UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF OIL PALM PROTOPLAST ISOLATION AND TRANSFORMATION METHOD FOR TRANSIENT EXPRESSION ASSAYS USING DSRED REPORTER GENE

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FBSB 2021 24

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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April 2021

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Genetic engineering is highly regarded as a forefront technology in agriculture. Various crops have been improved via this technology to increase the overall yield or specific product of targeted crops. Genetic engineering has also been carried out to address multiple issues that cause production losses, including drought conditions or susceptibility to pest and pathogen attacks. The steady progress and maturation of genome editing technology have allowed scientists to do this more precisely and efficiently. Nevertheless, multiple tools for the target crop, such as an established transformation method for optimal DNA delivery, a reliable transient expression system for preliminary evaluation of the targeting efficiency, and a proven tissue culture routine for regenerating genome-edited plantlets, are required. This current research was carried out to develop a high-throughput transient expression system for oil palm by utilizing protoplasts isolated from oil palm *in vitro*-derived leaves. First, seven transformation vectors that carry DsRED protein-encoding genes, each controlled by a different promoter, were constructed. Next, the isolation of mesophyll protoplasts was optimized by identifying the best parameters affecting protoplast yield and viability, such as enzyme combinations and procedures to obtain clean and viable protoplasts. By doing this, an efficient protocol for isolation of oil palm mesophyll protoplast that can produce up to 2.5 x 10^6 protoplasts/g FW with up to 94.78% viability was developed. Then, optimization for isolation of protoplasts from the mesocarp of the age around 12 weeks after anthesis (WAA) was carried out with previously optimized enzyme mixtures. After two hours of incubation time, 3.98 × 10^6 protoplasts/g FW with 85% viability were recovered. Five parameters affecting the polyethylene glycol (PEG)-mediated transformation efficiency were optimized, including DNA and polyethylene glycol (PEG) incubation time, concentrations

of DNA and PEG, and duration of heat-shock applied. This study has shown an increment in transformation efficiency of almost 56%. The developed transient expression system was tested with eight DNA constructs with DsRED as a visual reporter gene. This experiment indicated that the CaMV35S promoter drove significantly higher expression of DsRED in oil palm protoplasts than other plants constitutive, oil palm constitutive and tissue-specific promoter tested in this study. This advanced method will serve as a high-throughput transient expression platform in the current pipeline for oil palm genome editing.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENGOPTIMUMAN PENGHASILAN PROTOPLAS SAWIT DAN KAEDAH TRANSFORMASI SAWIT UNTUK ASAI PENGEKSPRESAN SEMENTARA DENGAN MENGGUNAKAN GEN PELAPOR DsRED

Oleh

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Kejuruteraan genetik diiktiraf sebagai salah satu pemacu utama kemajuan di dalam bidang pertanian. Pelbagai jenis tanaman telah berjaya ditambahbaik dengan menggunakan pendekatan ini dalam meningkatkan hasil tanaman. Selain itu, kejuruteraan genetik juga diaplikasikan dalam menyelesaikan pelbagai isu yang menyebabkan kerugian hasil tanaman termasuk kemarau dan kerentanan terhadap serangan perosak serta patogen. Kemajuan pesat dalam bidang teknologi penyuntingan genom membolehkan para saintis mengaplikasikan penambahbaikan tanaman melalui kaedah kejuruteraan genetik dengan lebih tepat dan berkesan. Walau bagaimanapun, pelbagai protokol bagi tanaman sasaran seperti kaedah transformasi bagi pemindahan DNA yang optimum, sistem pengekspresan gen sementara bagi menilai kecekapan sasaran sgRNA, dan rutin tisu kultur untuk regenerasi tanaman yang telah disunting perlu ada. Kajian ini bertujuan untuk membangunkan sistem pengekpsresan gen sementara sawit berdaya pemprosesan tinggi, berasaskan protoplas yang dihasilkan daripada daun *in vitro* sawit. Tujuh vektor transformasi yang membawa gen mengekod protein DsRED dengan setiap satunya dikawal oleh promoter berlainan telah dihasilkan. Seterusnya, penghasilan protoplas mesofil sawit dioptimumkan dengan mengenalpasti parameter yang mempengaruhi hasil dan kebolehhidupan protoplas seperti gabungan enzim dan prosedur mendapatkan protoplas hidup yang bersih dari bendasing. Dengan ini, protokol penghasilan protoplas mesofil daripada sawit yang efisyen dengan hasil sebanyak 2.5×10^6 protoplas/g berat segar dengan kebolehhidupan sehingga 94.78% berjaya dibangunkan. Seterusya, penghasilan protoplas sawit dari tisu mesokarpa pada usia 12 minggu selepas antesis dioptimumkan dengan menggunakan gabungan enzim optimum terdahulu. Selepas tempoh inkubasi selama 2 jam, 3.98×10^6 protoplas/g

berat segar dengan kebolehhidupan sebanyak 85% berjaya dihasilkan. Selepas itu, lima parameter yang mempengaruhi kecekapan kaedah transformasi dengan polietilena glikol (PEG) telah dioptimumkan termasuk tempoh inkubasi DNA dan PEG, kepekatan DNA dan PEG, serta tempoh kejutan suhu yang dikenakan. Kajian ini telah menunjukkan peningkatan kecekapan transformasi mendadak menghampiri 56% berjaya dicapai. Berikutan keputusan tersebut, sistem pengekspresan gen sementara yang dibangunkan telah diuji dengan lapan konstruk yang berbeza, setiap satunya dikawal oleh promoter berlainan yang membawa DsRED sebagai gen pelapor visual. Dapatan kajian menunjukkan CaMV35S memacu pengekspresan DsRED yang sangat tinggi di dalam protoplas sawit berbanding promoter konstitutif tumbuhan, promoter konstitutif sawit dan promoter spesifik tisu sawit yang lain. Sistem yang dibangunkan ini akan diaplikasikan sebagai pelantar pengekspresan gen sementara di dalam program penyuntingan genom sawit.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my main supervisor, Assoc. Prof. Dr. Noor Azmi Shaharuddin, for his great support, encouragements, friendships, and insights throughout my study at Universiti Putra Malaysia. Without his thoughtful instructions, comments, and help, it would be impossible for me to complete this study.

I sincerely thanked Dr. Abdul Masani Mat Yunus (Research Group Leader of Transgenic Technology Group, MPOB), for his kind but critical advice, comments, the experience shared, and guidance whenever sought. Thus, shaping me to be the better person I am today.

I would like to present honest thanks to my co-supervisor at UPM, Prof. Dr. Ho Chai Ling for her time, experience, and insight, enable my project to progress and finished within the stipulated period.

I would like to extend my gratitude to the Malaysian Palm Oil Board (MPOB) for allowing me to further my study and providing financial support. Special thanks to Dr. Ahmad Parveez Ghulam Kadir (Director General of MPOB), Dr. Zainab Idris (Deputy Director-General of MPOB), Dr. Mohamad Arif Abd Manaf (Director of ABBC), and Dr. Omar Abd Rasid (Head of Functional Biotechnology Unit) for allowing me to complete my thesis. My thanks also go to Pn. Rusnani Abd Majid and Pn. Suraya Harun (Training Unit, MPOB).

I extend my appreciation to members of Transgenic Technology Group (MPOB) and Tissue Culture Group (MPOB) for their assistance. Finally, I wish to express my deepest gratitude and appreciation to my family, especially to my father (MD Piji Ali) and mother (Ngamidah Samingan), for their continuous support and prayer. For my wife, Norfaezah Jamaludin, thank you for your love, support, sacrifices and encouragement throughout my study. And to my first born, Khayra Sumayya, I love you.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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BA	6-Benzylaminopurine
BiFC	Bimolecular fluorescence complementation
CaMV	Cauliflower Mosaic Virus
Cas9	CRISPR associate protein 9
CFDA	5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester
CRISPR	Clustered regularly interspace short palindromic repeats
DMRT	Duncan's multiple range test
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow fluorescent protein
FP	Fluorescent protein
GFP	Green fluorescent protein
GUS	β-glucuronidase
IPTG	Isopropyl β- d-1-thiogalactopyranoside
KSP	Kernel-specific promoter
LB	Luria Bertani
LSP	Leaf-specific promoter
MES	2-(N-morpholino)ethanesulfonic acid
MSP	Mesocarp-specific promoter
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RE	Restriction endonucleases
RFP	Red fluorescent protein
SEM	Standard error of the means

TCTP	Transnationally control tumour protein
UEP1	Ubiquitin extension protein 1
UEP2	Ubiquitin extension protein 2
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

CHAPTER 1

INTRODUCTION

The outstanding share the palm oil gathers in the global market has proved its potential in leading the world to combat the forecasted food insecurity. Current oil palm productivity (tonnes oil hectare^{-1} year^{-1}) was already high compared to other oil seeds, such as soybean, rapeseed, and sunflower (Zimmer, 2010). However, the extrapolated global population growth and food demand have required us to move forward and break the current yield barrier. The Malaysian palm oil industries have pursued efforts to improve oil palm via genetic engineering since the start of the 21st century. Since then, tremendous progress has been achieved in the field of omics, methods to deliver foreign DNA into oil palm, production of transgenic oil palm, to the complete sequence of *Elaeis guineensis* published for researcher and industry uses.

Several methods have been published for the DNA transformation of oil palm, including by biolistic (Hanin et al., 2020; Parveez and Christou, 1998), *Agrobacterium*-mediated (Izawati et al., 2012), polyethylene glycol (PEG)-mediated and microinjection of protoplast (Masani et al., 2014). Transgenic oil palm production has specific objectives such as improving the oil content, introducing novel resistance traits, or biological factory synthesizing novel products (*i.e.,* bioplastic). Genes introduced for these purposes require promoters to drive the expression in oil palm.

Several plant constitutive promoters are commonly used in generating transgenic plants, such as the Cauliflower Mosaic Virus 35S promoter (CaMV35S) and maize-ubiquitin promoter. However, the possibility of introducing multiple genes for the generation of a transgenic oil palm requires more promoter candidates as the overuse of a similar promoter could lead to homology-dependent gene silencing (Badai et al., 2019). Multiple studies to isolate the oil palm endogenous promoter were initiated. Several oil palm endogenous promoters have been isolated, including the constitutive promoter such as TCTP (Masura et al., 2011), UEP1 (Masura et al., 2010) and UEP2 (Masura et al., 2019) also various tissue-specific promoters for oil palm (Zubaidah et al., 2018). Evaluation of the performance of available promoters is required before selecting the most suitable promoter to control the expression of the desired transgene in oil palm.

The option of evaluating gene function in perennial crops via stable transformation is time-intensive and not viable due to its long lifecycle nature. Adopting a robust transient expression approach, on the other hand, provides the preliminary data and helps users to make better-informed decisions before

proceeding with the stable gene integration experiments. The transient expression has been used in oil palm to evaluate the activities of isolated promoters (Masura et al., 2019) and as a visual selectablemarker (Majid and Parveez, 2016). The use of a suitable visual reporter gene is essential in transient gene analyses.

β-glucuronidase (GUS) gene, originated from *Escherichia coli*, is regularly used for evaluation of novel promoters isolated from oil palm (Masura et al.,2011; Masura et al., 2010; Masura et al., 2019; Zubaidah et al., 2018; Zubaidah and Siti Nor Akmar 2010). Detection of *gus* gene activity can be easily carried out based on the development of blue colour spots upon the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic (X-Gluc), which is the substrate for β-glucuronidase. The assay requires many sample tissues, and its destructive nature means none of the evaluated samples could be regenerated. GUS assay appears problematic for those sample tissues that are difficult to obtain and those perennial crops with limited samples availability.

Another visual reporter commonly used for oil palm genetic engineering works is fluorescent protein (FP). The FP has been proven to be more efficient as direct visual screening can be carried out in the absence of substrates (Zhang et al., 2015). Green fluorescent protein (GFP), first isolated from *Aequorea* jellyfish, is the earliest and most commonly used FP (Chalfie et al., 1994). The potential of GFP as a visual reporter for the transformation of oil palm has been studied in advance (Parveez and Majid, 2018; Majid and Parveez, 2016). However, the latest study indicated that application GFP as a visual reporter in oil palm could interfere with the cycle of plant regeneration and toxic to the cells of oil palm (Parveez and Majid, 2018).

Red fluorescent protein (RFP) can be utilized to replace GFP. DsRED, a variant of RFP, is a 28-kDa fluorescent protein found in the coral of *Discosoma* genus (Bevis and Glick, 2002). Experimental findings have shown no evidence of its toxic impact on plant cells (Nietsch et al., 2017; Saha and Blumwald, 2016; Wu et al., 2016). Furthermore, DsRED has been proven to be more robust and accurate than the GFP-based reporter system in walnut (Zhang et al., 2015). The photostable characteristic and distinct DsRED red fluorescent signals differentiating between transformants and non-transformants improved the walnut reporter system previously based on GFP. To date, there is no published report on DsRED in oil palm; hence it is interesting to explore the impact of the promoter in driving transgenes in an oil palm system. The adoption of DsRED may provide a new role for FP in future oil palm genetic engineering works.

A protoplast-based transient gene expression system offers versatility and a time-efficient solution for high-throughput gene functional analysis (Page et al., 2019). Protoplasts are produced by removing the cell wall via enzymatic or

mechanical means. It has been suggested that a protoplast-based transient gene expression system could provide the solution in analysing the large scale of genes quickly (Page et al., 2019). The ability of protoplast to maintain its cell type characteristics after being isolated will be helpful in cells and tissue-specific gene functional analysis (Marx, 2016).

A few studies have been carried out on protoplast isolation from oil palm mature tissues (Masani et al., 2013; Sambanthamurthi et al., 1996). The isolation of protoplast from different oil palm tissues is required to observe the specificity of tissue-specific promoter driving transgene expression in its specialized tissues *in vivo*. On the other hand, it has been reported that mesophyll tissues were the best candidate for transient gene analysis tool due to the mature metabolite synthesis machinery it houses (Yoo et al., 2007). Hence, the protocols for protoplast isolation from oil palm mature tissue need to be developed and optimize. The optimized mixture of enzymes and procedures would result in a higher yield and viability of the isolated protoplasts.

One method available for direct transfer of DNA into protoplasts is by treatment with polyethylene glycol (PEG). This method has been less damaging to the plant cells than other protoplast transformation techniques such as electroporation and microinjection (Masani et al., 2014). Previously, Masani et al. (2014) have developed a protocol for the transformation of oil palm protoplast via PEG-mediated method. It is reported that the successful transformation rate was 5%. For a transient gene analysis platform to be reliable, it needs to be replicable with high efficiency. In that light, further optimization needs to be carried out. Optimizing parameters affecting the PEG-transformation would improve the transformation efficiency, thus improving the transient gene analysis platform's reliability.

There is no available transient gene analysis system utilizing protoplasts that was specifically developed for oil palm. This leaves a gap for a high-throughput transient gene expression platform to determine the activity of isolated endogenous promoter and the expression of isolated genes in oil palm. It also affects the pace of oil palm gene-editing progress. This system plays an essential role in evaluating the gRNA efficiency and preliminary proof-of-concept *in vivo* study. Developing a protoplast-based transient gene analysis system for oil palm would provide a versatile platform to study the gene function and its expression faster and more efficiently than other tools currently available for oil palm. In order to close this gap, an extensive project on the development of a transient gene analysis system utilizing oil palm protoplast was designed. This research was devised to provide a solution for the highlighted problems with the following objectives:

i. To construct transformation vectors carrying gene encoding red fluorescent protein (DsRED), each driven by a plant constitutive

promoter, an oil palm constitutive promoter or an oil palm tissue-specific promoter.

ii. To test the functionality of constructed transformation vectors in oil palm embryogenic calli and tobacco protoplasts.

iii. To optimize protoplast isolation from oil palm *in vitro* leaf and mesocarp tissues.

iv. To optimize parameters involve in the transformation of final constructs into oil palm protoplasts via PEG-mediated method.

v. To analyse the relative strength of different promoters introduced based on the red fluorescent signals under the fluorescent microscope.

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BIODATA OF STUDENT

Mohd Al Akmarul Fizree bin MD Piji was born in Tg Karang, Selangor. He received her early kindergarten education in Klang, primary education at S.K. Klang from 1996-2000 and S.K. Bukit Beruntung from 2000 onward. He continued his secondary education at S.M. Sains Selangor from 2002-2006.

He then pursued his study with Diploma in Microbiology at UITM Shah Alam from 2008-2011. He was then given a chance to further his undergraduate study in Bachelor of Science, majoring in Biotechnology and Management with Honours from 2011 to 2014. His final year project was Identification and Cloning of Riboswitches from *Burkholderia pseudomallei* under the supervision of Assoc. Prof. Dr. Firdaus Raih from Faculty of Science and Technology, UKM.

He was allowed to pursue his Master program at the Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), in 2017 while working as a research officer at Malaysian Palm Oil Board. He is a recipient of *Tawaran Kemudahan Latihan MPOB.* During his post-graduate program, he actively participated in the international conference as a poster presenter, international symposium, workshops, and seminars. He has published a manuscript in Scientia Horticulturae entitled Evaluation of DsRED Expression in Oil Palm Embryogenic Calli. While performing his duty as a research officer and further his study at the same time, he was also awarded with *Anugerah Perkhidmatan Cemerlang* and *Anugerah Khas Ketua Pengarah* (organizing PIPOC 2019) at *Majlis Penyampaian Anugerah Dalaman MPOB* 2019 (ADaM).

LIST OF PUBLICATIONS

- **Indexed Refereed Journal**

Fizree, P. M. A. A., Shaharuddin, N. A., Ho, C. L., Manaf, M. A. A., Parveez, G. K. A., and Masani, M. Y. A. (2021). Efficient protocol improved the yield and viability of oil palm protoplast isolated from in vitro leaf and mesocarp. *Scientia Horticulturae.*

Fizree, P. M. A. A., Shaharuddin, N. A., Ho, C. L., Masura, S. S., Manaf, M. A. A., Ahmad Parveez, G. K. A., and Masani, M. Y. A. (2019). Evaluation of transient DsRED gene expression in oil palm embryogenic calli. *Scientia Horticulturae*, *257*, 1–9.

- **Poster/ Conference Proceeding**

Fizree, M. P. M. A. A., Shaharuddin, N. A., Ho, C. -L., Manaf, M. A. A., Parveez, G. K. A., and Masani, M. Y. A. (2021). Optimization of protoplast isolation from oil palm in vitro-derived leaf and mesocarp. 4th International Conference on Molecular Biology & Biotechnology (ICMBB2021). Kuala Lumpur, Malaysia: Malaysian Society for Molecular Biology and Biotechnology.

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