

**ISOLATION AND CHARACTERIZATION
OF GA 20-OXIDASE GENE FROM SAGO PALM (*Metroxylon sagu*)**

**by
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification to this or any other university or institution of higher learning.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AP	Alkaline phosphatase
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
<i>bt2</i>	Brittle-2
C	Carbon
cDNA	Complementary DNA
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol, threo-2,3-dihydroxy-1,4-dithiolbutane
dNTPs	deoxyribonucleotide triphosphate
DIG	Digoxenin
EDTA	Disodium ethylenediaminetetraacetate
EGTA	Ethylene glycol tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FAO	Food and Agriculture Organisation of the United Nations
g	Gram
GAs	Gibberellins

GA 20-oxidase	Gibberellin 20-Oxidase
GSP	Gene Specific Primer
H	Hydrogen
IPTG	Isopropylthio- β -D-galactoside
IRRI	International Rice Research Institute
kDa	Kilo Dalton
LCDA	Land Custody and Development Authority
LB Medium	Luria Bertani Medium
LB broth	Luria Bertani broth
mcs	Multiple cloning site
ml	Millilitre
<i>Ms20ox</i>	cDNA GA 20-oxidase
<i>MsGenom20ox</i>	Genomic GA 20-oxidase
N	Nitrogen
ng	Nanogram
nm	nanometer
OD	Optical Density
O	Oxygen
pb	Pasangan bes
PVPP	Polyvinylpyrrolidone
PVP-10	Polyvinylpyrrolidone-10
PCR	Polymerase Chain Reaction

RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNase	Ribonuclease
RT-PCR	Reverse Transcript-PCR
RNA	Ribonucleic Acid
rpm	Rotation per minute
S	Sulphur
SDS	Sodium Dodecyl Sulfate
<i>sd-1</i>	Semi-dwarf
<i>sh2</i>	Shrunken-2
SSC	Sodium Citrate, Sodium Chloride Solution
TBE	Tris-Borate EDTA
TBS	Tris Buffer Saline
TTBS	Tween Tris Buffer Saline
TE	Tris EDTA
UV	Ultra violet
μg	microgram
μl	microlitre
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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ABSTRACT

GA 20-oxidase is one of the enzymes that are involved in the production of gibberellins. Gibberellins are plant hormones that are involved in controlling seed germination, stem elongation, leaf expansion, flower induction and development and growth of seed and fruit. Based on previously published conserved amino acid sequences of the plant GA 20-oxidase cDNA clones, oligonucleotide primers were used to amplify GA 20-oxidase gene from genomic DNA of sago palm. PCR amplification using this primer produced a single band with the estimated size of 500 bp in length. BLAST analysis has shown that this fragment was homologous with GA 20-oxidase gene. Based on the sequence obtained, several sets of gene specific primers were constructed and used to amplify the full-length gene both from genomic DNA and cDNA. The total size of GA20-oxidase gene obtained from genomic DNA and cDNA was 1332 and 1161 bp respectively. Comparison between genomic and cDNA sequence showed that, the GA 20-oxidase gene is comprised of two introns and three exons. In order to study the heterologous expression, GA 20-oxidase gene was cloned into pPCR-Script Amp SK (+) vector in sense direction and in correct reading frame, and was then transformed into *Escherichia coli* (*E. coli*). Protein was then extracted from *E. coli* and the fusion protein was analysed using western blot analysis. Heterologous expression was compared with endogenous GA 20-oxidase of sago palm. Expression activity was detected with polyclonal antibody which was induced and produced in a rabbit using short peptide sequence of sago GA 20-oxidase. Western hybridization analysis showed the presence of an intact 32 kDa band, for both crude protein samples obtained from sago tissue and *E. coli* containing sense GA 20-oxidase gene. No band was observed for protein extracted from negative control sample. This study indicated that the endogenous and heterologous products might be encoded by the same gene, GA 20-oxidase.

Pemencilan dan Pencirian Gen GA 20-Oxidase dari Palma Sago (*Metroxylon sagu*)

ABSTRAK

*GA 20-oksidadase adalah salah satu enzyme yang terlibat di dalam penghasilan hormon giberellins di dalam setiap tumbuhan. Giberellins terlibat di dalam pertumbuhan biji, pemanjangan batang, pelebaran daun, menggaruh pembungaan, dan perkembangan serta pertumbuhan biji dan buah. Berpandukan jujukan asid amino GA 20-oksidadase yang telah diterbitkan, primer oligonukleotida kemudiannya dibina dan seterusnya digunakan dalam mengandakan templat DNA genomik yang mengekodkan GA 20-oksidadase. Amplifikasi PCR berkenaan telah menghasilkan tetali tunggal DNA yang bersais 500 pb (pasangan bes). Keputusan analisa BLAST menunjukkan yang jujukan DNA tersebut adalah setara dengan gen GA 20-oksidadase. Daripada jujukan DNA yang diperolehi, primer-primer spesifik telah dibentuk dan kemudiannya digunakan untuk mengandakan gen lengkap daripada templat-templat genomik dan juga cDNA dimana masing-masingnya gen GA 20-oksidadase bersais 1332 dan 1161pb telah berjaya digandakan. Kajian ini juga menunjukkan bahawa gen GA 20-oksidadase adalah terdiri daripada dua bahagian intron and tiga ekson. Aktiviti pengepressan gen dalam *E. coli* kemudiannya dilakukan dengan mengklonkan unjuran bermakna (sense) cDNA berkenaan ke dalam vektor pPCR-Script Amp SK (+). Protein kemudiannya diekstrak dan aktiviti ekspresi disaringkan menggunakan antibodi poliklonal. Jalur protein bersais 32 kDa telah dikesan dalam sampel yang diperolehi daripada klon unjuran bermakna tadi. Di samping itu, protein yang diperolehi daripada tisu sago juga menunjukkan keputusan yang hampir serupa. Tiada jalur diperolehi daripada protein yang diperolehi daripada sampel kawalan. Kajian ini menunjukkan bahawa produk-produk endogenous dan heterologous berkenaan adalah dikodkan oleh gen yang sama iaitu GA 20-oksidadase.*

CHAPTER ONE

1.0 LITERATURE REVIEW

1.1 General Introduction

Sago palm (*Metroxylon sagu*) is one of the few tropical crops that can tolerate wet growing conditions such as peat swamps. In Sarawak, sago is grown as a starch crop by rural communities living along the coastal areas of certain districts. The total acreage of sago in Sarawak is about 65,000 ha of which, 45,000 ha are smallholders and 20,000 ha plantations. About 75% of the sago planting growing areas is located in the Mukah, Igan and Oya-Dalat districts of Sibu Division and Balingian (Tie *et al.*, 1991). There is also a substantial acreage of sago in the Pusa and Saratok districts of Sarawak. Sarawak, through CRAUN Research Sdn. Bhd. is intensively developing new areas and providing critical technical assistant for both plantation and smallholders.

The total production of Sarawak sago starch in the years 2009 and 2010 is considered to be quite stable with about 43,951 and 44,192 tonnes respectively. These gave a total export figure for the year 2009 and 2010 for sago starch of about RM62.57 and RM62.56 million respectively (Department of Statistics Malaysia, Sarawak Branch). The main export destination for starch for the past few years had not changed with West Malaysia as the main destination followed by Japan, Singapore, Sabah and Thailand. New importing countries need to be identified in order to increase export of this commodity. To achieve this however, a competitive quality of sago starch and flour needs to be produced. Therefore, intensive research activities needed to be carried out. To further support the development sago research, CRAUN Research Sdn. Bhd. had been authorized to undertake intensive research work on sago palm. The main aim is to transform the sago industry into

a modern sector in 21st century. To achieve this target, a systematic strategy including organizing brainstorming, partnership with research agencies and universities locally and abroad was conducted. Historically, research on sago started since 1970's, when the focus was more on agronomic aspects of sago plantation (Stanton, 1974). In early 90's however, this situation has changed, whereby seminars and symposium as well as colloquium on sago research and development have been intensively conducted. This was further supported by the availability of funding either from local government or universities.

Previous publications and presentations showed different field of research activities has been conducted. Various genes that are involved in starch biosynthetic pathway in sago palm have been isolated and published elsewhere (Au *et al.*, 2002; Salleh & Lau, 2003; Hwang & Salleh, 2005a; Hwang & Salleh, 2005b). The main issue in sago palm industry that needs to be addressed is its long maturity period. Sago palm takes about 10-12 years to reach maturity depending on soil conditions, while other starch producing plants, such as potato and cassava, mature much faster. For example, cassava takes only 6 months to reach maturity (Chulavatnatol, 2002).

To ensure sustainability of sago starch production, more intensive research work needs to be carried out. In most short maturing plant species, a conventional plant breeding technique is usually very successful for generating new elite plant varieties. This however is unsuitable for long maturing plants like sago palm, which is also hapaxanthic (producing flower only once at the end of its life). Another factor that hinders conventional breeding technique is the rare occurrence of flowering palms. The lack of flowering palms and the fact that sago palms were felled prior to flowering stage to extract its starch, makes it almost impossible to apply conventional breeding technique to produce new varieties. Therefore, a new approach needs to be explored and developed. A molecular breeding

technique has been identified as the best option for future sago research and development programme. This technique allows researchers to identify the potential genes that encode for the desired traits, and manipulate and transform into the host plant. This way, a new high yielding sago palm variety that matured earlier could be produced.

Apart from being exported to overseas, sago flour is also widely used in the local food industries. For instance, an estimated 11,070 tonnes were used annually for making vermicelli and noodle (Lee, 1987). A large amount was also utilized as feedstock for monosodium glutamate production in Malaysia (Zulpilip *et al.*, 1991). Sago flour is also used in the production of high fructose syrups, glucose, maltose and dextrose. In addition there is great potential for sago in animal feed industry. In Japan, sago starch is used in industrial adhesives, textile finishing, paper coating and also in the sweetener industries (Kainuma, 1977; Takahashi, 1986; Singhal *et al.*, 2008). There is also a strong demand for sago starch from Japanese food and beverage industries. About 60 - 70% of the 20,000 tonnes of sago starch consumed in Japan annually (Yatsugi, 1985; Ito *et al.*, 1986) is obtained from Sarawak.

1.2 Geographical Distribution of Sago Palm

Sago grows wildly or in semi-cultivated plantations in peat-land delta or riverine areas of Southeast Asia, especially in Papua New Guinea, Indonesia, Malaysia, Thailand and The Philippines with a total acreage of more than 3.75 million ha. Out of this, Indonesia has about 2.73 million ha followed by Papua New Guinea, Malaysia, Thailand and Philippines with 1.02 million ha, 45,000 ha, 3000 ha and 3000 ha respectively (Flach, 1997). The distribution of the sago areas in a particular country is dependent on the condition and location of peat land. Usually, the drier the area, the greater the number of productive sago stands, while less sago is found in areas located in deep and permanent flooding (Flach,

1997). Flach (1997) proposed that besides Moluccan Islands Indonesia, Papua New Guinea should also be considered as a centre of diversity for the sago palm. This is based on the gradual reduction of the number of sago palm varieties from the centre of Papua New Guinea and Moluccan Island, towards the borders of the area of dispersion. In Papua New Guinea, sago can be found growing in huge areas in the Sepik and Gulf provinces. In Indonesia, sago is mainly found in Irian Jaya, Bintuni, Lake Plain, Southern Irian and the Moluccan Islands (Flach, 1997). In Malaysia, Sarawak is the main sago-growing state especially in the Mukah, Igan, Oya-Dalat and Pusa-Saratok districts of Sibu division and Balingian (Anon, 1992).

There are two sago palm varieties presently utilized for starch production, namely *M. sagu* Rottb. and *M. rumphii* Mart. According to Takamura (1991), *M. sagu* is generally grown in the western part of the Moluccan Islands, Sulawesi, Java and Sarawak. On the other hand, *M. rumphii* is mainly found in the eastern part of the Moluccan Islands, Irian Jaya and Papua-New Guinea. Besides Papua New Guinea, Indonesia, Malaysia, Thailand and Philippines sago can now be found in other countries outside the region too. This was because, in 1985, sago palms were dispersed to other countries under the auspices of the Food and Agriculture Organisation of the United Nations (FAO) in Rome, Italy and in co-operation with Wageningen Agricultural University, in the Netherlands (Schuiling & Flach, 1985). Among the countries supplied are Costa Rica, Brazil, Africa, Zaire and Vietnam (Schuiling & Flach, 1985).

1.3 Plant Hormones

The growth and development of plant is controlled by the substances known as hormone or phytohormones. It determine the formation of flowers, stems, leaves, the shedding of leaves, and the development and ripening of fruit. It also shape the plant, affecting seed

growth, time of flowering, the sex of flowers, senescence of leaves and fruits. Apart from this, hormones also affect gene expression, transcription levels and cellular division. They are naturally produced within plants, though very similar chemicals are produced by fungi and bacteria that can also affect plant growth (Srivastava, 2002). These hormones although the production in plant are in a small amount, it is efficiently promote and influence the growth, development, and differentiation of cells and tissues (Opik & Rolfe, 2005). The plant hormones are required at a very specific times during it growth and development, and at specific locations. They also need to disengage the effects that hormones have when they are no longer needed. The production of hormones occurs very often at sites of active growth within the meristems, before cells have fully differentiated (Swarup *et al.*, 2007).

1.3.1 Classes of Plant Hormones

In general, there are five major classes of plant hormones (abscisic acid, auxins, cytokinins, ethylene and gibberellins), some of which are made up of many different chemicals that can vary in structure from one plant to the next. The chemicals are each grouped together into one of these classes based on their structural similarities and on their effects on plant physiology. Other plant hormones and growth regulators (brassinosteroids, salicylic acid, jasmonates, plant peptide hormones, polyamines, nitric oxide, strigolactones and karrikins) are not easily grouped into these classes; they exist naturally or are synthesized by humans or other organisms, including chemicals that inhibit plant growth or interrupt the physiological processes within plants. Each class has positive as well as inhibitory functions, and most often work in tandem with each other, with varying ratios of one or more interplaying to affect growth regulation (Rost & Weier, 1979). The five major classes of plant hormones are described in the following sections. Other plant hormones