UNIVERSITI PUTRA MALAYSIA

TRANSFORMATION OF TOBACCO (*Nicotiana tabacum*) WITH THE OIL PALM CINNAMYL ALCOHOL DEHYDROGENASE 1 (CAD1) GENE

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TRANSFORMATION OF TOBACCO (*Nicotiana tabacum*) WITH THE OIL PALM *CINNAMYL ALCOHOL DEHYDROGENASE 1* (*CAD1*) GENE

By

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Thesis Submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Bachelor of Science

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Abstract of thesis presented to the Department of Cell and Molecular Biology in fulfilment of the requirement of the degree of Bachelor of Science (Hons)

TRANSFORMATION OF TOBACCO (*Nicotiana tabacum*) WITH THE OIL PALM *CINNAMYL ALCOHOL DEHYDROGENASE 1* (*CAD1*) GENE

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Lignification in plants occurs via the lignin biosynthetic pathway that involves a series of enzymes; among these enzymes is the cinnamyl alcohol dehydrogenase (CAD). CAD catalyzes the conversion of hydroxycinnamaldehydes into monolignols. In oil palm, two copies of the *CAD* gene have been identified (*EgCAD1* and *EgCAD2*) and their functions are largely undetermined. Studying gene function in oil palm is generally time-consuming and technically difficult. Hence, the study of the *CAD1* gene from oil palm was performed via transformation of tobacco (*Nicotiana tabacum*) due to its relatively short life cycle and well-established transformation protocol. Tobacco leaf discs were used as target samples for *Agrobacterium*-mediated transformation of p*MDC32-EgCAD1* which carries a T-DNA region consisting of the oil palm *CAD1* gene. Phylogeny study of the *EgCAD* family was also performed to establish the relationship of *EgCAD1* with other members of the *CAD* family across different plant orders. The oil palm *CAD1* shares a common ancestor with their counterparts in the angiosperm and gymnosperm lineages. The *CAD* phylogeny showed that *EgCAD1* was clustered together with the genes from rice, pinus and banana, and they share the same recent common ancestor. The oil

palm *CAD1* was successfully integrated into the tobacco genome where positive PCR data showed a genomic region comprising the *EgCAD1* in the p*MDC32-CAD1* construct was amplified in the transformed tobacco plants. Normal phenotype of the transformed plants was observed. This study confirmed the successful integration of the *EgCAD1* into the genome of tobacco and the establishment of *CAD* family phylogeny. The findings serve as a basis for functional characterization of *EgCAD1* in the future.

Abstrak tesis yang dikemukakan kepada Jabatan Biologi Sel dan Molekul untuk memenuhi keperluan untuk ijazah Sarjana Muda Sains dengan Kepujian

TRANSFORMASI TEMBAKAU DENGAN GEN *CINNAMYL ALKOHOL DEHIDROGENASE 1* (*CAD1*) DARIPADA KELAPA SAWIT

Oleh

YEE MEI FOONG

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Pengerusi: **Mohd Puad Abdullah, PhD**

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Lignifikasi dalam tumbuhan berlaku melalui tapak jalan biosintesis lignin yang melibatkan satu siri enzim; di antara enzim ini adalah cinnamyl alkohol dehidrogenase (CAD). CAD memangkinkan penukaran dari hidrosicinnamyldehida ke monolignol. Dalam kelapa sawit, dua salinan gen *CAD* telah dikenal pasti (*EgCAD1* dan *EgCAD2*) dan fungsi gen ini masih belum ditentukan. Penyelidikan fungsi gen dalam kelapa sawit biasanya memakan masa dan sukar dari segi teknikal. Oleh itu, kajian gen *CAD1* dari kelapa sawit dilakukan melalui transformasi tembakau (*Nicotiana tabacum*) disebabkan oleh kitaran hidupnya yang singkat dan protokol transformasi yang mantap. Kajian filogeni keluarga *CAD* juga dilakukan untuk mengkaji hubungan *EgCAD1* dengan ahli-ahli lain keluarga *CAD* dan order tumbuhan yang berbeza. Cakera daun tembakau telah digunakan sebagai sampel untuk transformasi *Agrobacterium*-berantarakan p*MDC32-EgCAD1* yang membawa rantau T-DNA terdiri daripada gen *CAD1* kelapa sawit. Gen kelapa sawit *CAD1* berkongsi moyang yang sama dengan rakan mereka dari keturunan angiosperma dan gimnosperma. Filogeni *CAD* menunjukkan *EgCAD1* telah digolongkan bersama dengan gen daripada padi, *Pinus* dan pisang; dan mereka berkongsi moyang yang

sama. Gen *CAD1* dari kelapa sawit telah berjaya diintegrasikan ke dalam genom tembakau di mana data PCR positif menunjukkan rantau genomik yang terdiri daripada sempadan *EgCAD1* telah dapat diamplifikasikan dalam pokok tembakau. Tumbuh-tumbuhan yang telah ditransfomasikan mempunyai fenotip yang normal. Kajian ini mengesahkan kejayaan integrasi *EgCAD1* ke dalam genom tembakau dan penubuhan filogeni keluarga gen *CAD*. Penemuan ini menjadi asas bagi pencirian kefungsian *EgCAD1* pada masa akan datang.

Approval

This thesis was submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences and has been accepted as fulfillment of the requirement for the degree of Bachelor of Science (Hons). The member of the Supervisory Committee was as follows:

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Declaration

Declaration by undergraduate student

I hereby confirm that:

- this thesis is my original work;
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Declaration by Supervisor

This is to confirm that:

- The research conducted and the writing of this thesis was under supervision.

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Name of Chairman of

Supervisory Committee: _____

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LIST OF ABBREVIATIONS

α	Alpha
g	Gravitational acceleration
µg	Microgram
µL	Microliter
A. tumefaciens	*Agrobacterium tumefaciens*
BAP	Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
CAD	Cinnamyl alcohol dehydrogenase
Cl	Chloride
cm	Centimeter
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic acid
LB	Luria Bertani
M	Molar
MEGA	Molecular Evolutionary Genetic Analysis
$MgCl_2$	Magnesium chloride
mL	Milliliter
mM	Millimolar
MS	Murashige and Skoog basal Medium
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram
OD	Optical density
PCR	Polymerase chain reaction

rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TE	Tris-EDTA
Tm	melting temperature
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris-hydrochloride
U	Unit
UV	Ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Lignification in plants is gaining broadening interests from various agricultural and biotechnology fields due to its importance in garnering economic value especially in terms of recalcitrance of plant biomass. Currently, plant lignification is believed to be part of plant's responses towards biotic and abiotic stresses as well as plant developmental process (Humphreys and Chapple, 2002). Lignification in plants occurs via lignin biosynthetic pathway, where the deposition and polymerization of different types of monolignols form lignin (Whetten and Sederoff, 1995).

The lignin biosynthetic pathway involves several key enzymes; among them is the cinnamyl-alcohol dehydrogenase (CAD). It belongs to the oxidoreductase family and it catalyzes the reversible conversion of p-hydroxycinnamaldehydes to monolignols. In *Arabidopsis*, it is believed that *CAD-c* and *CAD-d* are the primary genes that supply coniferyl and sinapyl alcohols to biosynthesize lignin (Sibout *et. al.*, 2005). Sibout *et al.* (2005) discovered that a *cad-c cad-d* double mutant of *Arabidopsis* has limp floral stem and abnormal pattern of lignin staining, with 40% loss of lignin content in the mutant plant as compared to the wild type. In oil palm, *CAD* was first discovered for its role in the differentiation of the endocarp and mesocarp tissues of the dura variety (Bhasker and Mohankumar, 2001). This revealed the relationship of *CAD* from oil palm with other enzymes in the lignin biosynthetic pathway by comparing the expression levels of *CAD, PAL* and *POD* during the development of fruit shell (Bhasker and Mohankumar, 2001).

To date, several classes of *CAD* have been identified across different plant species. In *Camellia sinensis,* deep sequencing of the transcriptome revealed three types of *CAD* with only 20%-54% sequence identities. This showed that the *CAD* genes were of different classes, or exist in a multi-gene family (Deng *et al.*, 2013). However, the function of the each different classes of *CAD* is yet to be determined, and among different species. In *Oryza sativa,* twelve distinct genes were found to encode products that are highly similar to CAD. *OsCAD2,* an orthologue of *CAD* was found to participate in promoter-reporter gene fusions in the onset of lignification (Tobias and Chow, 2005).

Due to the nature of the *CAD* genes that tend to form a multi-gene family, identifying a specific *CAD* gene responsible for lignifications in oil palm is difficult. Previously, two copies of *CAD* gene namely *EgCAD1* and *EgCAD2* have been isolated from oil palm. The aim to single out the specific *CAD* gene for lignification in oil palm is made especially difficult when no prior information about the gene is available in the plant. One approach in which the gene function can be predicted is through phylogenetic analysis of the gene families from different groups of plant. Preliminary expression analysis of *EgCAD1* indicated that the gene is expressed in the oil palm root, leaf and stem. This information helps to narrow down the possible functional characteristics of *EgCAD1*. Since functional analysis of genes in oil palm is technically difficult and time-consuming; thus, tobacco is a good plant candidate for studying the basic function of *EgCAD1* in plant lignification. The current study encompasses the transformation of tobacco with *EgCAD1* and phylogenetic analysis of the *CAD* gene family with the following specific objectives:

i. To establish the phylogeny of the oil palm *CAD1* gene via maximum likelihood method;

ii. To transform tobacco leaf discs with the oil palm *CAD1* gene via *Agrobacterium*-mediated transformation.

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