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CLONING AND SEQUENCING OF β-MANNANASE GENES FOR GALACTOMANNAN BIOSYNTHESIS IN COCONUT (*Cocos nucifera* L)

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ABSTRACT

"Makapuno" coconut, an important coconut mutant, comes from Filipino have a high commercial value due to the accumulation of galactomannan in maturing Makapuno nut. This accumulation leads to many elite applications in coconut such as in food industry, medical, pharmaceutical and personal health. It is also considered as an important factor for providing high currency resource accompany with potential war material in the future. However, during last decades, coconut genome is not sequenced completely as well as the study of beta-mannanase gene in coconut is still very little. Thus, in this article, we designed primers OSBMannan-F and OSBMannan-R for targeting beta-mannanase gene through beta-mannanase gene in rice genomic. PCR products cloned into pGEM-Teasy vector and subsequently sequenced.

We were successful for amplifying beta-mannanase gene in coconut with approximately 400bp DNA fragment size. The sequences of cloned PCR products were sequenced. Nucleotide sequences in this report have been submitted to the Genebank database with accession number EU1198197 (coconut) and EU1198710 (rice).Sequence analysis with BLAST indicated that the sequence is relatively high similar to with others which derived from NCBI. The relatively high level of conservation revealed the high level of evolutionary contrainst and the importance of its function

Successful amplification for beta-mannanase gene in coconut by using primers, which designed on rice genome, it opens the way for applications to find target gene through another genome indirectly. To the best of our knowledge, the results presented here are the first characterization of beta-manannan gene in coconut. The further analysis is necessary for identifying nucleotide sequence and its functional site more exactly.

Key word: β-mannanase, galactomannan, Makapuno coconut, sequencing.

INTRODUCTION

Coconut (*Cocos nucifera* L) is an important element in tropical vegetable, especially along the seashore of widely scattered islands and islets in the Pacific Ocean. It provides the basic human needs and having a means of livelihood to millions of people living in the tropical belt. About 12 million hectares of coconut are grown worldwide with 96% of farmers being smallholder who have been suffering in the last two decades due to declining farm productivity and unstable markets for their traditional coconut product. Consequently, coconut farmers have a low average net income, they are living below the poverty line. Moreover, the majority of coconut product only utilized as fresh fruit, rural food system, building material, firewood and oil extraction. Coconut potential value was not appreciated correctly although it can be considered such as resource for high foreign earnings. Makapuno, an "important coconut mutant" comes from the Filipino, which has high commercial value mainly due to high accumulation of galactomannan in maturing Makapuno nut. Galactomannan occur in nature as the reverse polysaccharides in the endosperms of a wide range of legume seeds and is composed of a 1, 4-linked D-mannan backbone to which single

D-galactosyl units are attached to C-6 of some of the D-mannosyl residues. These polysaccharide partially purified form, find widespread applications as thickening and gelling agents in food industry. It has been demonstrated to perform other functions, such as modulation of the mechanical strength of the endosperm of seeds to facilitate radicle protrusion (Groot and Karssen, 1978) and to control water imbibition in early stages of germination (Reid and Bewley, 1979). It takes up proportionally high amounts of water and distributes it around the embryo, protecting it against loss of water (Reid and Bewley, 1979). They are most commonly found in the Leguminosae and Palmae families, but are also components of the endosperm cell walls of the Annonaceae, Ebonaceae and Loganaceae (Matheson, 1990), where they act as carbohydrate reserve (Reid, 1985) and may assist in protecting the embryo from desiccation (Reid and Bewley, 1979). Moreover, galactomannan are endosperm cell wall polysaccharide referred to as gums because these polysaccharides produce gels or highly viscous solutions at low concentrations in solvents, and thus, these polysaccharides havea myriad of applications in industry (Whistler et al., Introduction to industrial gums, In Industrial Gums: Polysaccharides and their derivatives, Whistler and BeMiller, eds., Academic Press, San Diego, pp. 1-19, 1993). This leads a promising product from coconut in commercial food preparation and industrial applications. Other industries where the potential of Makapuno is being towarded are in the pharmaceuticals, medical, and personal care industries; and it also become a "potential war" material in the future.

The synthesis of the gum galactomannan is catalyzed by the enzymes beta-mannannase and alpha-galactosidase. However, the last two decades, there has been progress and studies on the elucidation of the genes involved in galactomannan biosynthesis, very little is known.

In this context, the aim of the study is to identify beta-mannanase gene which participate in the process of galactomannan biosynthesis in coconut.

MATERIALS AND METHODS

Plant materials: Seeds of rice, *Oryza sativa* L, were sown directly in soil in planting trays. Seedling leaf material was harvested after 14 days and stored in 1.5 ml tubes at -20^{0}C. IR64, a widely grown *indica* variety in Asia, was obtained from IRRI Genebank. For coconut, One "makapuno" coconut sample (Makapuno type I – nut cavity is filled completely with soft endosperm) was obtained from the Philippine Coconut Authority research station in Guinobatan, Albay. Coconut fruit has maturity in 5-6 months old. The endosperm was harvested and stored in tubes at -20^{0}C.

Genomic DNA isolation

DNA was isolated from coconut endosperm and rice leaves using liquid nitrogen. The protocol was described by Xin'ai Zhao (2002). Grind samples in liquid nitrogen. Add 0.5 ml DNA extraction buffer [1% Sarcosyl, 100mM Tris-HCl (MW: 121.1), 100mM NaCl (MW: 58.44) and 10mM EDTA (MW:372.2)]. Add 0.5ml Phenol**:**Chloroform: Isoamylalcohol. Vortex and centrifugation at max speed, 10min, 4ºC. Transfer supernatant to a fresh 1.5 ml microfuge tube (optional: a second Phenol **:** Chloroform **:** Isoamylalcohol extraction. Add 100µl 3M Sodium Acetate and 1 ml Isopropanol (2- propanol) for precipitation of DNA. Centrifuge at max speed, 15min, 4ºC**.** Wash pellet in 70% pre-cooled EtOH. Dry and resuspend pellet in 100µl TE buffer. DNA qualtility characterized through spectrophotometry (1:500 dilution)

PCR and TA cloning

The PCR reaction was conducted in a final volume of 25 µl containing 5 µl 5X buffer with $MgCl_2$, 2 µl of 10 mM dNTP, 1 µl of each 5 mM primer, 1 µl genomic DNA and 0.1 µl of 5U/ µl Tag polymerase (Promega GoTag). Target genes were amplified with specific primers OSB Mannan F (5' AACGGCGAGTAATCTTGCTG 3') and OSB Mannan R (5'AGGTGGTTGGCGATGAAG

TC3'). Two primers, based on mannanase gene in rice genome, were designed to amplify the coding region of mannanase gene by using Primer3 BLAST. The reaction conditions for PCR included a denaturing step of 94^0 for 5 min followed by 35 cycles of 30 sec at 94^{o}C, 30 sec at 59^{o}C and 1 min 30 sec at 72^{o}C, ending with an elongation step of 5 min at 72^{o}C. After agarose gel 1% electrophoresis, PCR products was purified by using QIAquick gel

Extraction Kit (QIAGen, USA) and cloned into pGEM-T easy vector (Promega, Madison, WI), and was subsequently sequenced. Setting up ligation reactions contain 5 µl of 2X rapid ligation buffer plus T4 DNA ligase, 1 µl pGEM-T easy vector, PCR product and 1 µl T4 DNA ligase. Incubate the reactions 1-2 hour at room temperature. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 16^{o}C for 16h.

Transformation into JM 109 cells

For transformation, we used JM109 high efficiency competent cells. These cells are provided with the pGEM-T and pGEM-T easy vector systems II and compatible with blue/white color screening and standard ampicilin-resistant selection. JM109 cells were maintained on minimal medium plates supplemented with thiamine hydrochloride prior to the preparation of competent cells. The reaction of transformation the following as: 5µl of recombination plasmid and 50 µl of competent cells. Sit on ice about 20 min. Heat shock at 42^{0}C for 1 min after that place on ice for 2 min. Cells were treated with $CaCl_2$ solution followed by heat shock step at 42^{o}C which causes plasma membrane of bacterial cell become permeable and capable of uptake of plasmid DNA. To help cells recover after heat shock step, the cells were incubated in 950µl LB medium with SOC. For the transformation control, 1:10 dilution with SOC medium is used for plating. Incubate at 37^{0}C with gentle shaking for 1h 30min. Selection for transformants was conducted on the plates containing LB/ampicilin/IPTG/X-gal medium with 100 µg/ µl ampicilin, 0.5 mM IPTG and 80 µg/ml X-gal. Recombinant plasmids were extracted and digested by restriction endonuclease enzyme digestion EcoRI with approximately 400bp DNA fragments to test presence of insert. Total volume (20 µl) of digestion reaction contain 2 µl of 10X buffer, 4 µl plasmid DNA, 0.2 µl EcoRI per one reaction and 13.8 µl sterile distill water.

Sequencing and sequence analysis

The inserted PCR fragments were sequenced by a capillary automated ABI PRISM 3700 DNA sequencer (Macrogen, South Korea) until the whole inserted fragment was sequenced. Similarity searches of the sequenced DNA and deduced amino acid sequence and were done by BLAST and EMBL/Swiss Prot available online. A multiple-sequence alignment was performed using CLC combined Workbench 3.0.3. The species in the sequence alignment contain manannase gene derived from NCBI with accession number represent (*Bacillus licheniformis* FJ594484, *Bacillus subtilis* EU918394, *streptomyces sp*. EU399236, *Aspergillus sulphureus* DQ328335 and *Dictyoglomus thermophilum* AF013989). SignalP 3.0 Server (http://www.cbs.dtu.dk/services) predicted signal peptide. ScanProsite were performed to analyze functional site of the deduced amino acid sequence (http://www.expasy.org/prosite).

RESULTS

PCR amplification of beta-mannanase gene

Using primers OSBMannan-F and OSBMannan-R which designed on the basis of beta-mannanase gene in rice genome. We were successful in amplifying a 400-bp band from genomic DNA coconut. These PCR products were digested by EcoRI enzyme and cloned for sequencing.

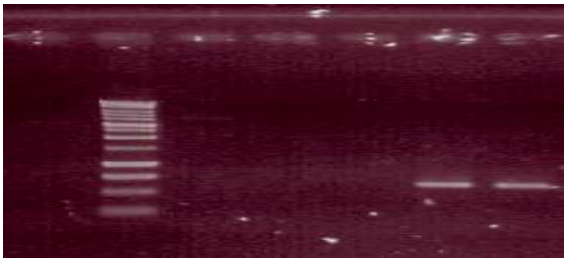

Fig. 1: PCR amplification products from rice and coconut genomic DNA. Primers which designed on beta-mannanase gene in rice genome, used to amplify the coding region of beta-mannanase gene. PCR product size is 400 bp. 1: Coconut, 2: rice

Sequence analysis of PCR products

After screening 1.000 recombinant plasmid, 3 clones were isolated and sequenced. The nucleotide sequence and analysis of cloned DNA fragments from genomic DNA of coconut and rice through PCR reaction to be identified and translated into amino acid sequence for consideration of homologous. The resulting sequences was submitted to Genebank database with accession number EU1198197. The

nucleotide sequence analysis of all clones showed an insert of approximately 950 bp for coconut and 1130 bp for rice. The clones had six open reading frame for rice and six other for coconut. There are five exon in rice located with exon 1 (13-756), exon 2 (758-775), exon 3 (781-948), exon 4(951-965) exon 5 (967-1128); and seven exon in range of nucleotide (13-728, 873-1176, 1186-2289, 2291-2308, 2314-2481, 2484-2498, 2500-2661) with one intron (729-872). The nucleotide sequence of rice and coconut were identical with beta-mannanase gene, *Bacillus licheniformis* FJ594484, *Bacillus subtilis* EU918394, *streptomyces sp*. EU399236, *Aspergillus sulphureus* DQ328335 and *Dictyoglomus thermophilum* AF013989. Figure 2 shows a comparison of nucleotide sequence of these clones with others. The prediction results of signal peptide showed that two putative domain have also been detected COG3934 (residue 125-250; 600-625) in coconut and COG3934 (residue 150-200; 320-350) in amino acid sequence**.** ScanProsite results showed that there existed many functional sites in the submitted sequence of rice and coconut such as arginin-rich region, cAMP- and cGMP-dependent protein, Protein kinase C phosphorylation site, Casein kinase II phosphorylation site, N-glycosylation site.

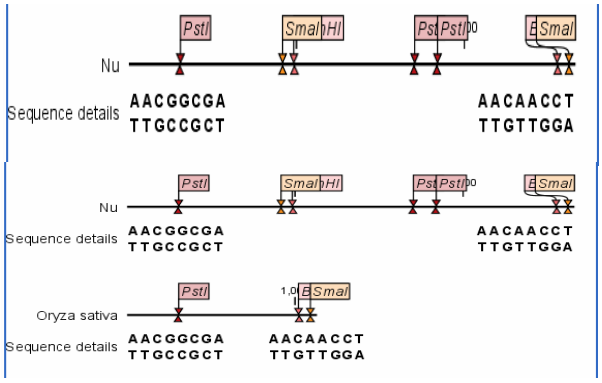

Fig. 2: the graphic shows cloning site in coconut nucleotide sequence (a) and in rice (b)

The cladogram shown in fig.3 depicted the phylogenetic relationships inferred from the complete nucleotide sequences available in NCBI database. The neighbor-Joining method in MEGA version 3.0 was used to construct the phylogenetic tree based on Poisson-corrected pairwise idstances between prorein sequences and node robustness was assessed using a bootstrap approach. The relatively small size of beta-mannanase protein sequence resulted in a reduction in the statistical significance of the bootstrap analysis of this tree. The tree shows that beta-mannanase gene from coconut (sequenced on SP6 promoter of pGEM-T easy vector) and *Oryza sativa*; coconut (sequenced on T7 promoter of pGEM-T easy vector grouped together, support its close relationship.

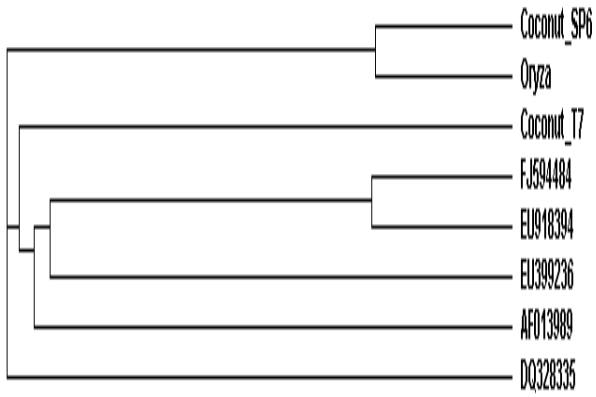

Fig. 3: The cladogram of beta-mannanase nucleotide sequences. The nucleotide sequences of rice and coconut and from *Bacillus licheniformis* FJ594484, *Bacillus subtilis* EU918394, *streptomyces sp*. EU399236, *Aspergillus sulphureus* DQ328335 and *Dictyoglomus thermophilum* AF0139

Phylogenetic analysis

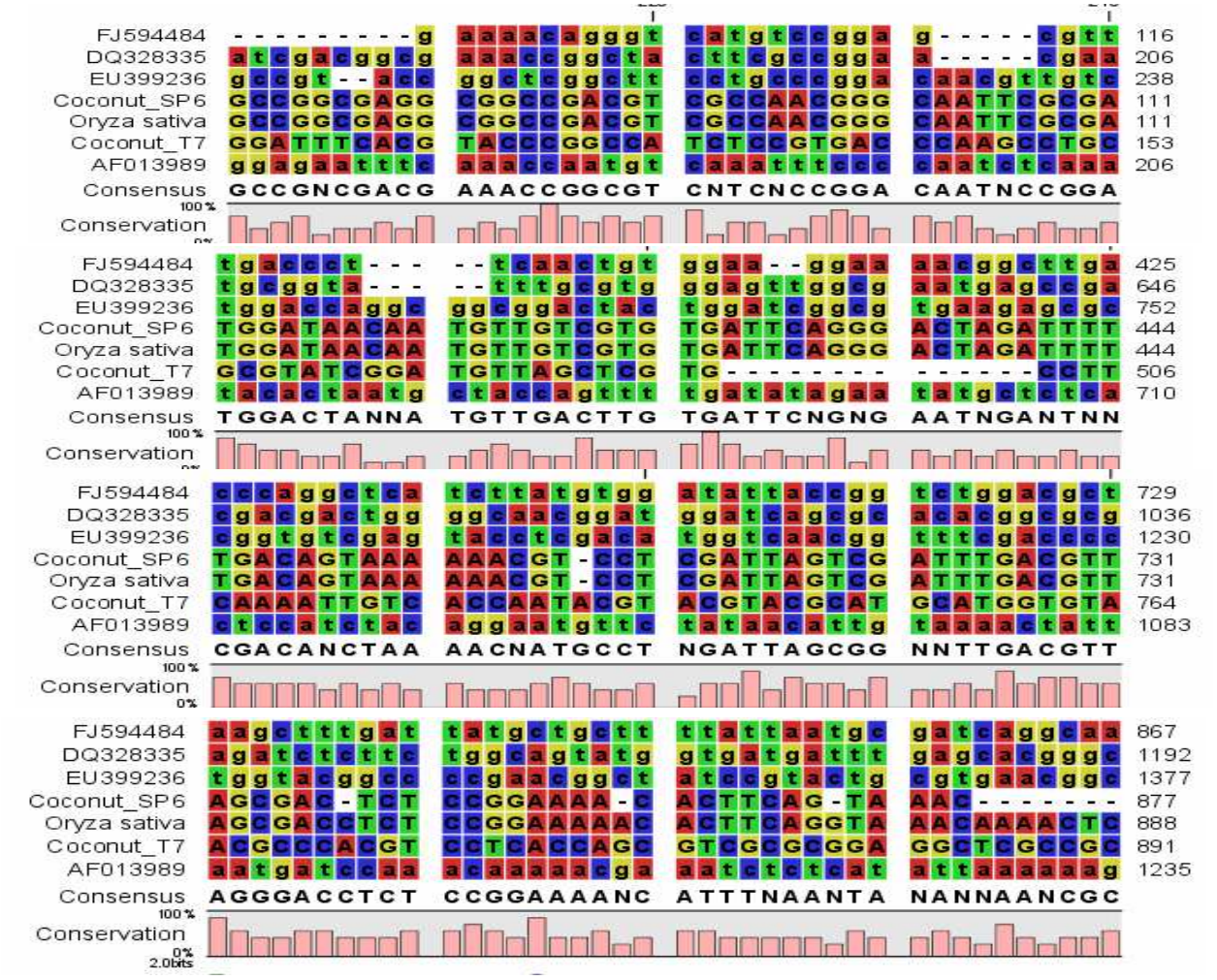

Fig. 2: Comparison of nucleotide sequence of rice and coconut with the others. GeneBank accession numbers are as follows: *Bacillus licheniformis* FJ594484, *Bacillus subtilis* EU918394, *streptomyces sp.* EU399236, *Aspergillus sulphureus* DQ328335 and *Dictyoglomus thermophilum* AF013989. the column conservation represents the homologous level between nucleotide sequence of species.

DISCUSSION

Through PCR reaction, we have been able to amplify coding region of beta-mannanase gene for a band pattern 400 bp with primer OSBManan-F and OSBMannan-R which designed on beta-mannnanase in rice genome. The primers OSBMannan-F and OSBMannan-R have Tm (58% and 57.9), GC% content (52.6% and 60%), respectively. Beside, with understanding and sequencing of coconut genome restrictedly, this is very important significance in studying for beta-mannanase target gene in coconut. Some genome of eukaryotic organism such as *Arabidopsis thaliana* etc. were sequenced completely, it allow us to ultilize primer design for searching beta-mannanase gene, identify similarity in different sequences supporting for understanding of its functional sites more exactly.

BLAST search similarity indicated that the obtained sequences from coconut and rice are relatively similar to nucleotide sequence of organisms, which contain beta-mannanase gene with more than 50%. This sequence encodes a protein of 864 aa in coconut and 364 aa in rice. By

results of signal peptide and ScanProstie of deduced amino acid sequences, two putative domains COG3934 have also been identified and existed many functional sites such as arginin-rich, protein kinase phosphorylation site and N-glycosylation site. Based on the above results, the isolated sequence was assumed to be homolog of beta-mannanase gene. On the one hand, our cladogram analysis reported close relationship in sequence of rice and coconut with those of others. The relatively high level of conservation among all sequence containing beta-mannanase gene were known, It suggest that the high level of degradation constraints and the importance conservation of its function.

CONCLUSION

Because beta-mannanase participate in galactomanan biosynthesis which was accumulated in makapuno coconut. They offer the much value in food industry, medical and personal health. However, at present, the studies about beta-mannanase is too little. We proposed that the further analysis and characterization of beta-mannanase gene expression would help to reveal nucleotide sequence and its functional sites. To the best of our understanding, the results presented here are that the first characterization of a gene encoding beta-mannanase in coconut.

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Dòng hóa và đọc trình tự các gen β-mannanase trong sinh tổng hợp galactomannan của cây dừa (*Cocos nucifera* L)

Dừa "Makapuno", là giống dừa đột biến quan trọng của Philippines, có giá trị thương trường cao do tích tụ được nhiều galactomannan trong quả dừa chín. Sự tích tụ này làm cho nhiều ngành công nghiệp phát triển theo như thực phẩm, dược liệu, y khoa và dịch vụ sức khỏe cho người. Nó còn được xem như nguồn vật liệu có tính hiện thực cao kết hợp được với vật liệu dung trong chiến tranh tương lai. Tuy nhiên, trong thập kỷ qua, bộ gen của cây dừa vẫn chưa được giải trình tự đầy đủ cũng như việc nghiên cứu beta-mannanase gene trong cây dừa còn rất ít. Trong báo này này, chúng tôi thiết kế cặp mồi OSBMannan-F và OSBMannan-R để đánh dấu beta-mannanase gene thong qua gen tương đồng beta-mannanase của bộ gen cây lúa. Sản phẩm PCR products được dòng hóa trong plasmid pGEM-Teasy và đọc trình tự ngay sau đó.

Chúng tôi khuếch đại được beta-mannanase gene trong cây dừa với kích thước DNA là 400bp. Trình tự sản phẩm PCR được dòng hóa đã cho kết quả để thảo luận. Nucleotide sequences được đăng ký trong Genebank database với số đăng ký EU1198197 (coconut) và EU1198710 (rice). Phân tích sequence nhờ BLAST cho thấy rằng trình tự này tương đồng với kết quả của NCBI.