Partial isolation of the starch branching enzymes [SBE] encoding gene from the plantain banana [Musa paradisiaca] genotype Raja

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Abstract. Banana fruit has the potential to be developed as a functional food in terms of its medicinal benefit. One of its features enabling such beneficial uses is its resistant starch characteristic. However, the starch resistance composition of the fruit will decrease during fruit maturation and food processing. Rationally, blocking of starch resistance decreasing process should maintain the beneficial characteristic of the banana fruit. This could be done by suppressing the expression of the SBE gene so that it can inhibit the formation of branching points in the amylopectin chain metabolism. Based on that consideration, we isolated the SBE gene involved in the formation of amylopectin in genotype Pisang Raja. In the initial step, the MaSBE_5458_F and MaSBE_5458_R primer combination was designed and successfully generated part of the sequence of the MaSBE5 gene. A total DNA sequence of 1,372 bp in length has been isolated, purified and characterized by sequencing technique. Blast analysis showed that the MaSBE_5458 gene sequence has a 97% similarity with 1,4-alpha glucan branching enzyme 1, chloroplastic/amyloplastic DNA sequence isolated from Musa acuminata subsp. malaccensis.

Keywords: SBE gene, resistant starch, functional food, Pisang Raja, amylopectin

1. Introduction
Banana is one of the important horticultural crops in world trade because of their contribution to the nutritional needs and public health. The genotype Raja [also popularly known as Pisang Raja] has a good opportunity to be developed as a functional food since it contains a high proportion of resistant starch. Resistant starch is a type of starch that is difficult to be digested during food processing in the human body. For that reason, such type of starch is recommended to be consumed by people with diabetes and obesity because of its low glycemic index [1].

The green banana fruit contains a high proportion of type II resistant starch. However, the type II resistant starch will decrease along with fruit maturation and food processing. An increasing proportion of resistant starch in natural ingredients can be done through modification of the starch biosynthesis pathway. That strategy is based on the well-accepted metabolism pathway, where most starches are composed of two types of glucose polymers, namely amylose and amylopectin. In amylose, the glycosidic bonds are formed in the form of α-1,4 glycosidic bonds, whereas in amylopectin there are two glycosidic bonds, namely α-1,4 glycosidic and α-1,6 glycosidic [2].
Resistant starch proportion is positively correlated with amylose content. The linear shape of the amylose molecule with a large number of hydroxyl groups allows it to more easily form hydrogen bonds with each other so that the hydrogen bonds that are formed become stronger. The existence of this hydrogen bond forms a helical structure in amylose, the presence of this bond makes amylose more difficult to hydrolyze by digestive enzymes [3]. Unbranched structures make amylose tightly bound so it is difficult to gelatinize and consequently difficult to be digested [4].

Increasing the resistant starch content in banana can be done by increasing the amylose content through genetic engineering on the metabolism of starch biosynthesis. The main enzyme involved in amylose synthesis is granule bound starch synthase [GBSS], while the enzymes involved in amylopectin synthesis are starch synthase [SS], starch branching enzymes [SBE] and starch debranching enzyme [SDBE] [5] [6]. Amylose content in starch can be increased by using two methods, namely increasing the expression [overexpression] of the GBSS gene or eliminating or suppressing the activity of genes associated with amylopectin synthesis such as SBE and SS [7] [8].

There are many studies on increasing amylose levels in some plants successfully using RNAi technology by inhibiting gene expression encoding for the formation of amylopectin synthesis such as SBE [9] [10] [11] [12] [5] [13] [14]. However, so far no report dealing with gene isolation, characterization, and modification of SBE in banana purposed to increase its amylose content. Here we report our initial result in isolating the SBE gene involved in the formation of the structure of amylopectin in banana genotype Raja.

2. Materials and Methods

2.1. Plants materials and Isolation of Genomic DNA
The young green and healthy leaf of banana genotype Raja was used for genomic DNA extraction using The Wizard® Genomic DNA Purification Kit [Promega-USA].

2.2. Specific Primer Designing
The specific primer for MASBE5 cloning was designed based on the gene sequence of GSMUA_Achr6T24120_001/MaSBE5 provided at https://banana-genome.cirad.fr/. The primer sequence position was determined using the primer - BLAST which is available on the website https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi. The primer was designated as MaSBE_5458_F and MaSBE_5458_R with an estimated product size of 1,307 bp. The detailed characteristic of primers designed in this study is listed in Table 1.

2.3. PCR-based Isolation of MaSBE5 Sequence
The total full length of MaSBE5 [cDNA sequence gene size] is 3041 bp, while the designed primer is expected to generate only 1,354bp product. The KAPA2G Robust PCR kits [Roche, Germany] was used to generate the expected fragment. The total of 25 µL PCR reaction contained 12.5 µL KAPA2G Robust DNA Polymerase, 2 µL of each primer [10 ng/µL], 1 µL genomic DNA [10 ng/µL], and 7.5 µL Nuclease free water. A touchdown PCR reaction was run with temperature ranged 70-55°C as described previously [15].

2.4. Cloning of MaSBE5 gene fragments
The MaSBE5 fragment produced from the PCR reaction was cloned into the pGEM®-T Easy Vector [Promega-USA]. The transformant DNA plasmid was then transformed into Escherichia coli strain DH5α competent cell. Using the blue-white selection technique, the putative transformants were further evaluated via colony PCR using T7/SP6 primers.

2.5. Sequencing and BLAST analysis
The sequencing of the cloned MaSBE5 fragment was performed using both primers. Sequence data were edited and analyzed using the BioEdit program [16]. Homology analysis of the MaSBE gene in
the nucleotide level was undertaken using the BLAST program which is freely available at http://www.ncbi.nlm.nih.gov/.

3. Results and Discussion

3.1. DNA Isolation
DNA isolation from the leaves of plantain banana of genotype Raja was successfully carried out and the result is visualized in Figure 1.

![Figure 1](Image1.png)

Figure 1. Genomic DNA isolated from *Musa paradisiaca* of Genotype Raja. M = Lamda[$\lambda$] DNA, 1-2 = DNA sample

The concentration of the $\lambda$ DNA marker used in this study is 100 ng/$\mu$L. The fragment intensity of the isolated DNA is predicted to be four-time brighter than $\lambda$ DNA, so the DNA concentration of the two samples should be 400 ng/$\mu$L. Based on that data, it is assumed that the quantity of isolated DNA should be sufficient for further analysis.

3.2. Primer, PCR, and Cloning of MaSBE_5458 Fragment
The specific primers combination was designed based on the sequence data GSMUA_Achr6T24120_001 which is available at https://banana-genome-hub.southgreen.fr/. The binding sites of the primer were determined to produce a single 1,354 bp PCR product. Primer characteristics are listed in Table 1.

| Name         | Nucleotide Sequence | Length [bp] | Tm °C | GC% | Product length [bp] |
|--------------|---------------------|-------------|-------|-----|--------------------|
| MaSBE_5458_F | GCC TCT CTA CGT GTA GGA AT | 20          | 53.4  | 50  | 1354               |
| MaSBE_5458_R | AGG TTT ATC GCA GAC ACC AC | 20          | 54.9  | 50  |                    |

PCR amplification successfully produced PCR product which is of 1,354bp in size [Figure 2A]. However, the generated amplicon also produced an additional faint fragment which is longer than the major expected product. Based on this, cloning steps were undertaken to further detail characterization.
The cloning experiment produced some white transformants [see Figure 2A]. In total 18 colonies were visible after overnight incubation at 37°C. Twelve colonies are white, indicating that those 12 colonies are transformants. In order to verify their recombinant status, a direct PCR colony was performed on all growing colonies. As expected, the 12 colonies produced fragment, while 6 colonies produced no amplicon [Figure 4]. For sequencing analysis, one of the putative transformants was selected.

3.3. Sequence analysis of MaSBE_5458 partial gene
The selected colony was subjected to the sequencing of the MaSBE_5458 partial gene. Sequencing was performed using both primers to cover the complete cloned fragment. Sequence data were further verified using the BioEdit program [16] to control its integrity. The forward primer MaSBE_5458_F produced a sequence of 1,462 bp in size, while the reverse primer MaSBE_5458_R produced a fragment of 1,198 bp in size. After the trimming and editing of both sequences, a contig was successfully constructed. An overlap segment cover 1,372 bp in size could be detected. The overlapping sequence segment is shown in Figure 4. Based on the constructed contig, a sequence of 1,372 bp in size could be verified. The length of the verified sequence is longer than that of expected

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Figure 2. PCR amplification [A] and cloning of PCR product [B]. M = 1 kb marker, 1-2 = sample

Figure 3. PCR colony of 18 samples after overnight incubation. M = 1 kb marker, 1-18 = samples of colonies amplification
before which should be 1,354bp. In order to address this issue, alignment analysis was performed using Clustal W Version 2.1 available at https://www.ebi.ac.uk/Tools/msa/clustalo/. In total 35 insertion-deletion [indel] events could be obviously identified, while base substitution is observed in 385 events [See Figure 5]. Many indel events could be observed in the low conserved area, while in the high conserved area only 4 indel events are existing. This is assumed to be an exon region. Figure 6 showed, that the exon 21 and 22 were obtained in the highly conserved area. These data were identified using WebDSV which is available on www.molbiotools.com.

Blast analysis of the MaSBE_5458 partial gene sequence showed a 97% similarity level with Musa acuminata subsp. malaccensis 1,4-alpha glucan branching enzyme 1, chloroplastic/amyloplastic.

**Figure 4.** An overlapping segment of sequence data generated from both primers MaSBE_5458F/R

**Figure 5.** Alignment analysis of the MASBE_5458 partial sequence with its reference [XM_009405458], yellow and green colored boxes are indel positions, while dark blue and red shaded sequence indicated binding site position of forward and reverse primers. The long insertion [green shaded color] are assumed to be intron sequence.

The reference sequence XM_009405458.2 has a length of 3,041 bp in size, while the isolated MASBE_5458 sequence covers only 1,372 bp. In order to cover the full length of the MASBE_5458 gene sequence, about 1,669 bp should be isolated. Therefore a chromosome walking strategy will be applied in future work.
4. Conclusion
The partial Starch Branching Enzymes [SBE] encoding gene from plantain banana [Musa paradisiaca], genotype Raja involves 1,372 bp is successfully isolated covering 2 exons. Blast analysis showed that the sequence has 97% similarities with XM_009405458 isolated from Musa acuminatasubsp.malaccensis.

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