Faculty of Resource Science and Technology

MOLECULAR CLONING AND CHARACTERIZATION OF PARTIAL cDNA ENCODING FOR SUCROSE PHOSPHATE SYNTHASE FROM KELAMPAYAN (*Neolamarckia cadamba*)

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DECLARATION

I hereby declare that this thesis is my original work except for quotations and citations, all of which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or any other institutions.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	Ampere
AGE	Agarose Gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary DNA
cm	Centimeters
CTAB	Cetyltrimethylammonium Bromide
℃	Degree Celsius
ddH_2O	Double-distilled water
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
LB	Luria Bertani/Broth
m	Meter
mg	Milligrams
ml	Milliliter
µl	Microliter
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information

ng	Nanogram
PCR	Polymerase Chain Reaction
Pi	Inorganic phosphate group
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SPS	Sucrose Phosphate Synthase
ssDNA	Single-stranded deoxyribonucleic acid
T_a	Annealing temperature
T_m	Melting temperature
TAE	Tris-Acetate EDTA
UDP	Uridinediphosphate
UV	Ultra-Violet

LIST OF TABLES AND FIGURES

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ABSTRACT

Sucrose phosphate synthase (SPS) is a plant enzyme that has a crucial role in the biosynthesis of sucrose. Sucrose is critical for the maintenance of cellular metabolism, cell wall biosynthesis and respiration. *Neolamarckia cadamba* or locally known as Kelampayan is a light hardwood trees and source of raw materials for manufacturing of plywood, paper and short-fibred pulp. The aim of this study was to clone and characterize the cDNA encoding *SPS* gene from *N. cadamba*. Total RNA was isolated from leaf samples and reverse transcribed into cDNA by RT-PCR. The *SPS* gene was then amplified by PCR. The PCR product was purified and sent for automated sequencing. The sequence analysis of nucleotides and amino acids revealed the presence of a 598 bp partial cDNA sequence. The deduced amino acid sequence of SPS was homologous to the other known SPS proteins suggesting that it was derived from *N. cadamba SPS* gene. The *SPS* sequences from the study can serve as a foundation for further insight to understand the role of SPS protein and also for Kelampayan tree improvement purpose.
Key words: cloning, *SPS* gene, RT-PCR, sequence analysis, *Neolamarckia cadamba*

ABSTRAK

Sukrosa fosfat synthase (SPS) adalah enzim tumbuhan yang mempunyai peranan penting dalam biosintesis sukrosa. Sukrosa adalah penting dalam pengekalan metabolisme sel, biosintesis dinding sel dan pernafasan. Neolamarckia cadamba atau dikenali sebagai Kelampayan adalah pokok kayu keras ringan dan sumber bahan mentah untuk pembuatan papan lapis, kertas dan pulpa pendek berserabut. Tujuan kajian ini adalah untuk mengklon dan mencirikan cDNA yang menyandi gen SPS daripada N. cadamba. RNA keseluruhan telah diasingkan daripada sampel daun dan ditranskipsi ke cDNA melalui RT-PCR. Gen SPS kemudian diamplifikasi oleh PCR. Produk PCR telah dimurnikan dan dihantar untuk sequencing. Analisis sequencing nukleotida dan amino asid mendedahkan kehadiran 598 bp cDNA separa. Amino asid SPS yang dianalisis adalah homolog kepada SPS protein yang diketahui dan ini menunjukkan bahawa ia berasal dari gen SPS N. cadamba. Sequence SPS daripada kajian boleh dijadikan sebagai asas untuk memahami lebih lanjut peranan protein SPS dan juga untuk tujuan peningkatan kualiti pokok Kelampayan.
Kata kunci: pengklonan, gen SPS, RT-PCR, sequence analisis, Neolamarckia cadamba

CHAPTER I

INTRODUCTION

Neolamarckia cadamba is a lightweight hardwood and is utilized for pulp in the making of low- and medium- quality paper and indoor light construction purpose. In addition, its fast growing rate makes it suitable for reforestation in watershed and eroded areas. It is also appropriate for windbreaks in agroforestry systems and extremely good for dipterocarp line planting by acting as a shade tree (Joker, 2000). The plant's barks and leaves are reported useful medically by acting as astringent, anti hepatotoxic (Kapil *et al*., 1995), wound healing, anthelmintic (Gunasekhran and Divyakant, 2006) and anti diuretic (Patel Divyakant *et al*., 2012).

It is crucial to ensure sustainable supply of raw materials in the local industry. Thus, the government established joint effort and smart partnership with the private sector to plant at least one million hectares of forest by 2020. Therefore, the formulation of The Forests (Planted Forests) Rules 1997 was created and it contains the procedures and guidelines on commercial forest establishments. Moreover, the continuous economic growth into rural areas and increasing dependency on renewable resources can be aided by agriculture and forestry (FAO, 2012).

There is an issuance of 43 Licences for Planted Forests (LPF) whereby 1.3 million hectares out of the 2.8 million hectares area covered was suitable for planting. Among the tree species identified for the project were *Acacia mangium* (Mangium) , *Acacia hybrid* (Acacia), *Hevea brasiliensis* (rubber), *N. cadamba* (Kelampayan), *Azadirachta excelsea* (Sentang), *Eucalyptus pellita* (Eucalyptus), *E. deglupta* (Eucalyptus), *E. grandis* (Eucalyptus), *Paraserianthes falcataria* (Batai) and *Shores macophylia* (Engkabang

Jantung) (PERKASA, 2012). In this regard, more study regarding the *N. cadamba* at molecular level is essential in order to cater with the increasing demand in agriculture and forestry areas.

In most of the plants, sucrose is the export form of the photoassimilate. The phloem is used to export most of the sucrose in mature leaves (source) to the plant organs (sink) that are net consumers of the photoassimilate (Komatsu *et al.*, 1996). Sucrose phosphate synthase (SPS) is the crucial enzyme in the sucrose synthesis pathway which catalyzes the following reaction:

fructose 6-phosphate + UDP glucose → sucrose phosphate + UDP

SPS is an allosteric enzyme and is triggered by binding of the substrate-similar glucose-6-phosphate and inhibited by Pi at the allosteric site (Doehlert and Huber, 1983). Regulation of the SPS activity is achieved by covalent protein phosphorylation (Huber *et al*., 1989; Siegl *et al*., 1990; Huber and Huber, 1991). Nowadays, the function and structure of SPS has also been analyzed at the molecular level. The cDNA clones for SPS were isolated and characterized in maize (Worrell *et al*., 1991), spinach (Klein *et al*., 1993) and sugar beet (Hesse *et al*., 1995). So far, there exists no information about molecular cloning and characterization of cDNA of SPS from *N. cadamba*. Therefore, the objective of this study was to clone and characterize the partial cDNAs encoding SPS from *N. cadamba*.

CHAPTER II

LITERATURE REVIEW

2.1 *Neolarmarckia cadamba* (Kelampayan)

N. cadamba belongs to the family of *Rubiaceae* (Nair, 2007). They can grow up to 45 m in height with trunk diameter of about 100 to 160 cm and without branches for more than 25 m (Lim *et al*., 2005; Peter, 2007). The flowers are globose and solitary, orange or yellow in colour (Acharyya *et al*., 2010). The fruits are small capsules of dense fleshy, yellow or orange infructescence with approximately 8,000 seeds. The fruits possess medicinal value as it can cure ulcers, diarrhea, fever and vomiting (Peter, 2007).

The distribution of *N. cadamba* covers a wide area whereby it grows naturally from Nepal eastward to Papua New Guinea. It is a typical pioneer that is fast-growing in the open areas commonly in the altitude of below 1,000 of the secondary forests (Chung *et al*., 2009). According to Chee (2000), after 8 to 12 years of planting, *N. cadamba* can be harvested for pulpwood, whereas it requires 15 to 20 years for sawlog production. Besides that, it is very light demanding and frost-intolerant. It can endure periodic flooding and grow on different type of soils (Joker, 2000). Additionally, it is suitable for the manufacturing of plywood, packing case, wooden sandals, toys and short-fibred pulp (Timber Technology Centre, 1999)

The optimization of timber production cannot be achieved without the access of good genetic stock, regardless of the subsequent silvicultural management quality. In the 2011 Kelampayan Tree Improvement Programme Workshop, the ultimate aim is the production of kelampayan that are adjusted to the local conditions with optimum growth and wood quality in order to accomplish economic benefits (The Star Online, 2011).

(a) **(b)**

(c)

Figure 2.1 *Neolamarckia cadamba.* (a) Flowers and leaves of *N. cadamba* (Photo adapted from http://www.flickr.com/photos/37118859@N02/4922773122/) (b): Trunk of *N. cadamba*. (Photo adapted from http://picasaweb.google.com/manglayang/HanjaJabonSamama#5488 813820177245106) (c): Trees of *N. cadamba*. (Photo adapted from http://agrowmania.blogspot.com/2012_06_01_archive.html).

2.2 Sucrose

Sucrose is crucial to plant metabolism and the most important metabolite in the plant cell walls growth and development. In vascular plants, it is the most translocated carbohydrate and has fundamental roles as carbon source and energy for non-photosynthetic tissues. During the importation of sucrose to sink tissues, the sucrose influx is essential for the maintenance of cellular metabolism, cell wall biosynthesis and respiration, converted to starch for future use (Sturm, 1999; Kutschera and Heiderich, 2002; Canam *et al*., 2006). Moreover, sucrose is a signal molecule in the gene expression regulation (Smeekens, 2000; Wiese *et al*., 2004), as a result this influences associated metabolic pathways and also morphological development (Lunn and MacRae, 2003).

Sucrose is the main compound for the translocation of photoassimilates from the leaves to non-photosynthetic tissues maybe because of its high solubility, low reactivity, and energy storage capacity (Akazawa and Okamoto, 1980; Giaquinta, 1980). After it is synthesized in the source tissues, the movement of sucrose will involves both the symplasmic and apoplasmic pathways to the phloem and this is the place where it is loaded by a proton-driven symporter (Riesmeier *et al.*, 1994). During the biosynthesis of sucrose in photosynthetic tissues, the enzyme that are involved, the triose phosphate, SPS and fructose-1,6-biphosphate are suggested to be responsible in the major rate-limiting steps (Kerr and Huber, 1987; Stitt, 1989; Neuhaus *et al.*, 1990). Synthesis of the sucrose also involves the coupled action of sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP).

2.3 Sucrose Phosphate Synthase (SPS)

Sucrose phosphate synthase (SPS) is a plant enzyme that has a crucial role in the biosynthesis of sucrose (Huber and Huber, 1996). In plants, SPS catalyzes the net sucrose synthesis and its activity is generally high in source tissues and low in sink organs (Huber and Huber, 1992). SPS activity is the controlling factor for sucrose synthesis and photosynthesis (Shinano *et al.*, 2006; Stitt *et al.*, 1988; Zuniga-Feest *et al.*, 2005) and the regulation of SPS is done by metabolites and reversible protein phosphorylation in photosynthetic and nonphotosynthetic tissues.

In leaves, modulation of SPS activity is done by phosphorylation with response to light or dark signals and end-product accumulation (Huber and Huber, 1996). An increased light and CO_2-saturated rate of photosynthesis under ambient conditions manage to enhance the SPS activity whereby the sucrose to starch ratio in leaves is increased (Galtier, 1993) together with the increased in the partitioning of fixed-C into sucrose (Micallef *et al.*, 1995). Nowadays, SPS activity can be manipulated and this has causes promising impacts on plant growth and resource allocation (Huber and Huber, 1996).

On the other hand, calcium, metabolites, and novel "coarse" control of the protein phosphatase that activates SPS appear to be involved in the regulation of the enzymatic activity of SPS. Besides that, osmotic stress of leaf tissue in darkness activates SPS, which may function to facilitate sucrose formation for osmoregulation (Huber and Huber, 1996).

In some species, alteration of the affinity for substrates and effectors is also involved in the light modulation of SPS that occurs by reversible protein phosphorylation (Stitt *et al.*, 1988.). According to Yu *et al.* (2007), they concluded that the sucrose content in plant sink organs is regulated by the level of SPS expression. Moreover, the finding of

five families of SPS genes in wheat (*Triticum aestivum L.*) and other monocotyledonous plants from the family Poaceae (grasses) is revealed and each of the SPS gene families in wheat showed different, but overlapping, spatial and temporal expression patterns, in most organs, at least two different SPS genes are expressed (Castleden *et al.*, 2004).

2.4 Sucrose Phosphate Synthase (SPS) in Wood Formation

The formation of wood determines the ecological and economical values of the trees whereas its quality is determined by the accumulation of biocides, for example, the phenolic substances. Sucrose is present in the sapwood storage part and its formation involves SPS. In plant, sucrose is the main transport form of carbon (Ziegler, 1975) sucrose and sucrose metabolism maybe having an important role not only during cambial differentiation (Sung *et al.*, 1993; Higuchi, 1997) but also during secondary differentiation process of wood tissues. The studies on SPS activity is done previously in the woody stem of both a gymnosperm, Scot pine (Uggla *et al.*, 2001), and angiosperms, including *Poplar*, *Robinia*, *Salix*, *Acer* and *Fagus* (Hauch and Magel, 1998, Magel *et al.*, 2001, Schrader and Sauter, 2002). Furthermore, greater SPS activity is observed in the middle and inner sapwood and in the bark and outer sapwood, which indicates spatial variation in *Robinia* (Park *et al.*, 2009). Besides, chilling has shows to increase the SPS activation state in *Poplar*, *Acer*, *Salix*, and *Fagus* (Schrader and Sauter, 2002).

2.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a method for amplifying defined sequence of RNA using enzyme *in vitro* (Rappolee *et al.*, 1988) into complementary DNA (cDNA) strands and enables the analysis of minute amount of RNA samples. Generally, RT-PCR reaction vessel contains a mixture of buffers, nucleotides, primers, thermostable polymerase and RNA from the specimen of interest. In order to successfully carrying out the RT-PCR, it involves the principles of denaturation of the double–stranded DNA, the annealing of primers and primer extensions (Schochetman *et al.*, 1988).

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Materials

Fresh young leaves tissues of *Neolamarckia cadamba* (Kelampayan) were collected and used for RNA extraction.

3.2 Primer Design

Data mining of primer was done through searching of data from GenBank. The primer specific to the sucrose phosphate synthase (*SPS*) gene was designed by the application of the software, Primer Premier 6.0 (PREMIER Biosoft International, USA). The Forward and Reverse primers were designed based on the conserved regions of the *SPS* genes from other woody species. They were subjected to multiple alignments using ClustalW Program (http://www.ebi.ac.uk/clustalw). The template used was *coffea canephora* since it is from the same family Rubiaceae with *N. cadamba*. The Forward primer sequence is 5'-TTACTTACGAGGCAGGTATC-3' (20 bp) with the T_m 55.75°C and the Reverse primer is 5'- CAGGAGGAATTACAGCCATT-3' (20 bp) with the T_m 55.75°C. The expected amplicon size is 646 bp.

3.3 Apparatus Treatment

All the apparatus that was required for RNA extraction such as the pipette tips, 15 ml Falcon tubes, 1.5 ml microcentrifuge tubes, glassware, mortar and pestle were treated with 0.1% DEPC treated water prior to autoclaving. The PCR tubes and microcentrifuge tubes were autoclaved before it is used.

3.4 Chemical Preparation

All the solutions required were prepared according to the manufacturer's instructions.

3.5 Total RNA Isolation from *Neolamarckia cadamba*

3.5.1 Chemicals and Reagents

The reagents that were used for total RNA extraction include liquid nitrogen, RLT buffer, β-mercaptoethanol, ethanol, RW1 buffer, DNase, 10 x DNase buffer, DEPC-treated ddH_2O and RPE buffer.

3.5.2 Total RNA Isolation Protocol

The total RNA of *N. cadamba* was extracted from the fresh young leaf tissue by using the RNeasy® Midi Kit (Qiagen, Germany). Firstly, 0.5 g of plant tissues was grinded in the liquid nitrogen into fine powder by using the pre-chilled DEPC-treated mortar and pestle. The finely-grind tissues were transferred to a clean 15 ml Falcon tube containing a mixture of 5 ml RLT buffer and 50 µl β-mercaptoethanol. The mixture was left to stand for 15 minutes. After that, it was centrifuged for 5 minutes with the speed of 3,000 rpm at 25℃ . The supernatant was transferred to a new clean 15 ml DEPC-treated autoclaved Falcon tube. Subsequently, before applying the samples to the RNeasy® Midi Kit Column, 0.5 volume of RNase-free 100% ethanol was added to the supernatant and mixed well immediately.

The sample was applied to an RNeasy midi column placed in a 15 ml tube. The samples were centrifuged for 5 minutes at 25°C at 3,000 rpm. The flow-through was discarded. Next, 2 ml of buffer RW1 was pipetted into the column and centrifuged for 5 minutes at 3,000 rpm at 25°C. The prepared mixture of 20 µl DNase I stock solution, 20 µl 10 x DNase buffer and 160 µl DEPC-treated ddH_2O was added onto the centre of the column and placed on the bench top for 15 minutes at room temperature. Then, 2 ml of buffer RW1 was added into the RNeasy column and was placed on the bench top for another 5 minutes. Later, it was centrifuged for 5 minutes at 3,000 rpm and the flow-through was discarded.

After that, 2.5 ml of buffer RPE was added into the column and centrifuged for 2 minutes at the speed of 3,000 rpm. The flow-through was discarded. Another 2.5 ml buffer RPE was added into the column and was centrifuged for 5 minutes at 3,000 rpm. The

column was transferred into a clean 15 ml Falcon tube. An amount of 100 µl of DEPC-treated ddH_2O was added into the centre of the column and it was left to stand for 1 minute before centrifugation at 3,000 rpm for 3 minutes. The elute was transferred to a DEPC-treated 1.5 ml microcentrifuge tube and labeled as „1st elute'. Following that, another 100 µl of DEPC-treated ddH_2O was added to the centre of the column and was left to stand for 1 minute before centrifugation at 3,000 rpm for 3 minutes. The elute was transferred to a new DEPC-treated 1.5 ml microcentrifuge tube and labeled as „2nd elute'. The total RNA that was extracted was stored at -80ºC for further analysis.

3.6 Agarose Gel Electrophoresis (AGE)

The total RNA isolated from the leaf was determined by running a 1.0% agarose gel. The agarose powder weighed 0.50 g was added into a conical flask containing 50 ml of 1x TAE buffer. Then, the mixture was heated at 300°C for 3 minutes in a microwave oven to ensure that the agarose powder dissolved completely in the mixture. A total of 3 µl total RNA was mixed with 1 µl of 1x loading dye, 3 µl of Lambda-*Hind* III (Promega, USA) was used as a marker. The gel was run under 50V, 55A for 60 minutes. Then, the gel was stained in ethidium bromide for 10 seconds and de-stained in distilled water for 30 minutes. The gel was later visualized under a UV transilluminator, Geliance 200 Imaging System (PerkinElmer, USA) for the detection of the presence of any bands.

3.7 RNA Purity and Quantification

The purity of the total RNA extracted was determined by using Thermo Scientific NanoDrop 2000 Spectrophotometer. With the sampling arm opened, 1 µl of RNA sample was pipetted onto the lower measurement pedestal. The sampling arm was closed and the spectral measurement was initiated using the operating software on the computer. The absorbance reading was recorded at 230 nm, 260 nm and 280 nm. The ratio of A_{260}/A_{280} and A_{260}/A_{230} were recorded to determine the RNA samples purity. The RNA concentration was calculated using the formula: [RNA] (ng/ µl) = A_{260} x RNA average extinction coefficient, where, RNA average extinction coefficient is 40 µg/ml.

3.8 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The synthesis of the first-strand cDNA was done by using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, USA) protocol. The RNA sample was pipetted into a DEPC-treated microcentrifuge tube and it was heated at 65°C for 10 minutes. Later, it was chilled on ice for 2 minutes. The oligo (dT) primer was added to the RNA and the solution was transferred to the tube of first-strand reaction mix beads and was incubated at room temperature for 1 minute. The contents of the tubes were mixed by vortexing the tubes gently. Next, it was incubated at 37°C for 60 minutes. The first-strand cDNA synthesized was kept in the freezer at -20°C for further analysis.

Table 3.1: Components for first-strand cDNA synthesis.

Component	Concentration	Volume/µl
Total RNA	-	11.3
DEPC treated water	-	20.7
Oligo(dT)	50 µM	1.0
Total		**32**

3.9 PCR Optimization

Gradient PCR was conducted in a MasterCycler Gradient Thermal Cycler (eppendorf, Germany) ranging from 35.2 °C to 55.7 °C. A total of 25 µl reaction volume was prepared in a PCR tube and the parameters used for the PCR reaction is shown as follows:

Table 3.2: Parameters used in PCR reaction.

Component	Final Concentration	Volume/µl
10 x PCR buffer	1 x	2.5
2 mM dNTP	0.2 mM	2.5
50 mM $MgCl_2$	1.5 mM	0.75
2.5 pmol/µl Forward primer	5 pmol	2
2.5 pmol/µl Reverse primer	5 pmol	2
cDNA	-	1
0.5 U/µl *Taq* Polymerase	1U/ µl	2
ddH_2O (ultra pure water)	-	12.25
Total		**25**

Following that, the mixture was heated to 95°C for 2 minutes, 35 cycles at 94ºC for 30 seconds, 45ºC ±10°C for 30 seconds and 72ºC for 30 seconds followed by a final cycle at 72ºC for 10 minutes. Later, the amplified RT-PCR products were examined on a 1.5% agarose gel along with 100 bp DNA ladder (BioLabs, New England).

3.10 PCR Product Purification

After gel electrophoresis, PCR product was purified by using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The DNA band was excised from the gel and the gel slice was place in a 1.5 ml microcentrifuge tube. An amount of 10 µl Membrane Binding Solution per 10 mg of gel slice was added. Then the mixture was vortexed and incubated at 55 °C until gel slice was completely dissolved. An equal volume of Membrane Binding Solution was added to the PCR amplification. Then, the SV Minicolumn was inserted into Collection Tube and the dissolved gel mixture or prepared PCR product was transferred to the Minicolumn assembly. It was incubated at room temperature for 1 minute. Later, centrifugation was done at 16,000 × g for 1 minute. The flowthrough was discarded and the Minicolumn was reinserted into Collection Tube.

Then, a total of 700 µl Membrane Wash Solution (ethanol added) was added. The column assembly was centrifuged at 16,000 × g for 1 minute. The flowthrough was discarded and the Minicolumn was reinserted into Collection Tube. Later, an amount of 500 µl Membrane Wash Solution was added and the column assembly was centrifuged at 16,000 × g for 5 minutes. The Collection Tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. The Minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube. An amount of 50 µl of Nuclease-Free Water was added to the Minicolumn. The column assembly was incubated at room temperature for 1 minute and centrifuged at 16,000 × g for 1 minute. The Minicolumn was discarded and DNA was stored at –20°C.