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Quality RNA Isolation and Efficient Construction of Rft1 (Peasy:Rft1) Gene

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ABSTRACT

***Introduction**- High-quality RNA isolation from a polysaccharide-rich plant such as rice for downstream application is challenging. Equally, successful gene cloning for molecular analysis is tricky as it produces negative transformants.*

***Objective**- Here we reported on qualitative and quantitative RNA, RT-PCR amplification of RFT1 gene from matured leaves of Malaysian upland rice, then construction of the gene.*

***Methodology**- The RNA was isolated from Wai cultivar leaves (indica sub-species) using three different methods, synthesized cDNA and RT-PCR amplification of RFT1 gene. A T-A cloning of the RFT1 gene into p-EASY T3 vector (pEASY:RFT1), chemical transformation into E. coliTrans1-T1 and screening of the transformed cells on selective Laix-plate, by colony PCR and RE analysis were observed.*

***Results**- The RNA integrity indicated that using guanidine isothiocyanate based method, precisely Trizol was the superlative (yield = 36.50±0.59 and purity = 2.04±0.09) for isolating quality RNA from upland rice leaves. The RT-PCR and bioinformatics analysis resulted in full-length RFT1 gene amplification; responsible for promoting flowering under LD condition. Both recombinant DNA construction and transformation were efficient with more than 50% white colonies due to deactivation of the lacZ reporter gene. Also, the gel analysis of the colony-PCR indicated an absolute RFT1 gene amplification with a single-band and double-bands (vector and insert) after RE analysis all at expected positions.*

***Conclusion**- These have shown the suitability of the RNA isolation protocols observed in producing quality RNA for downstream applications, first ever reported on full-length RFT1 gene isolation from Malaysian upland rice and successful gene construction (pEASY:RFT1).*

Keywords: *RNA isolation; Upland Rice; Matured Leaves; RFT1 gene; Construction.*

INTRODUCTION

Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA) are two core components of molecular biology i.e. the central dogma [1]. Intact and high-quality RNA isolation for downstream application from a polysaccharide rich plant such as rice is difficult due to so many sources of contamination and its instability [2, 3]. Generally, polysaccharide and other bioactive compounds interfere or co-precipitate in the presence of alcohol during RNA extraction, remains as RNA contaminants [4], as well as inhibiting the polymerase activity [5]. These complex compounds affect the quality of the RNA extracted from rice tissues and lessen its integrity for downstream applications. First-class RNA isolation is necessary and prerequisite step in gene expression studies such as complementary DNA (cDNA) synthesis, microarray analysis or cDNA library construction, amplification of actual gene (without interference of intron) from eukaryotic genome via RT-PCR (reverse transcriptase-Polymerase Chain Reaction), qPCR (quantitative PCR) and northern blot analyses [6, 7]. Diverse number of extraction protocols have been reported [8, 9] including; CTAB (Cetyl Trimethyl Ammonium Bromide) and modified CTAB, use of GT (Guanidine thiocyanate), SDS (Sodium Dodecyl Sulphate) or pure-zol [10]. All the above-mentioned methods aimed at obtaining high-quality RNA from starch-rich plant tissues.

Rice (*Oryza sativa*) is a cereal crop and polysaccharide-rich source [5], staple diet, a source of food for more than 70% of the world

populist and with speedy increasing global demand [11, 12]. It is model monocot plant for studies of genome organization, gene expression as well as transgenes behavior [13, 14]. Hence, it is essential to study more on the molecular traits of rice, particularly upland cultivars by considering their merit over wetland species; for plant molecular biology (precisely genetic transformation) and agriculture record. So far, little was reported concerning RNA and reproductive genes isolation from upland rice varieties, *indica* sub-species. Isolation and full-length amplification of such genes are the prerequisite in genetic transformation towards improving reproductive phase. *Rice Flowering Locus T1* (*RFT1*), is the second rice florigen after *Heading date 3a* (*Hd3a*) that promote flowering in rice growing at long-day (LD) environment [7, 15]. The gene regulates flowering period through a complex genetic network by translocation from leaf to shoot apical meristem (SAM) [15-19].

Subsequent to isolation, recombinant technology by joining purified foreign DNA into a vector (either cloning or expression) follows. Such is to get multiple copies or expresses the gene which all depend on the vector used. This allows scientist to discover proteins, explore on cellular activities after been changed and also study cell division. Using recombinant DNA technology, the gene can be clone into different vectors (construction) and direct its replication within the host cell. Similarly, the such construction via T-A cloning is one of the most efficient and simple method for the direct cloning of PCR products. The system is often desirable by facilitating the directional cloning and to isolate the ligated product after transformation using a specific orientation. Hence, RNA isolation with high quality, full-length amplification of *RFT1* responsible for flowering promotion in rice under the LD condition and then construction of the gene into a vector is of significant interest. It will provide an insight on the actual gene function and necessitate the development of transgenic rice with early flower production. This study was the first to report on the isolation of high-quality RNA from matured leaves of Malaysian upland rice (*indica* sub-species), RT-PCR amplification of *RFT1* gene and the gene construction. Three different RNA isolation protocols aimed at obtaining the best, amplification of full-length *RFT1* gene and efficient construction of the gene which will be used in genetic transformation was successfully achieved.

Materials and Methods

Plant Material and Growth Condition

Matured seeds of Malaysian upland rice cultivar Wai were collected from Sibu, Sarawak Malaysia. The seeds were planted in a pot with a soil mixture of 1:2 ration of sand – composed and grown the plant in the glasshouse of FBME, UTM-JB. The individual pots were watered between 2-3 days interval. The RNA isolation was carried out between 8-11 weeks old of growing period.

RNA Isolation Protocols

Prior to extraction analyses; mortar and pestle, spatula, pipette tips, 70% ethanol and nuclease free-water were all treated with 0.1% DEPC. The RNA was isolated from the cultivar matured leaves following three different extraction protocols. Firstly, the RNA was isolated using Trizol reagent (Sigma-Aldrich) following the manufacturers' instruction. The only alteration to the method was during phase separation were Chloroform and Phenol are used separate or in combination, just to obtain proper separation of the RNA. Secondly, Qiagen RNeasy mini kit was used according to manufacturer's instruction, and thirdly using TransGen EasyPure Plant RNA kit also as described by manufacturer's.

Determination of RNA Integrity (Quantity and Quality)

There are three quality control that are performed to determine the integrity of isolated RNA. That includes the concentration (yield), purity and quality (integrity). For concentration and purity of RNA; spectrophotometric absorbance at A_{260}/A_{280} nm were observed using Nanodrop™ 1000 spectrophotometer.

The quality of the isolated RNA was evaluated for 28S and 18S ribosomal RNA (rRNA) [20, 21] and assessed by gel electrophoresis on 1.2% (w/v) agarose. Prior to the analysis, all the gel items including gel-cast, tray, comb and gel-tank were soaked in 0.1 M NaOH for 20 minutes for proper sterilization. The agarose was dissolved in 1X TAE buffer (50X TAE 1 L stock: 242 g Tris-base, 57.1 mL Glacial Acetic Acid and 10 mL of 0.5 M EDTA) stained with SYBR safe (Invitrogen) using a micro-wave oven for 90 secs and allowed to cool to around 50 ^{o}C before pouring. The gel was run at 80V and 425A for 45 minutes, then visualized under the UV light using Gel documentation unit (Thermo-Fisher Scientific).

cDNA Synthesis, RT-PCR Amplification of RFT1 Gene and Sequence Analysis

Initially, DNase treatment was observed using DNase I Promega kit prior to cDNA synthesis. The treatment was to eliminate the remnants of gDNA from the isolated RNA. cDNA was synthesized from the DNase-treated RNA using GoScript™ reverse transcription system (Promega) according to manufacturer's instruction. RT enzyme and Oligo(dT)$_{18}$ primer was used to reverse transcribed 5µg of the intact RNA.

Reference gene; precisely, acting housekeeping (*Act1*) gene [22, 23] was earlier amplified by RT-PCR (reverse transcription PCR) in master cycler (Gradient Eppendorf) to determine the integrity of the synthesized cDNA. The thermo-cycling condition was; pre-denaturation at 94 ^{o}C for 5 min, followed by 30 cycles; denaturation for 60 sec at 94 ^{o}C, annealing for 30 sec at 53 ^{o}C and extension for 3 min at 72 ^{o}C, then final extension at 72 ^{o}C for 5 min and cooling at 4 ^{o}C. While for *RFT1* gene amplification, gene-specific primer (GSP) (F (5'tggctagct taaccttcctg3' and R (5'gtctaccatcacctgtaggt3') was used. The amplification was carried using the following profile: 94 ^{o}C for 4 min pre-denaturation, then 35 cycles; denaturation for 30 sec at 94 ^{o}C, annealing for 40 sec at 55 ^{o}C, extension for 1 min 20 sec at 72 ^{o}C, then final extension at 72 ^{o}C for 5 min and cooling at 4 ^{o}C. The quality of the amplicons was analyzed by gel electrophoresis (74 V and 425 A for 45 min) on 1 % (w/v) agarose gel in 1X TAE stained with SYBR safe (Invitrogen).

Wizard SV gel and PCR clean-up system kit from Promega was used to purify the gel band of the *RFT1* gene and later send for sequencing to 1st Base Sdn Bhd Company. The sequence was analyzed by nucleotide and protein BLAST for identification. Multiple sequence alignment of *RFT1* gene from the present study and other *RFT1* gene sequences from different cultivars obtained from GenBank (http://www.ncbi.nlm.nih.gov/) were carried out. The cultivar's *RFT1* gene sequence include that of Basmati 370 (BAH30236), Kemasin (AB838579.1), Pokkali (BAO03221), Bleiyo (BAJ53916) and Muha (BAJ53912).

Construction of RFT1 Gene into Cloning Vector (pEASY: RFT1) and Transformation

pEASY$^{(R)}$-T3 (3039 bp) cloning vector from Transgen-Biotech was used in the present study for construction. The vector is about 3039 bp with ampicillin resistance gene (Amp^{R}), pUC18 origin of replication and T-overhangs for easy cloning. The vector contains T7 and SP6 polymerase promoters (priming site) flanking a pUC18 polylinker region (multiple cloning sites) within the alpha-peptide coding region of the functional enzyme β-galactosidase (*lacZ* reporter gene). It possesses a dual *NotI* and *EcoRI* enzyme site for the release of the insert using single enzyme digest.

For PCR amplification of *RFT1* gene to be ligated into the vector, *Taq* DNA polymerase master mix was used and amplified using GSPs as shown above.The PCR product gel bands were purified using Wizard SV gel and PCR clean-up system kit (Promega). A purified full-length *RFT1* gene was ligated into the vector (construction) via T-A cloning system as described by the vector's manufacturer's instruction, resulting in '*pEASY:RFT1'*. Optimal conditions for ligation of insert and vector (I:V=4:1) were exercised at 5µL reaction volume, 10 min incubation time and higher incubation temperature up to 37^{o}C.

The resulting plasmid construct *pEASY:RFT1* was introduced into *E. coli* strain *Trans*1-T1 Phage Resistance chemically competent cell (Transgen-Biotech). The transformation was by heat-shock method for 30-40 sec at 42^{o}C as described by Sambrook and Russell [24], Li, Sui [25] and Chan, Verma [26]. After shaking the transformed cells in 250 µL LB (Luria Bertani) medium for 1 hour at room temperature, 50-100 µL of the transformed cells were spread on LB selective plate (LB plate supplemented with 50 µg/mL ampicillin, 120 µL of 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 40 µL of 100 mM IPTG (Isopropyl-β-D-1-thiogalactosidase)) called X-gal method or Laix-plate [24, 27]. Positive control (untransformed cell on the selective plate) and negative control (empty plate) were also provided respectively.

Screening of Positive Transformants

Positive transformants i.e. containing the constructed DNA fragments were identified by white-blue colony on Laix-plate, colony PCR and restriction endonuclease (RE) analysis. For direct PCR amplification, a 4-6 white/light blue colonies on the selective plates were picked and added into 20 mL autoclaved distilled water and then, used as a template for amplification of the gene construct using GSPs. The thermo-cycling condition was; pre-denaturation for 10 min at 94 ^{o}C, followed by 30 cycles; denaturation for 30

sec at 94 °C, annealing for 30 sec at 55 °C and extension for 2 min at 72 °C, then final extension at 72 °C for 5 min and cooling at 4 °C. The amplicon quality was analyzed on 1% (w/v) agarose by gel electrophoresis and visualized as described above.

Then, for RE analysis [28, 29], a 2-3 white/light blue colonies were inoculated into 5mL selective LB broth and shaken at 150 rpm, 37°C overnight. The recombinant vector was isolated from the overnight cultured using Invitrogen Pure Link™ Quick Plasmid Miniprep kit as described by the manufacturer. Single digestion of the DNA construct vector with *EcoRI* and *NotI* RE bought from New England Biolabs (NEB) were carried our as described by manufacturer's instruction intending to release the insert from the vector [30].The digested product was equally analyzed on 1% (w/v) agarose by gel electrophoresis and equallyvisualized as described above. Also, the cloned gene were sequenced.

Results and Discussion

Best RNA Isolation Methodology

The most important and prerequisite step in the nucleic acid (NA) downstream application is the isolation of high-quality RNA, which would serve as a key factor and an evidence to further the analysis. Once the RNA isolated is of higher integrity based on qualitative and quantitative examinations, the next is to secure its integrity by storing at -80 °C - -70 °C prior to subsequent analysis. We attempted to isolate qualitative and quantitative RNA following three different methods from matured leaves of Malaysian upland rice, cultivar Wai. All the three different protocols applied in this study produced quality RNA as successfully analyzed. Beginning by Trizol reagent; using such for isolation of RNA from various samples including bacteria and animal have been testified [31-33]. The technique has been widely used, yielded positive result and recommended for RNA isolation from plant samples [34-36]. In this study, the first incubation at room temperature after homogenization was observed on ice to prevent the possible degradation of the RNA. Then, the chloroform and phenol were used in separate and equally mixed together during phase separation step, but no result difference was observed.

Interestingly, during phase separation step all the three expected phases were well parted and high volume aqueous upper layer was collected. Hence, using Trizol reagent for high-quality RNA isolation from matured leaves of upland rice was achieved, with optimum concentration at A_{260}/A_{280} and purity higher than that of Wan and Wilkins [37] and Arif, Ma [10] as tabulated in table 1. Using such protocol based on guanidine isothiocyanate and chloroform/phenol extraction has been accepted, because a small amount of time is required to isolate quality RNA without sacrificing its quality as earlier reported by Chomczynski and Sacchi [33], reviewed by Simms, Cizdziel [32]. In addition, the Trizol reagent was integral and extraction method was efficient in yielding RNA with higher quality as confirmed in this research. Though, using such was tiresome if compared to mini kits, but yielded superlative after a little modification. However, the sticky, gel-like mixture produced during homogenization process is a major problem of using Trizol reagent which affects the RNA quality and efficiency as described by Wang, Wang [6], but no such was observed in the present study.

For Qiagen RNeasy mini kit; the isolation of the RNA was successful which gave higher purity (2.22±0.03) among the three protocols (Table 1). This corresponds with the finding of Arif, Ma [10] which indicated that mini kit was the second best after PUREzol. The methods (Trizol reagent and Qiagen RNeasy mini kit) are actually based on guanidine isothiocyanate and chaotropic to recuperate RNA. But, for later is with the help of β-mercaptoethanol to recover the RNA. In case of mini kit from TransGen, also gave satisfactory purity, but with lower concentration. Figure 1a, b, and c have shown the RNA integrity from the three different extraction methods based on 18S and 28S ribosomal RNA (rRNA) [10, 38]. By comparing the three isolation protocols observed, Trizol reagent was the best for isolating RNA from matured leaves of Malaysian upland rice (Wai cultivar). This rendered higher integrity RNA, yield and reproducibly. Then, followed by Qiagen and TransGen kit as shown in Table 1 and Figure 1a, b and c respectively. Also, similar to this was reported by Portillo, Fenoll [39], which described Trizol as the best method that extracted higher RNA yield and quality among other independent extraction protocols.

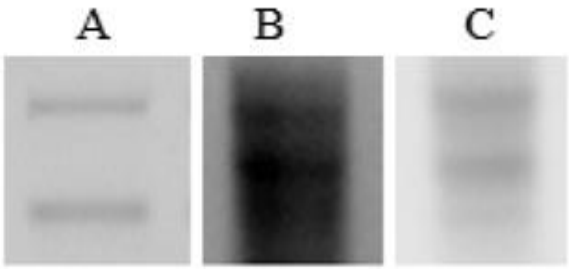

Figure1. *The 28S and 18S ribosomal RNA isolated from Malaysian upland rice matured leaves using three different extraction method; (A) isolated RNA using Trizol reagent, (B) Qiagen RNeasy mini kit and (C) TransGen EasyPure Plant RNA kit.*

Table1. *Total RNA purity, concentration and yield from Malaysian upland rice matured leaves via three different isolation protocols. The purity and concentration were based on Nano-drop spectrophotometric analysis, while yield was calculated against 100mg fresh weight leaf sample.*

Method	Purity	Concentration (ng/µL)	Yield (µg/100mg)
Trizol reagent	2.04±0.09	1,079.2±11.73	36.50±0.59
Qiagen RNeasy mini kit	2.22±0.03	705.9±9.08	28.36±0.37
TransGen EasyPure Plant RNA kit	1.91±0.05	167.1±9.64	9.85±0.42

Assessment of the RNA Quality and Quantity

Total RNA extraction success is absolutely depending on the purity, quantity and quality (integrity) of the isolated RNA from a given sample. The RNA purity and concentration was determined by Nano-drop 1000 UV spectrophotometer at absorbance ratio of A_{260}/A_{280} which enables accurate sample analyses. An A_{260} ratio is the maximum RNA absorbance ratio, while the absorbance at A_{260}/A_{280} is used to assess the RNA purity. Considerably, value ranges between 1.8-2.0 indicates that the RNA is pure [40]. This has justified that the RNAs isolated using the three different methods are all of high purity (table 1), which showed no significant contamination from either polysaccharide, polyphenols compounds or protein [6]. The outcome demonstrated the efficiency of all the techniques toward separating RNA from other macro-compound of the leaves. Even though all the method gave acceptable purity result; but Qiagen RNeasy was the best with 2.22±0.03, followed by Trizol and TransGen EasyPure Plant RNA kit (Table 1) [7].

Moreover, the concentration obtained was high from the first two protocols (Trizol and Qiagen RNeasy). Optimum concentration and yield above that of Arif, Ma [10] and Jhala Vibhuti, Mandaliya Viralkumar [5](2015) were obtained. The two guanidine isothiocyanate reagents gave the highest purity, concentration as well as yield. The result revealed that Trizol reagent produced high yield (36.50±0.59 µg/100mg), followed by Qiagen RNesay mini kit (28.36±0.37 µg/100mg). Portillo, Fenoll [39] reported that Trizol reagent is the best for RNA isolation from plant tissues compared to other methods. Therefore, these can be used as evidence toward producing pure and high concentrated RNA from *Oryza sativa* mature leaf, unlike from *Lycium barbarum*[41]. Hence, to further the RNA quality analysis, the total RNA integrity was validated based on 28S and 18S ribosomal-RNA (r-RNA) bands on agarose gel electrophoresis (Figure 1). The rRNAs are normally produced due to cleavage of a single RNA transcript and are used to assess the total RNA quality. Impact all the methods observed in this study produce band for 18S and 28S rRNA [10, 38].

Isolation and Characterization of RFT1 Gene

The RNA isolated via the diverse protocols observed are suitable for subsequent downstream application analyses. It was successfully used for cDNA synthesis and reverse transcription assay. cDNA synthesis uses to be the first downstream application after successful isolation of high-quality RNA. This process involves the use of reverse transcriptase (RT) enzyme and adapter or oligo$(dT)_{18}$ primer to synthesize double strand complementary DNA from a single strand RNA. In the present study, the cDNA was used as a template for reverse transcription amplification of the *Act1* reference gene. Universal *Act1* gene primers were used for the amplification and full-length gene were amplified (figure 2). *Act1* housekeeping genes are required for the maintenance of basic cellular function that is essential for the existence of a cell, regardless of its specific role in the living organism or

tissue(s) [22, 42]. In rice, the endogenous *Actl* transcript is plentiful in all tissues and development stages. Therefore, to investigate the physiological changes in gene expression analysis; comparative expression of actin gene is adequate for utmost reasons [23, 38]. Such reasons include; for an equal efficiency of enzyme reactions, which uses the non-regulated reference genes like 18S rRNA, 25S rRNA, tubulins and actin housekeeping gene. Though, the genes varied at certain experimental conditions as described by Schmittgen, Zakrajsek [43].

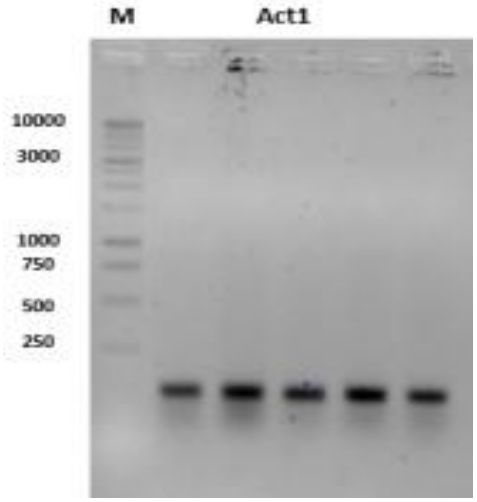

Figure2. *Agarose gel electrophoresis analysis of actin housekeeping gene from matured leaves of Malaysian upland rice cv. Wai. Lane 1: marker (1 kb ladder). Lane 2-6: actin housekeeping gene at approximately <150pb.*

Following the reverse transcription amplification of *RFT1* gene; the agarose gel electrophoresis result indicated that the gene was found at the actual expected position (figure 3). The gel band size was above 0.5 kb which correspond with that of finding of Ebana, Shibaya [44] and Ogiso-Tanaka, Matsubara [45]. They communicated that *RFT1* gene has 178 amino acids which are equivalent to about 534 nucleotides or more. Also, sequence analysis confirmed the gene to be *FRT1*. The nucleotide blast of the sequence indicated 99% identity with other cultivars from the database such as Kemasin. Moreover, the sequence was translated to amino acid sequence and analyzed for multiple sequence alignment with 5 other *RFT1* sequences from different cultivars of *indica* sub-species. The similarity index indicated that all the 6 sequences are highly conserved with almost 99% similarity as shown in figure 4. Hence, this conservation among the different cultivars may be due to their close evolutionary trend [15, 46]. This indicated that *RFT1* gene from the present research was fully amplified because of no gap or any other form of deficiency in the sequence.

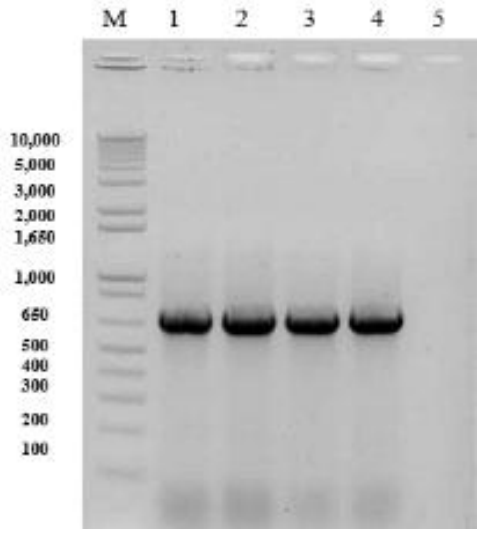

Figure3. *Reverse transcription PCR (RT-PCR) amplification and analysis of RFT1 gene from matured leaves of Malaysian upland rice, cultivar Wai. M; 1kb Plus DNA marker, Lane 1-4; RFT1 gene, and Lane 5; control.*

Figure4. *Comparison of RFT1 gene amino acid sequences from 6 rice cultivars. The gene sequences are from Wai cultivar of Malaysian upland rice and 5 others from NCBI data bank.*

NB: Stars; highly conserved region, double-dots; partially conserved region, single-dot; less conserved region and blank; non-conserved region.

Furthermore, the sequence alignment between Wai cultivar and other 5 cultivars from NCBI, revealed that nucleotide diversity difference is fairly much. This corresponds with the finding of Hagiwara, Uwatoko [47] and Ogiso-Tanaka, Matsubara [45] which indicated that defective *RFT1* found in *indica* cultivars and higher diversity of nucleotides (about 16 amino acids changes in *RFT1*); this implies that functional constraint was relaxed in *RFT1* after gene duplication. The authors also demonstrated that the haplotype diversity of *RFT1* and *Hd3a* was similar in cultivated rice. Even though the *RFT1* haplotype number is larger than that of *Hd3a* in the entire gene region, but smaller in the coding region.

Hd3a and *RFT1* are homologous genes, located close together and control the floral transition in rice mainly by dual regulation of *Ehd1*[48, 49]. Particularly, *RFT1* is the second rice florigen discovered and has been hypothesized as a hormone-like molecule for promoting flowering processes under LD condition [15, 16, 18, 50]. The gene is normally produced in the leaves and act as a mobile signal in the SAM of buds as well as growing tips. Nevertheless, understanding of the gene function in rice cultivars growing under LD condition Nations is still unclear. In addition, pieces of evidence indicated that overexpression of *RFT1* gene molecule with vascular-specific promoter or constitutive promoter results in an early-flowering phenotype under LD condition, while its suppression by RNA-interference (RNAi) procrastinate flowering occurrence as described by Komiya, Ikegami [15], Komiya, Yokoi [51]. This also implies that flowering under LD condition in rice is basically as a result of expression of this highly conserved florigen[52, 53].

Because of the above prove on *RFT1*, we tried to isolate the full-length gene from matured leaves of Malaysian upland rice, cultivar Wai, which could later be used for genetic transformation to induce early flowering. Molecular isolation, characterization and transformation of *RFT1* gene is of significant interest. This will necessitate the development of transgenic rice with early flower production. So far, this was the first to report on *RFT1* gene isolation, molecular activity, construction and transformation from Malaysian upland rice, *indica* sub-species. Interestingly, we are opportune to amplify the full-length *RFT1* gene from the matured leaves of Malaysian upland rice. Impact, this is the first ever reported from upland rice cultivars.

Construction of RFT1 Gene and Transformation Analysis

Successful cloning of the gene of interest into a vector remain a challenging task in molecular

studies, as it produces negative transformants. The ligation protocol observed in this research carries no T4 DNA ligase for the reaction, but it was effective and yielded a positive result with the *p-EASY* T3 cloning vector. Directional sticky-end ligation, precisely 'T-A cloning' was carried out based on the vector at hand, as well as considering its reporter gene and MCS. This development involves the joining of the two DNA molecule ends with a phosphodiester bond between the 5' phosphate of one nucleotide and the 3' hydroxyl of the other [54, 55]. In recombinant DNA technology; transformation is the next step after ligation of the foreign gene to the cloning vehicle where the recombinant vector is introduced into the bacterial cells. In the present study, an efficient transformation was achieved by the chemical method as described by Sambrook and Russell [24]. Using chemical transformation approach, bacterial cells are treated with $CaCl_2$; a divalent cation which makes them competent and allows the transient opening of the cells membrane [25, 26, 56, 57]. The method requires no special equipment to remove salts from the transformed DNA.

Subsequent to the successful introduction of the recombinant vector into the host cell, it is crucial to hand-picked the host cells that make up the DNA construct. In modern cloning strategies, such selection includes the growing of the bacterial cells on selective plates which permit the growth of only transformed cell, however, not necessarily containing the insert have been transformed to grow. Hence, such selectable media plates are prepared best on the plasmid anti-bacterial resistant gene that enables only transformants to grow on it. Similarly, the use of X-gal method (LAIX-plate) simplifies the process by given a clear difference between transformed cells with recombinant vector and those without the insert called negative transformants [58]. In this study, the transformed cells were spread on the LAIX-plate for selection of positive transformants. The white/blue colonies on the plates differentiate the insert possessing colonies from the non-insert possessing colonies, by displaying such coloured bacterial colony which occurred due to the *lacZ* gene deactivation [59].

The X-gal and IPTG in the LAIX-plate works by disrupting the α-complementation process of the vector that carries the *lacZ* gene sequence flanking an MCS. The MCS within the *lacZ* sequence is cut by RE enzymes (in case of a circular vector) so that the foreign gene would be inserted within the reporter gene, thereby disrupting it and thus production of α-peptide. Once the insert is ligated into the vector, it deactivates the reporter gene activity and permits the appearance of white colonies on the LAIX agar plates. Consequently, transformed cells containing the recombinant vector without the functional β-galactosidase may be formed as white or light blue on the plate. The compound is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, that spontaneously oxidizes and dimerizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo [29, 60, 61]. The blue colonies characteristic, hence, showed that the cells contain an uninterrupted *lacZ* gene or fully functional β-galactosidase enzyme, whereas the white/light blue colonies (X-gal is not hydrolyzed) indicated the presence recombinant vector in the bacterial cells [62].

According to Glover [63], insertion of DNA fragment into most cloning vectors destroy such reported gene integrity and recombinant can be identified by their phenotype as well as characteristics encoded due to inactivated gene displayed by the host organism. Also, additional examination and screening of positive transform ants from the resulting white colonies are required for more validation exercise. Therefore, to further determine the likelihood of the inserted gene in the transformed cells and the sensitivity of the direct PCR analysis, GSPs were used for the amplification as earlier reported by Mirhendi, Diba [28]. Figure 5A showed the gel electrophoresis result of the PCR, indicating the existence of the gene in the vector. The result revealed an absolute *RFT1* gene amplification and then, clearly demonstrated the efficiency of the amplification process (PCR) in determining the presence of the insert in the recombinant vector. This amplification of the constructed gene was effectively achieved in all the white and light blue colonies with no false positive reaction. Furthermore, this application of direct examining the existence of constructed vector from bacterial colonies is cost-effective, fast, simple and reliable [28, 64]. Hence, colony-PCR would be the most rapid, appropriate and routine method for determining the presence of insert gene in the recombinant vector. The technique is not time intensive and no additional steps needed such as in case of sonication, mechanical high-speed cell disruption or use of toxic chemical(s) [65].

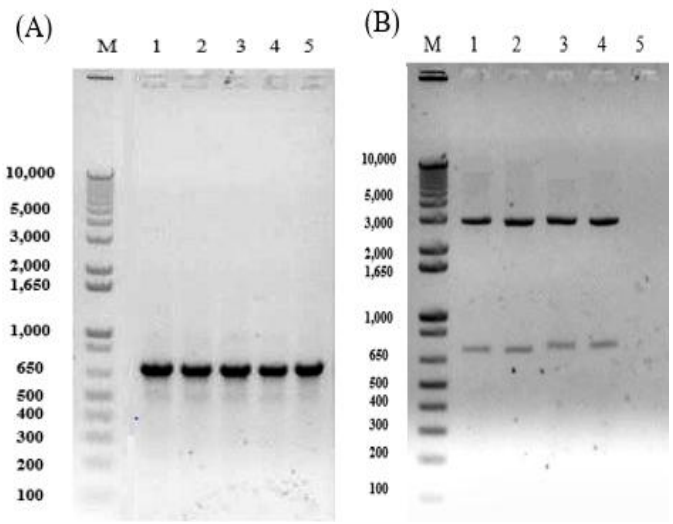

Figure5. *Positive transformants analysis. (A) Direct colony-PCR of transformed cells containing the pEASY:RFT1 construct using RFT1 GSP: M; 1kb Plus DNA marker, Lane 1-5; RFT1. (B) Restriction endonuclease analysis of pEASY:RFT1 construct. M; 1kb Plus DNA marker, Lane 1-2; recombinant vector single digest with Eco RI enzyme, Lane 3-4; recombinant vector single digest with Not I enzyme, and Lane 5; control.*

Evenly, RE digestion is examined for better confirmation of the presence of the insert in the constructed vector (*pEASY:RFT1*). In order to examine the existence of *RFT1* gene insert in the recombinant vector and efficiency of the vector in cloning acts; two separate single digestion using *EcoRI* and *NotI* enzyme were observed in this study. The selection of such restriction site was due to their dual appearance on the MCSs within the α-peptide coding region of the *lacZ* reporter gene. In fact, two separate bands at exact expected position of both *RFT1* gene insert and *p-EASY* T3 vector were observed on the gel after digestion as shown in Figure 5B, which indicated the presence of the insert in the vector. This corresponds with the finding of Mizuguchi and Kay [30], which communicated that multiple bands are seen on the gel after digestion of constructed vector. Normally, the plasmid-vector in possession of the foreign-DNA insert is isolated and digest with an enzyme(s) at appropriate sequences. It may be single and/or double digestion of the recombinant vector which depends on research aim. The RE recognizes its specific sequence on the DNA and cut the double strand at the exact position [63]. Such enzymes-cut led to the release of the insert from the recombinant plasmid and gave two separate bands on the agarose gel (figure 5B). This analysis (RE) is considered as the most accurate and reliable method of attesting the plasmid construct [28]. Also, signified the efficiency of the optimum ligation steps observed in this study and the vector's capacity in construction activity.

CONCLUSION

Polysaccharide and other bioactive compounds such as polyphenols affects the quality of RNA isolated from *Oryza sativa* tissues due to their abundance and also lessen its integrity for downstream applications. Rice; as a staple diet, source of food for many world populist with speedy increasing global demand; finding high quality RNA isolation method from such cereal crop and construction of the gene (*RFT1*) responsible for promoting flowering under LD environment like Malaysia for future use in promoting the flowering system will be a golden ticket. In this study, qualitative and quantitative RNA and full-length *RFT1* amplification were achieved. The construction of the gene into the cloning vector (*pEASY:RFT1*) was efficient and successful by producing lots of white colonies on Laix-plate, as well as providing a single band after colony PCR and double bands for RE analysis all at expected positions. Based on the three different RNA isolated methods observed; Trizol reagent was the best to produce high-quality RNA, then followed by Qiagen mini-kit. The vector used was efficient in carrying insert at optimal ligation condition. Using X-gal method, colony PCR and RE analysis was discovered the most suitable way for determining and confirming the existence of insert in the recombinant vector. Hence, this study has identified the suitable methods of RNA isolation from matured leaves of upland rice and amplified full-length *RFT1* gene. Also, constructed the gene and analyzed the gene presence via different systems. This discovery

was the first ever from Malaysian upland rice cultivar, which might serve as an evidence for future work.

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