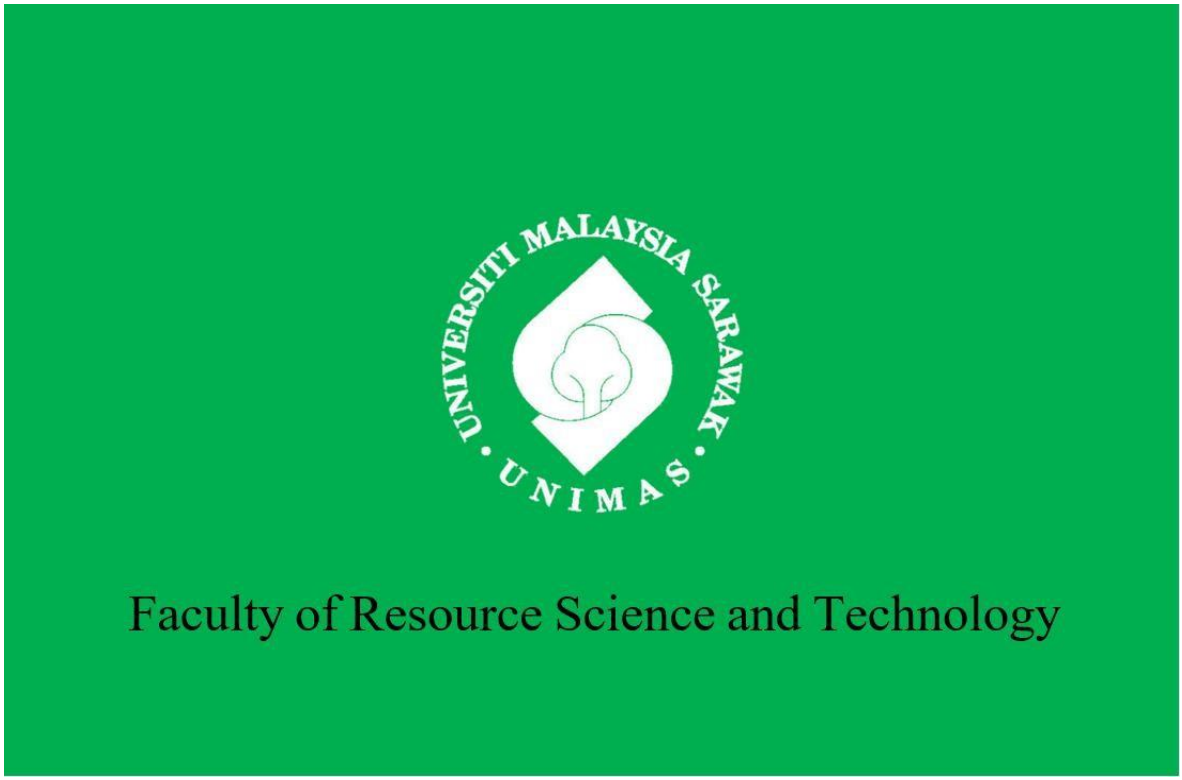

Expression Analysis of Differentiate Sago Palm Gene (Peroxidase A2 and Catalase Gene) via Amplified cDNA

Wai Chee Siong (71921)

Bachelor of Science with Honours
(Resource Biotechonology)
2022

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Wai Chee Siong

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

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Programme of Resource Biotechnology
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UNIVERSITI MALAYSIA SARAWAK

2022

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Expression Analysis of Differentiate Sago Palm Gene (Peroxidase A2 and Catalase Gene) via Amplified cDNA

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ABSTRACT

Metroxylon Sagu also known as sago plants can be found in Malaysia especially in Sarawak. The sago plants serve as starch sources for the locals. The sago plants have large economical value when the plants is trunking. But one day, scientist have found out that the trunking sago palms have diverse into another categories which are non-trunking sago palms. The non-trunking sago palms cause the economical to be reduce from time to time. The study is carry out through expression analysis to identify and differentiate the specific gene via cDNA amplification. Gene of Peroxidase A2 and Catalase was selected to design the primers. The experiment was using CTAB based method to extract the mRNA from the sago leafs. The primer selected purposely designed to apply. Expression level of the peroxidase A2 gene for trunking and non-trunking expressed equally. The expression of catalase gene cannot be expressed due to many reasons. Further research need to be done for confirmation of the related genes. CTAB based method is consume time but can get better results.

Key words: *Metroxylon Sagu*, peroxidase A2, catalase, expression analysis, amplified cDNA.

ABSTRAK

Metroxylon Sagu *merupakan tumbuhan sagu yang biasa dijumpai di Malaysia. Tumbuhan sagu berfungsi sebagai sember kanji bagi penduduk tempatan. Tanaman pokok sagu mempunyai nilai ekonomi yang besar apabila pokok sagu berbatang. Satu hari, saintis mendapati bahawa pokok sagu yang berbatang evolusi kepada pokok sagu yang tidak berbatang. Pokok sagu yang tidak berbatang menyebabkan ekonomi menurun dari semasa ke semasa. Kajian ini dijalankan melalui analisis ekspresi untuk mengenal pasti dan membezakan gen tertentu melalui amplifikasi cDNA. Gen peroksidase A2 dan katalase telah dipilih untuk membentukan primer. Eksperimen ini menggunakan kaedah berasakan CTAB untuk mengekstrak mRNA daripada daun sagu. Primer dipilih dan direka untuk digunakan. Tahap ekspresi gen peroksidase A2 untuk pokok sagu berbatang dan untuk pokok sagu yang tidak berbatang adalah sama. Ekspresi gen katalase tidak dapat dinyatakan boleh diganggu oleh banyak sebab. Kajian yang lanjut perlu dilaksanakan untuk pengesahan gen yang berkaitan. Kaedah berasaskan CTAB memakan masa tetapi boleh mendapatkan hasil yang lebih baik.*

Kata kunci: Metroxylon sagu, *peroksidase A2, katalase, analisis ekspresi, amplifikasi cDNA.*

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LIST OF ABBREVIATIONS

CTAB	Cetyltrimethyl Ammonium Bromide
cDNA	complementary DNA
mRNA	messenger RNA
NaCl	Sodium Chloride
PVP	Polyvinylpyrrolidone
TBE	Tris-Borate EDTA

CHAPTER 1

INTRODUCTION

1.1 Study Background

Sago plants have a scientific name *Metroxylon sagu* in Malaysia. Sago plant was mainly found in Malaysia, Indonesia, Papua New Guinea and Philippines (Mohammad Naim et al.,2016). Sago plants are easy to plant and do not need much attention and care. Sago plants are resistant to extreme weather changes and can grow well in wet and hot conditions. The example in Malaysia was one of the tropical forests that has a hot and wet environment for the whole year. Sago plants were easy to be planted but maturity duration needed more than 10 years. Sago plants is a plant which can convert carbohydrates to food starch and store in the trunk of the plants. Plantation of the sago plants can succeed in an acidic environment and clay soil to prevent growth of other plant species for nutrients competition.

Sago plants were important food sources. A single yield of sago plant can have a large amount of food starch and is the main diet for Eastern Asia. Sago plants have high economical value as any part from the plants can have different use either in industry, pharmaceutical, textile and food industry. Sago also wisely used in research as it can be applied as biosorbent, animal foods, microbiology use and fermenter (Lim et al.,2019). The dry food sources that form in sago plants can be stored for a long period. It is also mainly in the food security programs.

Sago plant was commonly used as a research target and had reports in the proteomic, genetic and transcriptomic fields. There were many genes that can be isolated from sago plants. Each function that occurs in sago plants has its own genetic arrangement. They were specific to each other. Transcriptomic field of sago plants analyse the genetic contains and construct the cDNA library to share the founds and DNA sequences. Sago plants had been founded diverse to trunking and non-trunking sago plants (Lim et al., 2019). The normal plant for sago plants is trunking whereas the abnormal type was non-trunking. The non-trunking sago plants cannot develop the trunks. Trunk for sago plants was important for starch to matured. The occurrences of non-trunking sago plants cause the production and economical value to decreased. The study of this topic is to differentiate the genetic sequences of the trunking and non-trunking sago plants. The peroxidase A2 gene and catalase gene sequences of sago plants need to be identified to understand the expression level of both diverse sago plants. The study also conducts expression analysis of desired genes through amplifying cDNA of sago plants. The method used was a modern method which was cheaper and high in reliability, low cost and easy to handle in a small lab.

The CTAB-based method was commonly used for plant cell biology (Wang & Stegemann, 2010). This method can work effectively with the polysaccharide contents. Plant tissue contains a high level of polysaccharide content. The plant cell walls are rigid and need to be degraded first to let the nuclei material inside to expose for the following extraction. To degrade the cell wall, the plant cells were snap freezing in the liquid nitrogen and used mortar and pestle to grind the leaf samples. Through the process of grinding in liquid nitrogen, the cell became fragile and brittle and the force applied to it made it break easily then with non snap freeze samples. The polysaccharide material of the plant samples can bind to nucleic acid and make it difficult to be isolated and purity to pure

RNA. The CTAB method can be effective to purify RNA quality and quantity. The RNA that is isolated with the CTAB method can have good quality. The RNA undergoes nanodrop to check for the concentration, purity and integrity. The RNA material can absorb the wavelength of 260 nm, proteins absorb the wavelength of 280 nm and chemical contaminants absorb the wavelength of 230 nm.

1.2 Problem Statement

When handled with leaf samples the size of powder forms varies from person to person. The leaf needs to be grind as fine as possible to obtain high concentration of genetic material. Due to the limitation of lab apparatus and materials most of the advanced methods could not be applied to the study.

1.3 Objectives

In this study, the level of expression of differentiated sago palm genes via amplified cDNA was analysed. The objectives were as follows

i) To study the extraction of total RNA.

ii) To study the cDNA conversion.

iii) To study the expression level of peroxidase A2 and catalase between trunking and non-trunking plants.

CHAPTER 2

LITERATURE REVIEW

The sago plant of trunking and non-trunking originated from the same family but somehow it diversified into 2 different categories. The expression analysis method is used to express the peroxidase A2 gene and catalase gene via amplification of cDNA. The study of this can know more about the method to conduct expression analysis. Beside that, this study also can find out the different genetic content between both types of sago plants. This study also can determine whether the peroxidase A2 gene and catalase have different expression levels. There are several major studies addressed in this section. Global gene expression analysis (Lovén et al.,2012), differential gene profiling of trunking and non-trunking sago palm (Edward et al., 2009) and comparing bioinformatic gene expression (Mantione at al., 2014).

Several previous studies and discussion on the difference between trunking sago plant and non-trunking sago plants.According to the proteomic field, trunking sago plants and non-trunking sago plants have differences in protein expression profiles. The transcriptomic of sago plant have applied by analysis the genetics of the sago plant and construct a cDNA library to share the found and DNA sequences. The sago plant had been distinguished into trunking and non-trunking plants (Edward et al.,2009).

2.1 The differences of gene in trunking and non-trunking sago plant

The sago plant has 2 main diversities. They were trunking and non-trunking sago plants and have research in this related field. The leaf samples were collected from 2

different plants which were trunking and non-trunking sago plants. Total RNA was isolated from the leaf samples. The method used was cetyltrimethylammonium bromide (CTAB) method and poly(A) mRNA isolated using Ambion MicroPoly(A) purist kit (Edward et al.,2009). To express the gene, 2 different genes were inserted into the GeneFishing DEG system. cDNA was obtained by reverse transcribing the mRNA. After that, the cDNA was amplified using 20 arbitrary primers. The amount of mRNA for cDNA was altered to the same amount for more stable comparison during the subsequent amplification. First strand cDNA synthesized from poly(A) mRNA using primer dt-ACP1 supplied in the SeeGene's GeneFishing kit and diluted by adding DNase-free water (Edward et al.,2009). The cDNA was used for the PCR reaction using dt-ACP2 and one of the 20 arbitbase pairrary primers. The result obtained is then PCR on agarose gel to get the pattern differential and band intensities of the product. The both expressed bands were then identified in size and band range between 100 to 700 base pairs. There were a minimum of 30 being targeted and cloned for sequencing. The bands will be visible when the amount of sample loaded is increased. When a large amount of PCR product for NT/ACP1 and T/ACP1 was electrophoresed for subsequent gel excise, the smear between 100bp and 200bp area was found to be a cluster of 3 bands sized about 100bp, 150bp and 200bp. Currently, at least 30 differential bands have been identified and more DEBs are expected to be identified when a mass PCR product is electrophoresed prior to gel excision of the targeted bands (Edward et al.,2009). The bands which remain faintly visible will be re-amplified prior to cloning. In the differential display analysis only 14 out of 20 ACP primers were used to obtain a differential transcript pattern.

2.2 Global gene expression analysis.

Gene expression analysis can give much information about the RNA through the genetics from cell and tissue. It is widely used in the molecular biology fields. In modern biology mostly are using computations to compare the difference of genes from the gene bank. There are over 750,000 expression data being set in the NCBI database (Lovén et al.,2012). During gene expression analysis, it can perform with many methods such as DNA microarrays, RNA-Seq, and methods that can measure the level of RNA species (Lovén et al.,2012). The most frequent method used was DNA microarray. It consists of a million individual oligonucleotide probes. The probes are fixed to the surface and have sequences that make them represent the RNA species. It is used for the relative level of RNA species which hybridize to the probe. After that parallel technologies use sequencing of RNA to derive cDNA populations. Isolation of RNA from the sources is the most common method that involves. Next introduce the same amount of RNA and analyse the data through normalizing the signal of the samples. When the amount of RNA and yield produced is similar, expression data should be accurate for the relative level. The genome wide analysis of the RNA expression may produce accurate assessment of changes in steady condition of RNA.

2.3 Comparing bioinformatic gene expression

The comparison on bioinformatic is the modern way biotechnologies use software. The gene expression have relationship between the genotype and phenotype. Bio-

technologies have used DNA microarray and RNA-Seq to link into this system. It is the comparing of the whole transcriptome gene expression profiling. RNA-Seq and microarray use widely in determine the DNA sequences. The most use which can solve nature is RNA-Seq. There are many generation of software invented base on the RNA-Seq method although it is more expensive than the microarray. Microarray for gene expression is widely use and have many references online also. Microarray is can say as old method than RNA-Seq method. The method used in microarray is poly A spike in method, cDNA synthesis, purify of RNA, preparation of hybridize samples, and extraction. RNA-Seq method, total RNA samples undergo poly(A) RNA , RNA fragment, single stranded cDNA, Double stranded cDNA, adapted ligated cDNA library and strand specific index sample for sequencing.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of CTAB Buffer

The CTAB buffer was prepared and composed of 4 grams of 2.0% CTAB, 4 grams of 2.0% PVP, 16.2 grams of 1.4M of NaCl, 8 ml of 20mM of 0.5M EDTA (pH 8.0) and 20 ml of 100mM 1M TRIS (pH 8.0). All the material were mix together with precise amount and distilled water until 200 ml. The 1% beta-mercaptoethanol only added into the samples before use (Untergasser, 2022).

3.1 Extraction of mRNA

Leaf samples were prepared and wiped with 70% alcohol for disinfection. The leaf samples were cut into small pieces and weighed 1.2 grams then transferred into pre-cooled mortar. The liquid nitrogen was added several times separately into the mortar that contains leaf samples and grind into very small tiny small pieces. After that, large forces were applied by grinding the leaf samples into powder form as tiny as possible. The more tiny of the samples, the more pure and high quality of mRNA extracted. The powder form leaf samples was then transferred into a nalgene tube that labeled. Volume of 15 ml CTAB and 150 µl beta-mercaptoethanol added in and vortex for 10 seconds and incubated for 5 minutes. Volume of 15 ml chloroform was added into the nalgene tube and shaken up and down vigorously at room temperature for 5 minutes. Centrifuge the sample nalgene tube with 13200 rpm at 4℃ for 5 minutes. Next 10 to 13 ml of supernatant was collected and transferred to a new nalgene tube followed by 15 ml of chloroform and mixed vigorously for 5 minutes at room temperature. Centrifuge the mixture with 13200 rpm at 4℃ for 5

minutes. After the centrifugation, 8 to 10 ml of supernatant was collected and transferred to a new nalgene tube followed by ice-cool isopropanol in 1 to 1 ratio and mixed to evenly incubate in room temperature for 10 minutes. The mixture was then centrifuged with 13200 rpm at 4°C for 15 minutes.

The supernatant poured into the waste beaker carefully and washed the internal nalgene tube with 1 ml ice-cool 70% ethanol. The solution inside the nalgene tube transferred to a new 1.5 ml microcentrifuge tube and centrifuged with 13200 rpm at 4°C for 2 minutes. Next, the supernatant was poured into the waste beaker carefully and pipette mix in 1 ml of 70% ethanol. Mixture was then centrifuged with 13200 rpm at 4°C for 2 minutes. The supernatant then poured into the waste beaker carefully and the tube was added in HCL-Tris buffer and 2 µl RQ1 RNase-free DNase by pipette mix. Incubated the mixture in room temperature for 30 minutes. Next 100 µl of 20 mM EDTA pH 8.4 was added in by pipette mix and put it on ice for 2 minutes. Centrifuge the mixture with 13200 rpm at 4°C for 15 minutes. Transfer 540 µl of the solution to 2 new microcentrifuge tubes each followed by 540 µl of ice-cool isopropanol to each tube. The mixture was pipette and incubated at room temperature for 10 minutes. Next, the mixture was centrifuge with 13200 rpm at 4°C for 15 minutes and the supernatant was poured out to the waste beaker carefully. Next 1 ml of 70% ethanol is added in by pipette mix and centrifuge with 13200 rpm at 4°C for 2 minutes. The supernatant was poured into the waste beaker carefully and added 1 ml of 70% ethanol. The mixture centrifuge with 13200 rpm at 4°C for 2 minutes. After that, the supernatant was discarded into the waste beaker carefully and air dry the pellet residue. Next the dry pellet residue was resuspended with a 100 µl TE buffer.

The final sample was then checked with gel electrophoresis by microwaving the mixture of 0.25 gram agarose gel powder with 25 ml 1X TBE buffer. Etbr solution was

added with a volume of 6.6 µl and poured into the mold with a comb to let it cool down and harden. Mix 2 µl loading dye and 10 µl sample then loaded into the gel. The first column loaded with a ladder for comparison. The power source was turned on and the parameter set as 140 Volt and 25 minutes. After that the RNA band was checked under the UV light for the results.

The sample is then subjected to a nanodrop check for the RNA concentration in ng/ul, A260, 260/230 and 260/280. The higher the ng/ul the better the RNA concentration. The A260, 260/230 and 260/280 should have a minimum of 1.8 for better RNA purity.

3.2 cDNA conversion

The mRNA samples were converted to cDNA through PCR process and cDNA reverse transcription kit. Volume of 10 µl of sample was pipetted into PCR tube. Next, 10 ul of master mix of cDNA reverse transcription kit was added into the samples. The master mix contains 2 µl 10X RT buffer, 0.8 µl 25 X dNTP mix, 2 µl 10X RT random primer, 1 µl MultiScribe™ Reverse Transcriptase and 4.2 µl Nuclease-free water. The mixture was mixed gently using a vortex machine and centrifuge the mixed mixture. After centrifuge, the mixture went through the PCR step with a pre-set parameter for about 35 cycles.

3.3 Primer Design

The primer was targeted on peroxidase A2 gene and catalase gene. The both gene primer was designed by using Primer3 software. The sequence that was provided was inserted into the Primer3 software and ran it with desired parameter setup. The parameter was set with primer length 18-24 base pairs, GC content 40-60%, Tm difference was between 0-5°C. The forward primer and reverse primer for both genes was obtained.

3.4 cDNA amplification

The cDNA sample was amplified using Promega GoTaq@ DNA polymerase kit. Volume of 1 µl cDNA template and 14 µl modified GoTaq@ DNA polymerase master mix was pipetted into a new PCR tube. The modified GoTaq@ DNA polymerase master mix contains 9.6 µl 5X Green GoTaq reaction buffer, 0.3 µl dNTP mix, 0.45 ul self-design reverse primer, 1.13 µl GoTaq DNA polymerase, 0.72 µl $MgCl_2$ and 7.95 µl Nuclease-free water. The mixture was mixed gently using a vortex machine and centrifugation. After the centrifuge process, the mixture was processed to PCR step with pre-set parameter setup for 45 cycles.

3.5 Gel electrophoresis

The 1% agarose gel was prepared by using 0.25 grams of agarose powder and 25 ml of 1X TBE buffer. The agarose powder and TE buffer was transferred into the Erlenmeyer flask and mixed well. The flask was then heated with a microwave machine until bubbling. The flask was then cooled down with running tap water until it became

warm. Volume of 6.6 µl of EtBr solution was pipetted into the agarose gel solution and swirled gently until the solution became colourless. The mixture agarose gel solution was poured into a casting tray inserting the comb. The mixture agarose gel solution was left at room temperature until it fully cooled down and solidified. After that remove the rubber and comb of the casting tray and put the agarose gel into the buffer tank. The buffer tank was filled with 1X TBE buffer solution until it fully covered the gel surface. Volume of 5 µl of 1kb DNA gene ladder and 5 µl of amplified cDNA was loaded into the well and ran the gel electrophoresis with 140V for 25 minutes. The mRNA samples obtained also using gel electrophoresis method to determine the presence of mRNA and overall concentration and purity.

CHAPTER 4

RESULTS

4.1 mRNA extraction nanodrop

The results showed the mRNA extraction using the nanodrop machine. The results for each samples are stated in the Table 1. The table contain of concentration, A260, 260/230 ratio and 260/280 ratio.

Table 1

Summarize of Nanodrop Value of Trunking and Non-trunking Sago Palms Samples

Samples Aspects	Concentration (ng/µL)	A260	260/230	260/280
Trunking 1	133.277	3.3319	2.857	1.883
	204.722	5.1181	3.383	1.866
Trunking 2	306.434	7.6608	2.582	1.933
	359.275	8.9819	2.650	1.932
Trunking 3	340.979	8.5245	2.636	1.914
	347.101	8.6775	2.728	1.922
Non-trunking 1	175.693	4.3923	2.918	1.937
	262.666	6.5667	2.758	1.936
Non-trunking 2	364.638	9.1159	2.775	1.917
	376.909	9.4227	2.736	1.916
Non-trunking 3	450.794	11.2699	2.572	1.923
	596.866	14.9217	2.544	1.946