RESEARCH ARTICLE

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Isolation and Analysis of Sucrose Synthase (SUS) Gene Fragment Originated from "Kopyor" Coconut Mutant

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"Kopyor" coconut is a naturally occurring coconut mutant having abnormal endosperm. The content of sucrose in the "Kopyor" coconut endosperm is twice as much as normal one. Fragment of sucrose synthase (SUS) gene, one coding for sucrose synthase enzyme involved in the sucrose biosynthesis, was isolated from "Kopyor" coconut and its sequence were analyzed. The size of DNA fragments of SUS gene originated from "Kopyor" coconut (CnSus1A, CnSus1B, CnSus2A, and CnSus2B) were 746, 738, 780, and 687 bp, respectively. Analysis of DNA sequences of CnSus1A, CnSus1B, CnSus2A, and CnSus2B showed features of non-grass monocotyl of SUS gene. Gene specific primer pairs (CnSUS1A, CnSUS1B, CnSUS2A, and CnSUS2B) were designed based on sequences of SUS gene fragments and they could be used for analyzing variability of SUS gene among normal and mutant coconut.

Keywords: Sucrose Biosynthesis Gene, Gene Specific Primers, Coconut Mutant.

1. INTRODUCTION

The "Kopyor" coconut from Indonesia is a rare naturally occuring coconut mutant found in Java Island. This unique coconut mutant may also be known as Makapuno coconut in the Philippine. Although morphologically similar to normal coconut, "Kopyor" coconut mutant exhibited unique features, such as the fruit has soft, crunchy and loose endosperm.

Unlike normal coconut, fruit of the "Kopyor" coconut has normal embryo but the embryo unable to normally germinate under natural conditions. The inability to normally germinate under natural conditions may be due to the inability of the embryos to utilize food reserved during germination process. Food reserves for germination process of the coconut embryos are mostly stored in endosperm in the form of galactomannan. Galactomanan stored in the endosperm cell walls as food reserves in the form of cell wall polysaccharides.[1]

Degradation of galactomannan results in formation of galactose and mannose that can be used as carbon and energy sources during the early stage of coconut embryo germination.[2,3]

Galactomannan is a polysaccharide compound deposited in the cell wall of coconut fruit endosperm. In mature coconut fruit, 61% of total polysaccharide existed in the endosperm is galactomannan.[4] In the case of "Kopyor" coconut fruit endosperm, α-D galactosidase as one of enzymes that degrade galactomannan was absence[5] resulting in interfering of normal galactomannan degradation.

Previous experiment indicated endosperm of "Kopyor" coconut fruit contained different composition of carbohydrate and sugar than that of normal one.[6] Total sucrose content of "Kopyor" coconut fruits endosperm was reported twice as much as that in normal fruits while in the coconut milk of "Kopyor" coconut fruits were eight times higher than that in normal fruits. Endosperm of "Kopyor" coconut was also reported to contain 62.3% of carbohydrates and 30.7% of fat. On the other hand, endosperm of normal coconut contained more fat than carbohydrates.

Sucrose synthase (SUS) is an enzyme involved in biosynthesis of carbohydrates in storage organ. Increased sucrose synthase activity was observed during last stage of coffee fruit endosperm development and the increased pattern was correlated with sucrose accumulation in developed seeds.[7] In various studies, synthesis of sucrose occurred at the same times as the degradation of galactomannan into mannose and galactose.

Isolation and characterization of sucrose synthase gene from "Kopyor" coconut has never been reported. The objectives of this research were to develop degenerate primers for SUS gene, isolate fragment of SUS gene using PCR and analyze DNA sequences of the PCR amplified product, and develop SUS gene specific primers for further diversity analysis of "Kopyor" coconut.

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2. MATERIALS AND METHODS

2.1. DNA Extraction, Primer Design and PCR Amplification

Newly emerged young leaf samples were obtained from coconut trees producing "Kopyor" fruit and used as primary sources for isolating total genomic DNA of coconut. Leaflet at the second and third position of the distal side of the young leaves were taken from "Kopyor" coconut provenances at the Margoyoso and Tayu sub-district, Pati, Central Java, Indonesia. Every location, ten (10) "Kopyor" coconut provenances were selected randomly.

As much as 50–100 mg of each fresh leaf "Kopyor" coconut samples were cut into pieces and ground in Eppendorf tubes using tissue grinder (Retsch MM301) at frequency of 300 hertz for three minutes. Homogenized tissues were incubated overnight in lysis buffer containing RNAse and filtrated using filter collumn. Subsequently, the procedures for DNA extraction were conducted according to manufacture steps as described in Plant Genomic DNA Mini Kit (Geneaid, Geneaid Biotech Ltd. http://www.geneaid.com).

Degenerate primers to amplify DNA fragment of SUS gene were designed based on known SUS sequences available in the GenBank DNA databases. The SUS gene from Oryza sativa (accession No. EF122480) was used to obtain other accession of SUS DNA sequences from DNA database (http://www.ncbi.nlm.nih.gov/; http://www.comphio.dfci.harvard.edu/tgi/; http://www.ebi.ac.uk/embl) using BLAST (http://www.ncbi.nlm.nih.gov/). A number of SUS gene accessions originated from both monocotyle and dicotyle plant species were selected and the DNA sequences were retrieved from GenBank. The selected accessions were having at least 70% identity with rice SUS sequences and 1.000–2.000 bp in length. Multiple allignment of selected SUS DNA sequences were conducted using ClustalW (http://www.ebi.ac.uk/embl), output of multiple alignment was exported to GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc), and used to identify the exon and intron parts of the SUS gene. Degenerate primers were design around highly conserved regions of the SUS exon.

Amplification of SUS gene fragments was by standard PCR amplification using SUS degenerate primers and total genomic DNA of "Kopyor" coconut as template. The total volume of PCR amplification reaction was 15 ul and consisted of genomic DNA (1 ul), a pair of degenerate primer (0.75 ul), dNTPmix (2.7 ul), 10× buffer (1.5 ul), $MgCl_2$ (Fermentas, 0.15 ul), and Taq DNA polymerase (RBC, 0.075 ul). Amplification was conducted by one cycle of pre-denaturation at 94 °C for 4.0 minutes, followed by 40 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 52 °C–55 °C for 45 seconds, and primer elongation at 72 °C for 90–120 seconds and ended by final extension at 72 °C for 5 minutes, followed by cooling at 16 °C for 10 minutes. The product of PCR Amplification were evaluated by using agarose (1%) gel electrophoresis at 300 Am and 200 V for 20 minutes.

2.2. DNA Sequencing and Its Analysis

Positive and clear PCR product amplified from a number of "Kopyor" coconut DNA were bulk into one mixture and the DNA was purified from contaminants following procedures for PCR clean up kit of Gel/PCR DNA Fragments Extraction Kit (Geneaid). Purified DNA fragment of PCR products were ligated and cloned into pGEMP-T (Promega). The ligated recombinant plasmids were introduced into *E. coli* DH5 competent cells. The transformed bacterial colonies were cultured on 800 ml liquid and shaked on orbital shaker at 220 rpm. After one hour at 37 °C, the cultures were spread on LB medium plates containing IPTG and X-gal. The desired *E. coli* colonies carrying recombinant plasmid were selected based on the blue and white colors of the colonies. The presence of recombinant plasmid in the selected *E. coli* colony was confirmed by PCR.

Selected white bacterial colony was inoculated onto 5 ml of liquid LB medium and incubated on an incubator shaker (220 rpm) at 37 C for 16 hours. The recombinant plasmid was isolated from bacterial cells following procedures for high-speed plasmid mini kit (Geneaid). Relative concentration of isolated recombinant plasmid were determined by comparing with standard DNA marker in 1% agarose gel electrophoresis. Recombinant plasmid positively identified as carrying the right size of DNA fragment were send to 1st Base Pte. Ltd. (http://www.base-asia.com/) for DNA sequencing.

Determined DNA sequences were read using Chromas Lite version 2.01 software (http://www.technelysium.com.au) and were cleaned from plasmid sequences. The remaining DNA sequences were exported to GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc) to determine positions of the primers, intron and exon. To determine the identity of the amplified DNA fragment, the DNA sequences were blasted against all accession in GenBank DNA database using BlastN (http://www.ncbi.nlm.nih.gov/). In addition, the combined exons were translated into amino acid sequenced and blasted against known protein sequences using query-translated db (TBLASX) available at http://www.genebee.msu.su/blastnew/blastform.php?program=tblastx. Multiple alignment of amino acid sequences translated from determined DNA sequences and selected accession from database were done using ClustalW (http://www.genebee.msu.su/clustal/advanced.html) and the outputs were exported into GeneDoc. Phylogenetic analysis for both DNA and translated amino acid sequences were also conducted in the format of Phylip-Phylogram.

3. RESULTS AND DISCUSSION

Sucrose synthase (SUS) is the enzyme involved in the synthesis of sucrose which catalyzes the reversible reaction: UDP-glucose + fructose ↔ sucrose + UDP.[8] Two pairs of degenerate primers SUS gene were resulted in this research, namely SUS1 and SUS2 with nucleotide sequences as shown in Table I. All degenerate primer pairs could amplify DNA SUS gene with annealing temperature of 52 °C. Figure 1 showed SUS1 and SUS2 degenerate primers could amplify total genomic DNA template from "Kopyor" coconut with PCR products between 650–700 bp.

Table I. Degenerate primer pairs and expected product size of SUS gene fragment amplified through PCR using total genomic DNA of "Kopyor" coconut mutant.

Size of PCR products (bp)	Sequences of the degenerate primers	Primer codes
650–700	5′-GGTTATCCTGATACYGGNGGNCA-3′	SUS1-F1
	5′-ACAAGGTTTCCATCACTRTARTTNCC-3′	SUS1-R1
650–700	5′-GATCCCAAGTTCAACATTGTNTCNCC-3′	SUS2-F2
	5′-CCAAATGCTTCATARAANGCNGG-3′	SUS2-R2

Note: N = A/G/C/T, R = A/G.

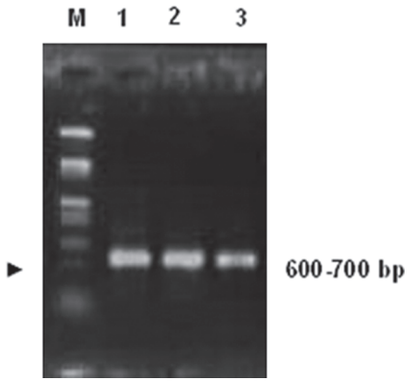

Fig. 1. DNA fragments amplified by PCR using degenerate primer pair of SUS2 and total genomic DNA of "Kopyor" coconut mutant from Sub-Distric Tayu (1) and Margoyoso (2). PCR amplified product using degenerate primer pair of SUS1 and total genomic DNA of "Kopyor" coconut mutant from sub-distric Margoyoso (3).

Degenerate primer pairs of SUS1 produce two kinds of nucleotide sequences of DNA fragments, CnSus1A with the size of the DNA fragments of 746 and CnSus1B with a length of 738 bp (Fig. 2). SUS2 primer pair also produced two kinds of nucleotide sequences of DNA fragments with a length of 780 (CnSus2A) and 687 bp (CnSus2B).

The differences between CnSus1A with CnSus1B or CnSus2A with CnSus2B was on the length of the fragments. It is caused by variations in the length of the intron regions, exon size remains the same between the fragments. The overall size of exons 7 and 8 (exons intact) were 96 and 174 bp. This size of CnSus1A and CnSus1B demonstrated compliance with exon size sucrose synthase of citrus plants (Citrus SUS1 and Susa).[9] At the position of exon 12 of the CnSus2A and CnSus2B contained one intron insertions. Inserts one intron gene insertion is typical structure of SUS at monocotyledonous where it is not present in exon 12 of SUS dicotyledonous plants.[9] As it is known that the area can be translated to the SUS genes from various plant consists of 806 to 812 amino acid.[9,10] The length of this amino acid is divided into 13 exons for dicotyledonous plants and 15 exons for

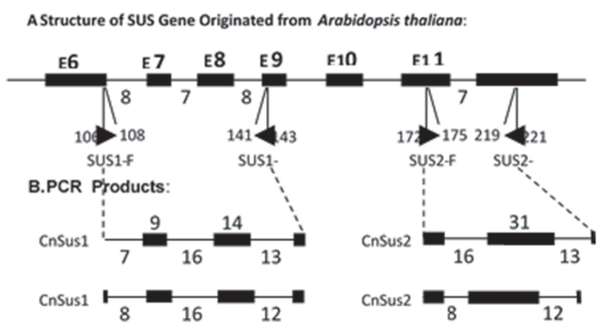

Fig. 2. Partial structure of SUS gene originated from *Arabidopsis thaliana* (GenBank accession No. NM122090) and PCR product generated using *degenerate* primer. (A) Exon and intron portion of SUS Gene of *A. thaliana* and location of the designed degenerate primer pairs (forward/F and reverse/R) in this research. (B) Amplified DNA fragments yielded through PCR using degenerate primer pair of SUS1 (SUS1-F and SUS1-R) or SUS2 (SUS2-F and SUS2-R) and template of total "Kopyor" coconut mutant genom. Relative position of the PCR product CnSus1A, CnSus1B, CnSus2A and CnSus2B against SUS gene originated from *A. thaliana* were noted.

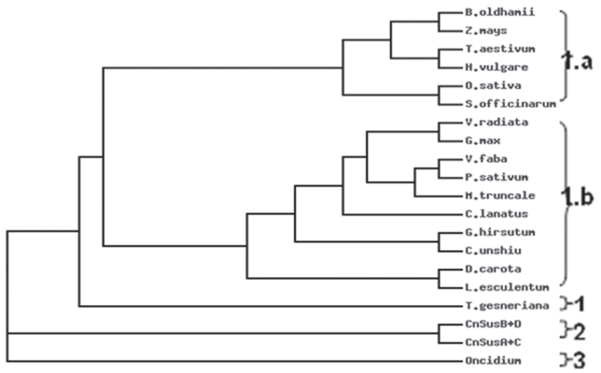

Fig. 3. Dendrogram constructed based on translated amino acid sequences of putative fragment of sucrose synthase from "Kopyor" coconut mutant CnSus1A, CnSus1B, CnSus2A and CnSus2B and a number of SUS sequences available in GenBank database (*Tulipa gesneriana*, accession No. X96938; *Triticum aestivum*—AJ000153; *Saccharum officinarum*—AF263384; *Oryza sativa*—Z15028; *Hordeum vulgare*—X69931; *Zea mays*—L22296; *Bambusa oldhamii*—AF412037; *Oncidium*—AF530567; *Vigna radiata*—D10266, *Glycine max*—AF030231; *Hordeum vulgare*—X69931; *Citrus lanatus*—AB018561; *Vicus faba*—M97551; *Pisum sativum*—AJ012080; *Medicago truncale*—AJ131943; *Gossypium hirsutum*—U73587; *Citrus unshiu*—; *Lycopersicon esculentum*—L19762; and *Daucus carota*—X75332.

monocot plants. Excess two exons in monocot plant caused by intron insertion in exon 6 and exon 12.

Sucrose synthase in plants are generally found in the form of isoforms and at least encoded by two genes, for example 2 genes in the coffee plant (*Coffea arabica*),[7] 3 genes in citrus (*Citrus unshiu*),[9] and 4 genes in bamboo.[10] Dendrogram of "Kopyor" SUS gene made based on the nucleotide sequences of amino acids derived from each DNA fragment. It divided into 3 groups (Fig. 3), namely group 1 was monocot group consisting of *B. oldhamii, O. sativa, S. officinarum, Z. mays, T. aestivum* and *H. vulgare*. Group 2 was SUS of dicotyledonous plants, such as *Citrus lanatus, Vicus faba, Pisum sativum, Medicago truncale, Gossypium hirsutum, Citrus unshiu, Lycopersicon esculentum* and *Daucus carota*. Group 3 consisted of SUS to "Kopyor" coconut fruit (CnSus1A + CnSus2A and CnSus1B + CnSus2B) and *Oncidium*. The interesting thing is SUS of "Kopyor" coconut separated from other monocotyledonous such as *B. oldhamii, O. sativa, S. officinarum, Z. mays and T. aestivum*.

SUS of plants classified into four different groups namely: group monocots, dicots SUS1 group, group dicotyl Susa, and the new group.[11] SUS genes in plants monocots further divided into 3 groups: group SUS1 grasses, SUS2 grasses, non-grass and SUS.[9] Based on phylogenetic analysis of amino acid sequences (Fig. 4), sucrose synthase: CnSus1A + CnSus2A and CnSus1B + CnSus2B entered into a non-grass SUS group is one group with *Oncidium*. It seems SUS of "Kopyor" coconut genome more closely related to species of orchids than grasses.

4. CONCLUSION

The various DNA fragments of Sucrose synthase in "Kopyor" coconut could be found from the exon 6 to 12 with typical of monocotyledonous plants. "Kopyor" SUS divided into three groups where the "Kopyor" SUS separated from other monocots. The nucleotide sequences of CnSus1A, CnSus1B, CnSus2A, and CnSus2B can be used as a template for designing specific

primers. SUS gene-specific primers of "Kopyor" coconut can be used for molecular analysis on the types of kopyor and normal coconut.

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