmes used were: (i)SBE 54: initial denaturation at 94°C for one minute and 30 cycles of amplification (94 °C for 30 seconds, 54.3°C for 30 seconds, 72°C for 45 seconds) with a final cycle of 5 minutes at 72°C, (ii) SBE 60: initial denaturation at 94°C for one minute and 30 cycles of amplification (94 °C for 30 seconds, 60°C for 30 seconds,72°C for 45 seconds) with a final cycle of 5 minutes at 72°C, (iii) *SBE* 50: initial denaturation at 94°C for one minute and 30 cycles of amplification (94 °C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds) with a final cycle of 5 minutes at 72°C. Subsequently, PCR programmes SBE 54, SBE 60 and SBE 50 were used for amplification with SBE primer pairs 1, 2 and 3 respectively.

Agarose Gel Electrophoresis and Cloning: For each PCR experiment, amplification (PCR) products were separated alongside a molecular weight marker (11.0 kb plus pst 1 lambda DNA) by 1% agarose gel electrophoresis in 1xTAE (Tris acetate EDTA) buffer ran at 500 volts for one hour. The gel was stained with 1% ethidium bromide for 30 seconds before washing for 45 secs and visualized under UV light. Gel photographs were scanned through a Gel Doc System (Gel Doc. 2000, BioRad, California, USA). Purification of DNA extracted from agarose gel was done as described by Sambrook *et al*. [15]. The cloning of DNA fragment was done using the pDRIVE vector (QIAGEN, USA). A six µl ligation was conducted under room temperature for five minutes with the following composition: PCR product (DNA) 1 µl, pDRIVE vector 1 µl, diluted salt 1 µl and water 3 µl. The ligate was kept on ice before electroporation.

DNA Sequencing and Sequence Analysis: Automated Sanger DNA sequencing method, which uses dideoxy nucleotide chain terminators to stop DNA *in vitro* synthesis, was used to obtain the nucleotide sequence of the amylose genes at Iowa State University DNA laboratory, USA as described by Salehuzzaman *et al.* [13]. The compositions of primers used for nucleotide sequencing are: M13F-5'-GTAAAACGACGGCCAG-3' and M13R-5'-CAGGAAACAGCTATGAC-3'. Sequencing was done from both 5' and 3' ends of the plasmids covering the vector and insert region. Sequences were edited using Editseq programme of *Lasergene* DNA sequences analysis software. Nucleotide sequences were analysed using *Lasergene* DNA sequence analysis software version 6.0, CLC DNA analysis workbench version 5.1, Genscan web-based server and BLAST programme. BLAST searches were conducted at NCBI database as described by Altschul *et al*. [16]. Sequence alignment was performed by *Megalign* and *clusta* IV programmes.

Phylogenetic Analysis: Sequence searches of the non-redundant and unfinished genome databases at NCBI were conducted with the isolated sbe I ORFs as query sequence. The ClustalX package was used to create an alignment of the sequences that was then submitted to a neighbour-joining analysis to generate a branching pattern. The phylogenetic tree was displayed using the CLC TREEVIEW program. Eighteen sbe I sequences of higher plants (and their accession numbers) included in the study were: Apple (DQ115404), barley (AY304541), cassava (X77012), poplar (EF203424),wheat (AF286317), sorghum (AF169833), sweet potato (AB194722), Kidney bean (AB029549), lotus (FJ592190), mungbean (AY667492), potato (X69805), Maize (NM_001111900), rice (AF136268), red rice (AY886062), cocoyam (EF552588), sago palm (DQ202304), soybean (BK007878)

RESULTS

Amplification, Isolation and Characterization of SBE I Clones: Amplifications of SBE I gene, as indicated by the presence of bands, occurred at the three annealing temperatures for all the three primer pairs (Fig. 1). Distinct band was obtained with primer pair *SBE* 1 at 55°C annealing temperature while 60°C was the optimum annealing temperature for primer pair *SBE* 2. Primer pairs *SBE* 3 gave two distinct bands at 50°C annealing temperature estimated to be 0.85 kb and 0.55 kb. The estimated product size for primer pairs *SBE* 1 and *SBE* 2 were 1.0 and 2.4 kb respectively. Figure 2 is the outcome of optimized PCR amplification of SBE I gene using primer pairs SBE1 (at 55°C), primer pair *SBE* 2 (at 60°C) and primer pair *SBE* 3 (50°C) in 50 µl reaction volume. Single and big bands were obtained with primer pairs *SBE* 1 and *SBE* 2 while two bands amplified with primer pair *SBE* 3.

Three independent clones (pOYE 303-6-1, pOYE 303-6-2 and pOYE 303-6-3) obtained from the PCR product of primer pair *SBE3* were sequenced and sequence comparison showed that the insert in the clones were similar at both nucleotide and amino acid levels and thus clone pOYE 303-6-1 were subjected for further analyses. The sequence of the insert in pOYE303-6-1 has been submitted to *GenBank* and published under the accession number HM046982. The insert in pOYE303-6-1 (HM046982) contained 502 nucleotides and 36.85% GC content and encoded a polypeptide of 35 amino acids with molecular weight of 3.90 kDa and pI of 4.8. BLAST search with HM046982 retrieved *Manihot esculenta sbe* I cDNA (accession X77012) with 100% identity. HM046982 was rich in glutamic acid (14.3%), glycine (8.5%) and alanine (8.5%).

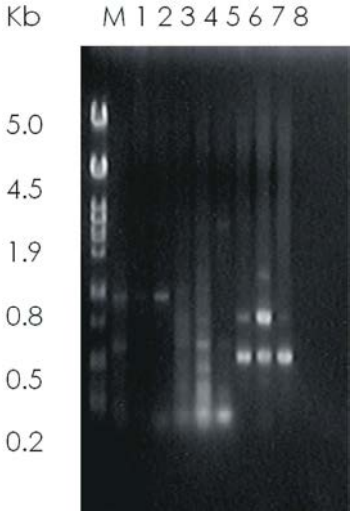

Fig. 1: Amplification of *sbe I* by PCR using three primer pairs at three annealing temperatures. M = DNA marker,1 = *sbe* primer pairs 1 at 45°C,2 = *sbe* primer pairs 1 at 50°C, 3= *sbe* primer pairs 1 at 55°C,4= *sbe* primer pairs 2 at 45°C, 5=*sbe* primer pairs 2 at 50°C,6= *sbe* primer pairs 2 at 55°C, 7 =*sbe* primer pairs 3 at 45°C, 8= *sbe* primer pairs 1 at 50°C, 9=*sbe* primer pairs 3 at 55°C. Molecular weight markers are indicated on the left in kb.

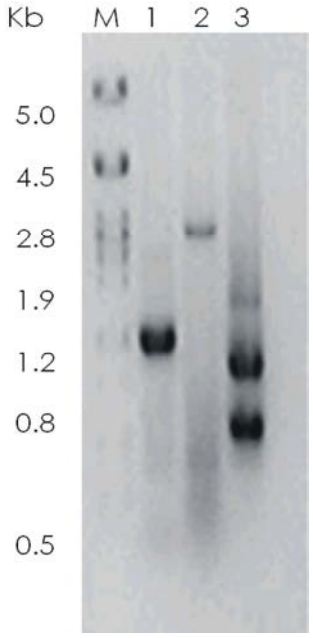

Fig. 2: Amplification of *sbe* I gene by PCR by three primer pairs. M = DNA marker,1=sbe primer pairs 1 at 55°C, 2= sbe primer pairs 2 at 60°C, 3= sbe primer pairs 3 at 50°C.Molecular weight markers are indicated on the left in kb.

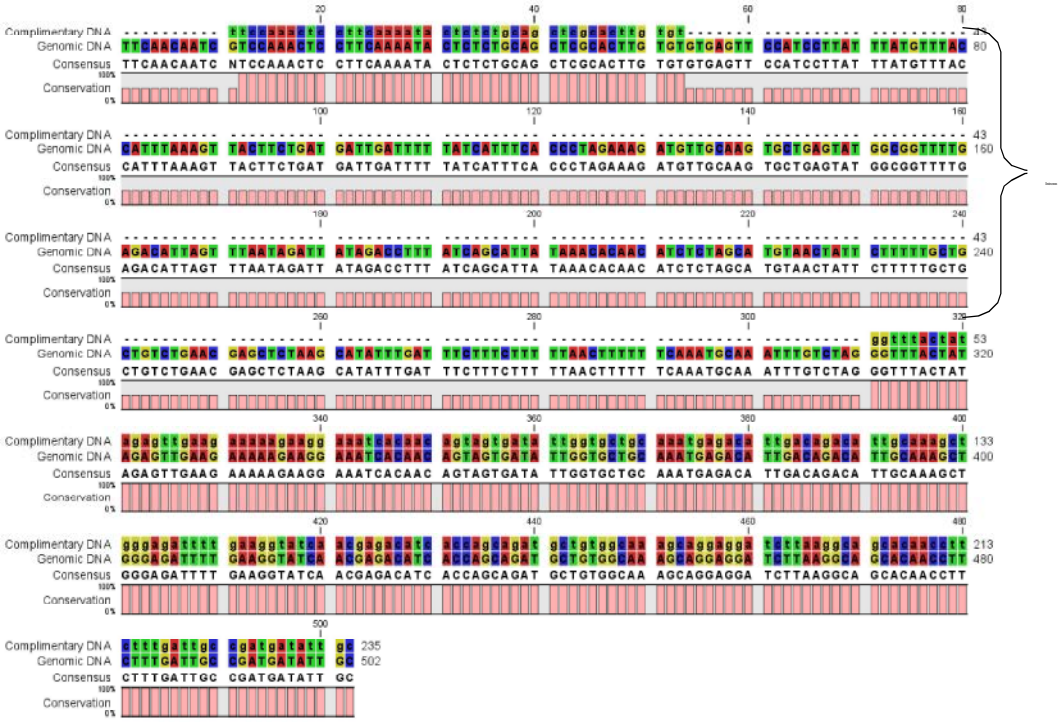

Fig. 3: Nucleotide sequence alignment of cassava SBE I cDNA (X77012) and genomic DNA (HM046982)

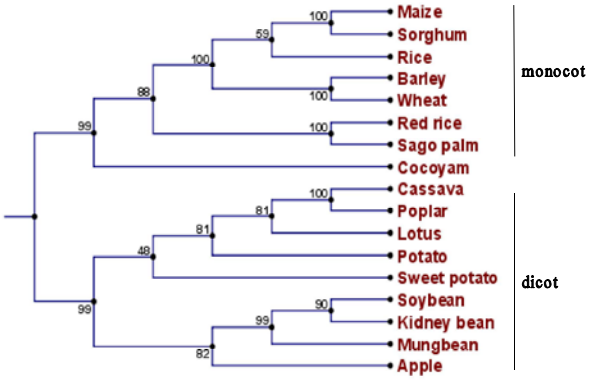

Fig. 4: Phylogenetic relationship of higher plants based on of SBE I sequences. The tree was constructed on the basis of available SBE I sequences in the *GenBank* using neighbourhood-joining method from bootstrapped data sets. The number of bootstrap replicates is indicated next to each branch.

***In silico* Identification of Introns:** The location of a putative intron identified by sequence alignment between the sbe I isolated genomic sequence (accession HM046982) and *sbe* I cDNA sequence (accession X77012) deposited in the *GenBank* were nucleotide 52-310 (Fig.3). The intron was 51.6% of HM046982 and made up of 41.7% T, 26.6% A, 16.6% C and 15.4% G. The putative intron location was further confirmed by gene prediction method using GENSCAN web server. The intron sequence contain splice site consistent with the consensus sequence 5'-GT _____AG-3' [17].

Phylogenetic Study: The phylogenetic analysis of a total of 17 SBE I proteins (Fig. 4) indicated that SBE I is grouped into two main groups: monocot and dicot. Dicot sbe I are further divided into two subgroups, cassava belongs to dicot subgroup A with poplar, lotus, potato and sweet potato. Cassava SBE I shared 85% nucleotides with poplar SBE I and 80% nucleotides with potato. A close analysis of the phylogenetic tree revealed that two gene duplications has occurred in the evolution history of SBE I proteins in plants. The first duplication occurred during the divergence of monocot and dicot, while the second duplication took places in dicot group to produce two subgroups of SBE I.

DISCUSSION

The successful PCR amplification of SBE I gene confirmed that the genome of cassava variety TMS 4 (2) 1425 encodes SBE I gene. It further points to the fact that the composition of oligonucleotide primer pairs designed for amplification of SBE I gene was specific for the gene.

The expected amplification product size of SBE I primer pairs was 246 bp. However, the observed amplification product size was 502 bp. Since genomic DNA was used as template DNA for PCR amplification and the SBE I oligonucleotide primer pairs was derived from cDNA, the difference in predicted and observed PCR product size could be attributable to the presence of introns in genomic DNA. Oligonucleotide primer pairs have been previously used to amplify starch gene in cassava [12, 13] and SBE I gene in wheat and rice [8, 9, 18]. Intron sequence has been established to account for differences in length between genomic and cDNA sequences [19]. Out of the three primer pairs designed for amplification of SBE I gene, genomic clones were obtained from only primer pair *SBE3*. The sequences of inserts of three genomic clones of SBE I determined had 100% identity at both nucleotide and amino acid levels. The homology of the sequences of the three clones suggested that SBE I gene in cassava has no isoform. It further implies that cassava SBE I gene has not experienced natural or induced mutation to produce polymorphism at nucleotide level. Previously, the cDNA encoding SBE I in cassava has been cloned and expression pattern determined [7, 12]. Our work is the first attempt to describe the genomic arrangement of SBE I gene in cassava.

From the pairwise alignment of SBE I cDNA (X77012 from the *GenBank*) and genomic DNA (HM046982 from this study) sequences, three main information were obtained: first, the identity of the clones was confirmed to be SBE I gene. Second, the location of intron in the SBE I genomic sequence and finally, the region of the SBE I gene cloned which is the 3' portion of the enzyme gene corresponding to +2357 to +2500 nucleotide of the cDNA sequence (accession X77012). This is the first identification of intron sequence in cassava SBE I gene which will have wide applications on gene expression studies and starch modification. For instance, one of these introns is a component of a gene construct for cassava modification in our cassava transformation programme [20]. The intron sequences can be employed to silence or overexpress SBE I gene in cassava or other plants for enhanced starch functionality. A gene construct containing an intron was employed in antisense technology to produce amylose-free cassava [2]. Similarly, RNA interference technology with intron was used to obtain low-amylose potato lines by Hofvander [10]. Intron also found use in expression studies of other genes in cassava or other plants. For example, Intron containing hygromycin gene construct produced stable transgene expression in cassava [21]. The introns can also be used for expression studies of other genes in other plants as the significance of introns in gene expression has recently been established [19].

Phylogenetic analysis provides information on relatedness of organisms based on the homology of their protein or DNA sequences. The phylogenetic relationship of cassava SBE I gene with other higher plants' SBE I gene previously reported by Salehuzzaman *et al*. [12] was expanded by including newly identified sequences from higher plants. The analysis showed that duplication plays a significant role in the emergence of subgroups of SBE I. Gene duplication events are the primary source of genetic novelty leading to speciation [20]. He explained further that after a duplication event, one daughter gene retains the preduplication function, while the other one, accumulates deleterious mutation and is eliminated, or, in some rare cases, survives by gaining a new function. The isolated cassava SBE I sequence, HM046982, belongs to dicot subgroup A with poplar, lotus, potato and sweet potato. This is not expected as cassava and poplar plants belong to the different plant families. Implication of this finding on future biotechnological research on SBE I gene is that any effective techniques for the gene modification in poplar plant can be used for cassava with or without modification. Such techniques include SBE I isoform discovery, gene cloning, downregulation and overexpression studies and functional characterization.

CONCLUSION

The study described partial genomic organization of SBE I in cassava. The study identified one intron in the genomic arrangement of SBE I. The isolated cassava SBE I sequence, HM046982, belongs to dicot subgroup A with poplar, lotus, potato and sweet potato. The introns will find applications in genetic modification of cassava for enhanced starch quality and in genetic manipulation of other crops.

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