GENOMIC WALKING OF HEAT SHOCK PROTEIN 70 AND CYSTEINE PROTEASE IN SAGO PALM

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Genomic Walking of Heat Shock Protein 70 and Cysteine Protease in Sago Palm

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This project is submitted in partial fulfillment of the requirement for the degree of
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Declaration

I hereby declare that the thesis hereby submitted is my original research work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature: [Signature]

(Tie Chui Ping)

Date: 36.05.2011
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AP</td>
<td>Adaptor Primer</td>
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<tr>
<td>CTAB</td>
<td>Cetyltriethylammonium bromide</td>
</tr>
<tr>
<td>CysProt</td>
<td>Cysteine Protease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GSP</td>
<td>Gene Specific Primer</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat Shock Proteins 70 kilodalton</td>
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<tr>
<td>Hsp70</td>
<td>Heat Shock Proteins 70 kilodalton</td>
</tr>
<tr>
<td>NP</td>
<td>Nested Primer</td>
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Genomic Walking of Heat Shock Protein 70 and Cysteine Protease in Sago Palm

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Abstract

The word sago is originated from Javanese which means starch containing palm pith. It is an important crop with great potential for commercial plantation. Heat Shock Proteins (hsp) are synthesized rapidly when a cell is confronted with adverse environment. Hsp70 functions as a molecular chaperon by stabilizing the proteins and prevent them from becoming irreversibly denatured. Cysteine protease is also believed to have involved in signaling during hypersensitive response. On the other hand, promoters play an important role in the control and regulation of the timing and location of gene expression. In this project, the upstream region of hsp70 gene and Cysteine protease gene in sago palm was isolated using a primer-based genome walking method and the isolated fragments were sent for sequencing. Bioinformatics analysis was carried out to analyze the sequences and the result revealed that the isolated fragments are not the promoter region of the desired genes..

Key words: Sago, genomic walking, Hsp70, cysteine protease, promoter

Abstrak


Kata kunci: Sagu, genomic walking, Hsp70, Cysteine protease, promoter
1.0 Introduction

Metroxylon sagu with its local name balau in Sarawak is a plant which stores starch in its trunk (McClatchey et al., 2006). According to McClatchey et al. (2006), Metroxylon sagu which has been grown commercially in Malaysia, Indonesia, the Philippines, and New Guinea is one of the most important economic species for local society to produce sago starch as well as conversion to animal food or fuel ethanol. In Sarawak, sago palm has great potential for commercial plantation and is mainly grown on peat soils. Some of its advantages include that it is economically acceptable, relatively sustainable, environmentally friendly, uniquely versatile, vigorous and crucial in promoting socially stable agro forestry systems (Flach, 1997). The starch produced by sago palm can be used in various ways. For example, the oxidized starches are used for paper industry coatings, the di-aldehyde starches are used in paper industry and the starch ether and esters are used for medicinal application (Flach, 1997). Heat Shock Proteins (Hsps) are rapidly synthesized when the cells are confronted with environmental variation which perturbs an organism’s physiological system (Tomanek & Sanford, 2003). The increase and accumulation of the Hsps provides added protection to the stressed cell. This ability is critical to survival. Examples of stresses that will induce Hsps to be synthesized and accumulated include an increase in temperature, exposure to pollutants such as various metals, hypoxia, anoxia, amino acid analogues, parasitism, predation, or competition and a large number of agents or treatments which result in reduced ATP level (Tine et al., 2010). The heat shock response is commonly referred to as the “stress response” due to large number of adverse conditions leading to increased Hsp expression.

Hsps are named based upon their molecular weight. For example, Hsp70 refers to heat shock proteins of size 70 kilodaltons. Hsp70 is constitutively expressed in most tissues and functioned as essential housekeeping gene by stabilizing heat-labile proteins and
prevent them from becoming irreversibly denatured or forming inappropriate aggregations which will eventually cause the cell to death (Garavaglia et al., 2009; Tine et al., 2010).

Cysteine protease is a type of endopeptidases with cysteine serves as the active site residue (Grudkowska & Zagdanska, 2004). “A defense mechanism in plants against insects is resolved in maize genotypes by induction of a cysteine protease without involvement of a cysteine protease inhibitor” (Gudkowska & Zagdanska, 2004, p.617). Besides, Hoorn and Jones (2004) also stated that cysteine protease is believed to involve in signaling during hypersensitive response.

To regulate the expression of a certain gene, promoter plays a very essential role. Promoter is the DNA region usually located upstream of the coding sequence of a gene or operon which binds and directs RNA polymerase to the correct transcription start site and therefore controls and regulates the timing and location of gene expression (Anwar et al., 2008). Understanding regarding promoter sequence is essential when the expression of a newly introduced gene is targeted to modify the characteristics of plants (Chawla & DeMason, 2003). Therefore, it would be much advantageous to us if we successfully identify the promoter sequences of hsp70 gene in order to regulate its expression.

In this research, a primer-based genomic walking method was used to obtain the promoter sequence of hsp70 gene and also cysteine protease gene in sago palm. Information regarding the isolation and sequencing of promoter sequence of hsp70 gene and cysteine protease gene in sago palm is very limited and therefore this research is aimed to help in facilitating the regulation of hsp70 gene and cysteine protease gene in sago palm. However, after the analysis of isolated fragments through bioinformatics analysis, the fragments are not the promoter regions of the desired genes.
2.0 Literature Review

2.1 Definition and Functions of Genomic Walking

It is critical to isolate and characterize unknown DNA sequences flanking known regions especially for the analysis of upstream and downstream non-coding regions. In such case, genomic walking is often used. Genomic walking is a rapid and reliable molecular biology technology to determine an uncharacterized DNA sequence flanking a known sequence region without going through arduous and prolonged genomic library preparation and screening with cDNA or probes obtained from prior screening (Guo & Xiong, 2006; Reddy et al., 2008). According to Ashoub and Abdalla (2006), genomic walking method is so powerful that it is used to identify unknown flanking sequences either in plants or animals where little information about sequences to be amplified is required.

"It may be used for identification of regulatory sequences outside cDNA coding regions and gaps in genome sequencing projects, or for mapping of insertional mutagenesis events produced by retroviruses and transposable elements" (Leoni et al., 2008, p.229). Commonly, genomic walking method is used to amplify promoter of certain genes. For example, Pennisetum and Salicornia promoter (Reddy et al., 2008), pea promoter (Chawla & DeMason, 2003) and shark Po promoter (Fors et al., 1990) had been successfully cloned and sequenced by using genomic walking technique.
2.2 Limitations of Genomic Walking Method

There are a lot of PCR-based methods which have been developed for defining flanking sequences from known genomic loci including inverse PCR (Ochman et al., 1988), ligation-mediated PCR (Tsuchiya et al., 2009), vectorette PCR (Arnold & Hodgson, 1991), nested PCR (Ashoub & Abdalla, 2006; Chawla & DeMason, 2003) and etc. Each method has its own advantages and limitations.

In inverse and ligation mediated PCR-based genomic-walking methods, the rate of success relies on successful DNA circularization or ligation to specifically designed adaptors (Guo & Xiong, 2006; Reddy et al., 2008). However, they reported that the above methods always remain low-throughput which means that they require a large amount of starting material but always result in very small DNA fragments or no DNA amplification at all. Reddy et al. (2008) also stated that it is always difficult to efficiently circularize or ligate oligonucleotide cassettes to genomic DNA fragments. Nevertheless, adaptor-specific PCR or vectorette PCR methods also always result in small DNA fragments (Ashoub & Abdalla, 2006). Therefore, these methods can be laborious and non-economic. In this research, a primer-based genomic walking with the use of nested PCR (Ashoub & Abdalla, 2006) was applied to identify and determine the promoter sequence of hsp70 gene in sago palm.

According to Guo & Xiong (2006), nested PCR is a powerful method which can be employed to suppress the PCR artifacts and to obtain more specific products by reducing false positive products or "off targets". Nested PCR is often used in reducing or eliminating undesired products while at the same time dramatically increasing sensitivity. (Roux, 1995).
2.3 Genomic Walking Using Nested PCR

Guo and Xiong (2006) had successfully amplify flanking sequences of known genomic loci of two highly divergent photosynthetic organisms, *Rhodobacter capsulatus* and *Heliophilum fasciatum* by using specific primers for a particular genomic locus which were exactly matched to a known sequence region from either of the two genomes and walker primers containing partial degenerated nucleotides mixed with specific ones. In nested PCR, product from the first round of PCR was used as template for the second round of PCR whose product in turn served as template for the third round of PCR. Three nested locus-specific primers and a common partially degenerated primer were use in successive rounds of PCR. However, the utility of degenerate primers always results in non-specific amplification of PCR products. In this research, different specific primers were used for successive PCR to reduce the non-specific amplification because the spurious PCR products are unlikely to contain binding sites for the inner specific primers. One single prominent final product was resulted from nested PCR. At the same time, no restriction digestion and ligation were needed. Moreover, the requirement of a small amount of starting genomic DNA (20ng) confers another advantage in situations when the amount of starting DNA is a limiting factor such as DNA extracted from environmental samples or non-culturable prokaryotic organisms.

Ashoub and Abdalla (2006) had also reported on their success to amplify up to 4kb of a potato leafroll virus full-length infectious clone. Firstly, the DNA was digested with restriction endonucleases which formed 3' overhangs. Phosphorus groups were presented at the 5' nucleotide. Then, single-stranded DNA primers with 3' sequences complementary to the tetra-nucleotide overhangs were annealed and ligated with the digested DNA. The presence of the phosphorus group presented was essential for the ligation. After ligation, a DNA library with 5' overhangs at both sites will be formed. These 5' overhangs will serve
as sites for AP in the first round of PCR but the binding sites for the AP will only be formed as a result of the GSP 1 extension in the first cycle of the PCR. The second round of PCR using NP and GSP 2 produced smaller and more specific products. No proprietary reagents are needed for this method. Moreover, it is economical and therefore suitable for either routine research or practical courses.

Chawla and DeMason (2003) had also reported that they had successfully cloned promoter sequences of pea gene using nested primer-based genome walking where adaptor primers and gene specific primers which are nested were used. Isolated genomic DNA was digested by blunt end cutting enzymes such as DraI, PvuII, EcoRV and StuI. Then, the DNA was purified and ligated with adaptors. A pair of GSPs was designed. The product from primary PCR reaction which used outer AP and the outer GSP 1 was diluted and used as a template for a secondary walk with the nested adaptor and nested GSPs. The major PCR products obtained were gel extracted and sequenced and aligned with the help of the GCG program. The upstream regions of PsPIN1 and PsPK2 were isolated, sequenced and analyzed.

2.4 Bioinformatic Analysis Using PlantCARE and PLACE

Chawla and DeMason (2003) had successfully analyzed sequences of upstream regions of PsPIN1 and PsPK2 which are orthologs of Arabidopsis PIN1 and PINOID genes by using PlantCARE and PLACE program, (Higo et al., 1999). Those programs revealed the presence of multiple presumed auxin-responsive elements. They also successfully identified the Unifoliata gene promoter which is the ortholog of Arabidopsis LEAFY.
3.0 Materials and Methods

3.1 Preparation of sago leaves

The young sago leaves were collected from UNIMAS plant house and wiped with 70% ethanol to prevent contamination. Then, the sago leaves were washed with distilled water and left to be air-dried. After that, 0.1g of sago leaves was weighed and the midribs were removed before the sago leaves are cut into small sections.

3.2 Genomic DNA isolation

The Cetyltriethylammonium bromide (CTAB) method which is described by Doyle and Doyle (1990) with slight modification was used. One milliliter of CTAB buffer was preheated with 2μl of β-mercaptoethanol at 65°C for 30 minutes. Then, sago leaves were grinded in the presence of liquid nitrogen and 1ml of CTAB buffer. The mixture was put into a 1.5ml centrifuge tube and incubated in water bath for one hour at 65°C followed by addition of 400μl of chloroform:isoamylalcohol (24:1). The mixture was mixed gently and centrifuged at 13000rpm for 5 minutes. The upper aqueous layer was transferred to a new 1.5ml centrifuge tube. Then, equal volume of ice-cold propan-2-ol was added. The tubes were incubated at -20°C for overnight.

The mixture was centrifuged at 13000rpm for 2 minutes on the next day. The supernatant was discarded. After that, 1ml of wash buffer was added to the pellet and the mixture was centrifuged at 13000rpm again for 5 minutes. The supernatant was discarded and the air-dried pellet was dissolved in 100μl of TE buffer and stored at -20°C. Then, 5μl of DNA was analyzed on 1.5% agarose gel treated with ethidium bromide. Then, the
stained DNA was illuminated with U.V. light using U.V. transilluminator and the gel was documented using documentation system.

### 3.3 DNA Quantification

A total of 5µl of DNA was diluted in 495µl of distilled water and placed in a quartz cuvette. The absorbance of diluted DNA sample at wavelengths 230nm, 260nm, 280nm and 320nm ($A_{230}$, $A_{260}$, $A_{280}$ and $A_{320}$) was measured. The ratio of $A_{260} : A_{280}$ and $A_{260} : A_{230}$ were subsequently calculated based on the reading showed by the spectrophotometer. Total concentration of DNA and total amount of DNA extracted were also calculated by the spectrophotometer.

### 3.4 PCR primers

Suitable primers are critical in genomic walking and the following primers were obtained commercially from First Base Laboratories Sdn. Bhd. The sequences of the primers were shown in Table 3.1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>OHP AatII</td>
<td>5'-GAA TTC GAG CTC GCC CGG GAT CCT CTA GAA CGT-3'</td>
</tr>
<tr>
<td>OHP KpnI</td>
<td>5'-GAA TTC GAG CTC GCC CGG GAT CCT CTA GAG TAC-3'</td>
</tr>
<tr>
<td>OHP PstI</td>
<td>5'-GAA TTC GAG CTC GCC CGG GAT CCT CTA GAT GCA-3'</td>
</tr>
<tr>
<td>AP</td>
<td>5'-GAA TTC GAG CTC GCC CGG GAT-3'</td>
</tr>
<tr>
<td>NP</td>
<td>5'-GCT CGC CCG GAT CCT CTA GA-3'</td>
</tr>
<tr>
<td>Hsp70</td>
<td>5'-CAT CGA AGA CGG TGT TAG TGG G-3'</td>
</tr>
<tr>
<td>CysProt</td>
<td>5'-GAG ATG CCT TTC CCC GTT GCC-3'</td>
</tr>
</tbody>
</table>
3.5 Genomic Preparation

First, 50μL of extracted DNA was treated with 1μL of RNase A to get rid of RNA. The mixture was then incubated at 37°C for 30 minutes. Then 10μL of RNase A treated DNA are digested individually with 2μL of AatII, KpnI and PstI in a final volume of 20μL for 16 hours at 37°C. Then, the restriction endonucleases were inactivated at 65°C for 10 minutes. Then, 10μL of the digested DNA from each reaction were ligated with 2μL of the appropriate OHP in the presence of 4μL 1X T4 DNA ligase buffer and 4μL of T4 DNA ligase in a final volume of 20μL. The reactions were incubated overnight at 4°C for primer annealing and ligation.

3.6 PCR amplification

The PCR condition described by Ashoub and Abdalla (2006) with slight modification was used. PCR reactions were carried out using 1μL of the above DNA libraries in the presence of 10μL GoTaq Green Master Mix; 1μL, each, of the AP and hsp70 primer in a final volume of 20μL. Samples were subjected to 35 cycles of PCR with 5 seconds of denaturation at 95°C, 15 seconds of annealing at 60°C, and 1 minute and 15 seconds of extension at 72°C. The cycle was preceded by 3 minutes of denaturation at 95°C and followed by 5 minutes of final extension at 72°C.

The second round of PCR were carried out following the conditions described above using 1μL of PCR product from the first round of amplification as template in the presence of 1μL, each, of NP and hsp70. Finally, a 10μL sample of the PCR product were analyzed on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide, and photographed. The protocol was repeated by replacing the hsp70 primer with Cysteine Protease primer.
3.7 DNA fragments extraction from agarose gel

Extraction of DNA fragments from agarose gel was performed using the GF-1 Gel DNA Recovery Kit. The protocol for this extraction was undertaken according to manufacturer’s recommendations.

3.8 Sequence Analysis

Then, the purified DNA was sent to First Base Laboratories Sdn. Bhd. for sequencing. After the results of sequencing were received, bioinformatics analysis was carried out to identify the intron, exon as well as genomic sequence within the sequences isolated. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the sequences (Chawla and DeMason, 2003).
4.0 Results and Discussions

The DNA extracted from sago palm was examined on 1.5% agarose gel (Figure 4.1). The quality of the extracted DNA was fine to proceed with the incubation with RNase A and genomic preparation. Although smearing could be seen, it was then reduced after incubation with RNase A to get rid of RNA because only DNA was needed to proceed (Figure 4.2).

![Figure 4.1: Agarose gel (1.5%) picture of extracted genomic DNA. M1 is the 1Kb Ladder (Fermentas). Lane 1 to 5 refers to the genomic DNA.](image)

Sample in Lane 4 (Figure 4.1) was selected to continue. From Figure 4.2, it is obvious that the smearing shown in lane 1 is reduced as shown in lane 2. Sample treated with RNase A was then incubated with restriction enzymes and then ligated with appropriate OHPs.
Figure 4.2: Agarose gel (1.5%) picture of extracted genomic DNA and RNAsé A treated genomic DNA. M1 is the 1kb ladder (Fermentas). Lane 1 refers to extracted genomic DNA and lane 2 refers to RNAsé A treated genomic DNA.

After that, first round of PCR was carried out using AP and Hsp70 primers. Samples were subjected to 35 cycles of PCR with 5 seconds of denaturation at 95°C, 15 seconds of annealing at 60°C and 1 minute and 15 seconds of extension at 72°C. Then, the cycle was preceded by 3 minutes of denaturation at 95°C and followed by 5 minutes of extension at 72°C. PCR carried out using PstI digested DNA as template did not produce any band. However, PCR carried out using AatII and KpnI digested DNA as templates each produced a distinct band as shown in Figure 4.3.
Figure 4.3: Agarose gel (1.5%) picture of products from first round of PCR using AP and Hsp70 primer. M2 is 100bp Ladder (Fermentas). Lane 1 refers to product from first round of PCR using AP and Hsp70 primer with AatII digested DNA as template while Lane 2 with KpnI digested DNA as template.

Another set of PCR was carried out under the exactly same conditions but replace the Hsp70 with CysProt primer. Electrophoresis was carried out and the agarose gel (1.5%) picture of the products shows that only PCR carried out using PstI digested DNA as template produced bands (Figure 4.4). PCR carried out using AatII and KpnI digested DNA as template did not produce any band.
Figure 4.4: Agarose gel (1.5%) picture of products from first round of PCR using AP and CysProt primer. M1 is 1kb Ladder (Fermentas). Lane 1 refers to product from first round of PCR using AP and CysProt primer with PstI digested DNA as template.

Second round of PCR using NP and gene specific primers was then carried out but the products were seriously smeared compare to the first round of PCR (Figure 4.5 and Figure 4.6). According to Roux (1995), the appearance of smearing could be due to high number of cycles or when the level of starting template is too high. He also reported that the first and second rounds of amplification can be terminated after 20 or so cycles rather than the usual 30-35 for better results. By performing such modification which has been proposed by Roux (1995), the chances of generating undesired high molecules and smears could be minimized for better results.
Figure 4.5: Agarose gel (1.5%) picture of products from second round of PCR using NP and Hsp70 primer. M2 is 100bp Ladder (Fermentas). Lane 1 refers to product from second round of PCR using AatII digested DNA as template while Lane 2 with KpnI digested DNA as template.

Figure 4.6: Agarose gel (1.5%) picture of products from second round of PCR using NP and CysProt primer. M2 is 100bp Ladder (Fermentas). Lane 1 refers to product from second round of PCR using PstI digested DNA as template.