AGROBACTERIUM-MEDIATED TRANSFORMATION OF *RICE FLOWERING LOCUS T1* GENE IN MR219 RICE SHOOT APEX

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ABSTRACT

Rice is one of the staple foods that contributes significantly to global food security. In Malaysia, rice occupies the commanding height of staple food basket. However, local rice production fails to satisfy domestic demand as domestic expansion of rice production is hampered by the low yield of Malaysian rice, its long reproductive phase and poor amenability to genetic transformation. Biotechnology methods employed to improve the Malaysia rice proved unsatisfactory due to its recalcitrance to transformation. This study sought to address these challenges by identifying the most efficient and rapid methods for *indica* rice transformation up to transgenic recovery. For this, *RFT1* gene was isolated from Malaysia upland rice (cultivar Wai) and constructed into pCAMBIA1305.2 expression vector. The construct was further mobilized into *Agrobacterium tumefaciens* LBA4404. MR219 Malaysian wetland rice was used for shoot apex induction. Five-day-old shoot explants were used for transformation with *A. tumefaciens* LBA4404 harbouring *pCAMB::RFT1* construct. The transformants were regenerated and analyzed for transient expression of *RFT1*, *hpt* and GUS. Stable integration of transgene and GUS were validated by PCR amplification and histochemical analysis. The RT-PCR and bioinformatics analyses showed that full-length *RFT1* gene was isolated and shared 99% nucleotide and 80% amino acid identity with other rice cultivars. *pCAMB::RFT1* construct was successfully developed and transformed into MR219 wetland rice. Optimum shoot apices regeneration frequency of 71.64±0.74% was recorded in MS medium supplemented with 3 mg/L TDZ and genetically transformed. The molecular analysis of the transgenic rice confirmed the integration of the *RFT1* transgene, *hpt* and GUS gene into the T_0 plant. Histochemical and PCR analyses of established transgenic MR219 also confirmed the presence of the transgenes. Total transformation efficiency was recorded in the range of 12.5±5.37% to 17.5±4.91%. This is the first report of full-length *RFT1* gene isolation and transformation into MR219 shoot apex. Findings from this study could serve as a new procedure for genetic manipulation and a fundamental stage for producing transgenic rice.

ABSTRAK

Padi adalah antara makanan ruji yang menyumbang utama kepada keselamatan makanan global. Di Malaysia, padi menduduki tempat tertinggi dalam kempulan makanan ruji. Walau bagaimanapun, pengeluaran padi tempatan gagal memenuhi permintaan dalam negeri kerana perluasan penghasilan padi terhalang oleh hasil padi Malaysia yang rendah, fasa pembiakannya yang panjang, dan kebolehmampuan untuk transformasi genetic yang rendah. Kaedah bioteknologi yang digunakan untuk menambahbaik padi Malaysia terbukti tidak memuaskan kerana sifatnya yang kurang daya untuk transformasi. Kajian ini bertujuan menangani cabaran-cabaran ini dengan mengenalpasti kaedah transformasi padi *indica* yang paling cekap dan pantas sehingga perolehan transgenik. Untuk itu, gen *RFT1* telah dipencilkan daripada padi tanah tinggi Malaysia (kultivar Wai) dan dibina ke dalam vector pengekspressan pCAMBIA1305.2. Konstruk itu telah dipindahkan seterusnya ke dalam *Agrobacterium tumefaciens* LBA4404. Padi sawah Malaysia MR219 telah digunakan bagi induksi apeks pucuk. Eksplan pucuk berusia lima hari telah digunakan dalam transformasi menggunakan *A. tumefaciens* LBA4404 menggandungi konstruk *pCAMB::RFT1*. Transforman telah ditumbuhkan semula dan dianalisa untuk ekspresi sementara *RFT1*, *hpt* dan GUS. Integrasi stabil transgen dan GUS telah disahkan melalui amplifikasi PCR dan analisa histokimia. Analisa RT-PCR dan bioinformatik menunjukkan bahawa gen penuh *RFT1* telah dipencilkan dan berkongsi identiti 99% nukleotida dan 80% asid amino dengan kultivar padi lain. Konstruk *pCAMB::RFT1* telah berjaya dibina dan ditransformasi ke dalam padi sawah MR219. Kekerapan penghasilan semula apeks pucuk optimum sebanyak 71.64 ± 0.74% dicatatkan di dalam media MS yang ditambah dengan 3 mg/L TDZ dan telah dilakukan transformasi genetik. Analisis molekul padi transgenik mengesahkan selitan transgen *RFT1, hpt* dan gen GUS ke dalam tumbuhan T_0. Analisis histokimia dan PCR bagi MR219 transgenik tertubuh juga mengesahkan kehadiran transgen. Jumlah kecekapan transformasi yang dicatatkan adalah dalam julat 12.5±5.37% ke 17.5±4.9%. Ini merupakan laporan pertama kali pemencilan gen penuh *RFT1* dan transformasi ke dalam apeks pucuk MR219. Penemuan dalam kajian ini boleh menjadi kaedah baru untuk manipulasi genetik dan peringkat asas penghasilan padi transgenik.

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LIST OF ABBREVIATIONS

Amp	-	Ampicillin
ANOVA	-	Analysis of variance
AS	-	Acetosyringone
ATP	-	Adenosine Tri Phosphate
BM	-	Branch Meristem
CaMV	-	Cauli flower mosaic virus
CAT	-	Chloramphenicol Acetyl Transferase
cDNA	-	Complementary deoxyribonucleic acid
CI	-	Consistency Index
CTAB	-	Cetyl Trimethyl-Ammonium Bromide
DEPC	-	Diethylpyrocarbonate
dH_2O	-	Distilled Water
DN	-	Day Neutral
DNA	-	Deoxyribonucleic acid
Ehd1	-	Early heading date 1
FM	-	Floral Meristem
FT	-	Flowering Locus T
gDNA	-	Genomic deoxyribonucleic acid
GFP	-	Green Florescence Protein
GPR	-	Glycine-Rich Protein
GUS	-	β-glucuronidase
Hd1	-	Heading date 1
Hd3a	-	*Heading date 3a*
HPT	-	Hygromycin Phospho-Transferase
hyg^R	-	Hygromycin
IM	-	Inflorescence Meristem
IPTG	-	Isopropyl-β-D-1-thiogalactosidase
kan^R	-	Kanamycin
KIN	-	Kinetin

LB	-	Left Boarder
LB media	-	Luria Bertani Medium
LB-G	-	Luria Bertani Glucose
LD	-	Long-day
MEGA	-	Molecular Evolutionary Genomic Analysis
mRNA	-	Messenger Ribonucleic acid
MS	-	Murashige and Skoog
NOS	-	Nopaline Synthase
NPT	-	Neomycin Phospho-Transferase
OCS	-	Octopine Synthase
OD	-	Optical Density
OsGI	-	*Oryza sativa Gigantea*
PCR	-	Polymerase Chain Reaction
PEBP	-	Phosphatidyl-Ethanol-amine-Binding Protein
PGR	-	Plant Growth Regulator
PPT	-	Phosphinotricin acetyltransferase
RB	-	Right Boarder
RE	-	Restriction Enzyme
RFT1	-	*Rice flowering locus T1*
RI	-	Retention Index
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal Ribonucleic acid
RT	-	Reverse Transcriptase
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
SAM	-	Shoot Apical Meristem
SD	-	Short Day
SM	-	Spikelet Meristem
SPSS	-	Statistical Package for the Social Sciences
T-DNA	-	Transferred Deoxyribonucleic acid
TDZ	-	Thidiazuron
TrE	-	Transformation Efficiency
Ti plasmid	-	Tumor inducing plasmid

UN	-	United Nation
Vir	-	Virulence
X-gal	-	5-bromo-4-chloro-3-indolyl β-D- glucuronic acid

LIST OF SYMBOLS

µL	-	Micro litre
mg	-	Milligram
G	-	Gram
L	-	Litre
v	-	Volume
V	-	Voltage
A	-	Current
%	-	Percentage
mL	-	Milli litre
µM	-	Micro Molar
w/v	-	Weight per Volume
rpm	-	Rotation per Minute
^{o}C	-	Degree Celsius
kb	-	Kilo base
bp	-	Base pairs

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Rice (*Oryza sativa* L.) is an essential cereal, suitable as a food source, and widely cultivated and used as a staple food in many population centres around the world. It is the most extensively cultivated cereal crop after wheat and enjoys attractive agronomic importance. Rice is cultivated worldwide due to its diversified flowering time. It is a facultative short day (SD) plant but also produce flower during non-inductive long-day (LD) condition (Itoh and Izawa, 2013a; Izawa, 2007). In addition, rice is apposite for flowering development analysis due to its relatively small genome size ($\geq$ 430 Mb) and diploid origin (2n = 24) (Sahoo *et al.*, 2011; Sankepally and Singh, 2016). It is a model species for studies on gene expression, genome organisation and transgenes behaviour (Manimaran *et al.*, 2013).

The species has 3 sub-species including *Indica, Japonica* and *Javanica.* About 80% of the world rice production is *indica* which is cultivated under tropical and sub-tropical environments as slender and long-sized grain (Din *et al.*, 2016). In Malaysia, many types of *indica* rice are cultivated, including upland and wetland cultivars (Sohrabi *et al.*, 2012). Examples of Malaysian upland rice cultivars include Lamsan, Selasi (Shahsavari *et al.*, 2010), Hitam Wai (Arifa *et al.*, 2016) and Panderas (Din *et al.*, 2016). Examples of wetland cultivars cultivated in Malaysia include MR219, MR232 and MR220 (Htwe *et al.*, 2011; Zuraida *et al.*, 2011). The uplands are cultivated in Sabah and Sarawak and some parts of Peninsula Malaysia under naturally well-drained soil, without surface water accumulation and phreatic water supply. The wetlands are farmed only in Peninsula Malaysia under flooded soil conditions.

Research on Malaysia upland rice has been neglected due to their low yield, long reproductive phase, and poor amenability to genetic transformation (Din *et al.*,

2016). Wetland varieties, particularly MR219, are high yielding cultivars and verified for their genetic transformation (Htwe *et al.*, 2011; Zuraida *et al.*, 2013; Zuraida *et al.*, 2012). The MR219 is a developed cultivar having resistance to abiotic stresses including drought, salinity and acidity. However, MR219 has proven to be susceptible to bacterial and fungal diseases such as leaf blight and blast (Fook *et al.*, 2015; Tan *et al.*, 2017). Nevertheless, the flowering development and productivity of this rice cultivar can be improved via genetic transformation of its florigen. This alteration may significantly increase rice productivity and decrease massive importation of rice into Malaysia. In recent times, this biotechnological approach has become an important tool in gene function studies, grain production and stress tolerant analysis (Tan *et al.*, 2017; Zhu *et al.*, 2017).

Naturally, the reproductive phase of rice is triggered by both florigens (Itoh and Izawa, 2013b) and environmental signals during its photoperiod (Albani and Coupland, 2010; Nuñez and Yamada, 2017). The florigens include *Heading date 3a* (*Hd3a*) under SD condition and *Rice Flowering Locus T1 (RFT1*) under LD condition which are highly conserved (Komiya *et al.*, 2009). Specifically, *RFT1* gene regulates flowering by translocation from the leaf to the shoot apical meristem (SAM). The gene interacts with transcriptional factor *flowering locus D* (*FD*) and functions as a mobile signal for switching on the flowering process (Itoh and Izawa, 2013b; Komiya *et al.*, 2008). Itoh and Izawa (2013b) reported that *Heading date* 1 (*Hd1*) and *Early Heading date1 (Ehd1)* act as floral regulators in transcriptional regulation and promote the activation of the *RFT1* gene. To date, no biotechnology-experimental evidence on the gene isolation and transformation in Malaysia rice has been reported. Similarly, its expression pattern is still not fully understood.

Genetic transformation is the best biotechnological strategy for developing transgenic rice (Yaqoob *et al.*; Zhu *et al.*, 2017). The system generates plants with improved traits and phenotype that are unachievable by a conventional breeding system. As reviewed by Birch (1997), mediated *Agrobacterium* transformation system appears to be the most promising transformation system for the production of transgenic cereal crops including rice. However, *indica* rice transformation remains difficult due to the cultivars' genotype-dependence, tissue culture recalcitrance,

transformation to transgene integration and regeneration to transgenic recovery (Tan *et al.*, 2017). Therefore, the present research identified suitable and viable explant that would allow successful integration of transgene during transformation, followed by regeneration to final recovery of transgenic rice.

In tissue culture, suitable explant identification was employed to achieve efficient gene transfer and transformants regeneration. Previously, several explants were considered for rice transformation, namely: embryogenic callus (Rahman *et al.*, 2011) and immature embryo (Hiei and Komari, 2008). Callus has less regeneration potential after transformation due to variety-dependence, while immature embryos are available only at certain periods in the year and are difficult to handle (Dey *et al.*, 2012). In this research, young rice shoot apex was used for successful transformation via *Agrobacterium*-mediated transformation system and transgenic recovery. The merit for shoot apex transformation includes being genotype-independent, direct regeneration of transformants, maintained cultivar integrity and ease of handling (Clement *et al.*, 2016; Dey *et al.*, 2012; Fook *et al.*, 2015).

To comprehensively dissect the naturally occurring LD flowering development in rice in pursuance of this research, a detailed study of the *RFT1* gene transformation and expression is necessary in order to fully understand its gene molecular mechanisms. However, construction is the basis for understanding the florigen mechanism and its flowering regulation by the application of genetic engineering. The current research is the first to isolate and construct the *RFT1* gene from upland rice, which is then transformed into MR219 cultivar. Therefore, *RFT1* gene isolation and genetic transformation would allow the development of new rice lines, facilitate elucidation on the gene mechanism, and provide insight into the molecular basis of rice growing at LD temperate regions.

1.2 Problem Statement

Gene isolation and appropriate construct development for genetic transformation to produce a high volume of transgenic variety(s) with predictable

transgene expression are the major technical challenges of *indica* rice transformation research. Till date, no report on the genetic transformation of Malaysian rice using any florigen has been reported. *RFT1* florigen was only isolated from wetland rice and was rarely transformed. The gene isolation from upland rice and its transformation would provide insight into the production of local transgenic rice and enhance stable integration of the transgene for phenotype improvement. Besides, the molecular mechanism underlying the rice flowering development in the nation and the responsible florigen gene is also incompletely understood. These tricky issues need urgent attention with regards to understanding the flowering regulation network of the country's rice cultivars. Such ends can only be achieved via the successful transformation of the flowering gene using suitable and viable explant. However, the identification of suitable explant via tissue culture prior to its transformation, its *in vitro* regeneration potential after the transformation, and its productive transformation technique remain problematic.

Despite the good characteristics of some Malaysia *indica* rice cultivars, little has been reported about its efficient genetic transformation mainly due to its recalcitrance and less amenability to transformation. However, most of the transformation methods are dependent on embryogenic callus or immature embryo culture as explant source, while their regeneration capacity and transformation efficiency are limited. Thus, these regional *indica* cultivars have a high risk of contamination during culture, thus limiting their regeneration efficiency after the transformation. This study sought to address the problems of these fewer viable-explants, their inefficient regeneration and unsatisfactory transformation (genotype-dependence) by providing a viable alternative with efficient regeneration and transformation potentials. Hence, the choice of MR219 variety for successful transformation would be very beneficial due to its abiotic stress resistance and susceptibility to diseases.

1.3 Significance of the Research

Isolation and construction of florigen remain the basis for understanding the flowering regulation mechanism of rice cultivars through the application of genetic transformation. Precise characterisation of the molecular nature of the photoperiod sensitivity in *RFT1* gene is the key factor to provide an insight into the molecular basis of day length recognition and mechanism behind the breeding system of rice in Malaysia. Further, the exact understanding of the molecular function of the floral regulator would allow the identification of critical targets for the development of local rice lines in the nation. Hence, to attain that, it is important to establish an efficient tissue culture by identifying a suitable explant for the transformation activity and transgenic regeneration protocol.

A successful transformation of *RFT1* gene into MR219 cultivar and transgenic regeneration to acclimatization will be of paramount importance. It will improve local rice transformation following the provision of viable explant and efficient transformation procedure, as well as produce prolific or stress-resistant lines after stable expression of the transgene in the transgenic variety line. In fact, the established method followed in this research will generate young viable-explants containing actively-dividing cells with high regeneration and transformation potentials. These better-quality varieties could lead to the improvement of local rice cultivars and reduce the persistent problem of rice importation into Malaysia. Thus, genetic engineering intervention through shoot apex transformation could break the barrier of genotype-dependence and establish a regenerable protocol for local transgenic *indica* varieties. Additionally, this solution could provide useful insights into the transformation of other rice cultivars.

1.4 Research Objectives

I. To isolate full-length *Rice Flowering Locus T1* (*RFT1*) gene.
II. To develop *RFT1* construct into a plant expression vector for transformation of Malaysia rice MR219.

III. To optimize the transformation of *RFT1* gene into Malaysia rice MR219 shoot apex.

IV. To characterize *RFT1* gene expression pattern in the transgenic MR219.

1.5 Scope of the Research

Genomic deoxyribonucleic acid (gDNA) and ribonucleic acid (RNA) was isolated from mature leaves of Malaysia upland rice, cultivar Wai. The full-length *RFT1* gene was amplified from the gDNA and complementary DNA (cDNA) by polymerase chain reaction (RT-PCR) analysis. Both the gene amplicon and sequences were analysed for full-length amplification. *pGMT:RFT1* construct was transformed into *E. coli* DH5α, while *pCAMB::RFT1* expression construct was transformed into *Agrobacterium tumefaciens* LBA4404. *In vitro* shoot apex induction for transformational purposes was carried out from mature seeds of MR219 Malaysia wetland rice. *Agrobacterium*-mediated transformation of the rice shoot apices with *Agrobacterium* cells harbouring the *pCAMB::RFT1* construct and regeneration of transgenic plant were established. The genotype of the transgenic rice by PCR amplification of the *RFT1*, *hpt* and GUS gene was determined. The transgenic rice was regenerated and acclimatized, the phenotypic characteristics at the vegetative stage were observed, and the transformation efficiency determined. Stable integration of the transgenes in the T_0 plant was confirmed by GUS assay and PCR validation.

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