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Isolation, Cloning, and Sequence Analysis of the Full-Length *RFT1* Gene from Malaysian Upland Rice (*Oryza sativa*, subsp. *Indica*, Cultivar Wai)

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Abstract

Rice Flowering locus T1 (*RFT1*) is the second rice florigen that promotes flowering during non-inductive long-day conditions. In this study, the full-length *RFT1* gene was isolated from the matured leaves of Malaysian upland rice (designated as Mu-*RFT1*), Wai cultivar of *indica* sub-species, and construct *pGMT:MuRFT1* was developed and successfully transformed into *E. coli* DH5α. The resulted gene sequence and phylogenetic relationships of ten other *indica*, two *japonica* rice and a single outgroup species were analyzed. The reverse transcription PCR and bioinformatics analyses demonstrated that the full-length Mu-*RFT1* shared 99 % nucleotide and 80 % amino acid identity with the other rice cultivars. Furthermore, the phylogenetic tree analysis revealed that the Mu-*RFT1* gene is closely related to Kemasin and shares common evolutionary ancestor characteristics including amino acid homology (99 %). This is the first study on the full-length *RFT1* gene isolation, cloning, and sequence analysis from upland rice, Malaysian cultivar. It also provides useful information on the phylogenetic relationship of the gene and its molecular evolution as well as designing a scientific breeding system for producing a novel variety of rice.

Keywords: Upland rice, *Rice Flowering locus T1* (*RFT1*) gene, Cloning, Phylogenetic tree, Sequence analysis.

1. Introduction

Rice (*Oryza sativa*) from the *Poaceae* family is the most important cereal crop and staple diet for many people worldwide. It is a model monocot plant widely used for genome organization and gene expression studies (Bajaj and Mohanty, 2005). The sub-species named *indica* comprises both the wetland and upland rice cultivars. The upland cultivars have numerous advantages including its potential as an alternative option for wetland rice and the possibility to be grown on dry-land or in rain-fed conditions. It is known to thrive on the surface, accumulated or phreatic or unbunded water supplies. The upland rice cultivar accounts for almost 80 % of rice cultivated worldwide, but contributes only to 12% of the global rice production due to the low yield and poor management practices (Din *et al.,* 2016). The *Oryza sativa* species is cultivated worldwide due to its diverse characteristics of flowering time. The florigen molecular and environmental signals such as photoperiod and temperature are the major factors that trigger floral induction processes (Liu *et al.*, 2018).

Scientific evidence from the molecular approach has indicated the existence of diverse evolutionary genes in rice that are uniquely acquired for promoting flowering. The flowering time in rice is basically determined by the expression of two essential yet highly conserved florigens including *Heading date 3a* (*Hd3a*) under short-day (SD) conditions and *Rice Flowering Locus T1 (RFT1*) under long-day (LD) conditions (Komiya *et al.*, 2009). The *RFT1* gene is the rice LD florigen precisely situated at 11.5 kb away from the *Hd3a* on chromosome six. The *RFT1* gene regulates heading date through a complex genetic network by translocation from the leaf to the shoot apical meristem (SAM). It generally interacts with the transcription factor *flowering locus D* (*FD*) using *RFT1*-GFP fusion from the leaves to the shoot apex. It reportedly functions as a mobile signal through a protein or mRNA to switch on the flowering process in rice cultivars (Itoh and Izawa, 2013; Komiya *et al.*, 2008). However, the *RFT1* gene has not been fully isolated from the upland rice variety of *indica* sub-species.

Additionally, understanding the gene function in rice cultivars growing under LD conditions is still unclear. Therefore, it is essential to further study the flowering system of upland rice, particularly the *RFT1* gene regulation. Arif *et al.* (2016) have only previously examined the integral RNA and *RFT1* protein isolation from the upland rice variety of *indica* sub-species but with no report on the full-length isolation. Therefore, this study presents the first report on the isolation, construction into

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cloning vector, and sequence analysis of full-length *RFT1* gene from the Malaysian upland rice. The study of the isolation of the full-length *RFT1* gene and its construction is of a significant interest, and will provide insights into the intrinsic gene function and its regulation mechanisms after plant transformation. Equally, the construction of a phylogram tree is important in the evolutionary studies of the gene. It is critical for inferring and clarifying the biological evolution and relationships between species.

2. Materials and Methods

2.1. Plant Material

Mature seeds of the Malaysian upland rice, cultivar Wai, were collected from Sibu, Sarawak Malaysia. The seeds were planted in pots based on the sandy soil to a compost ratio of 1:2, and were grown in the glasshouse of the Faculty of Science, Universiti Teknologi Malaysia, Johor Bahru, Malaysia.

2.2. RNA Isolation and cDNA Synthesis

The total mRNA was isolated from the 8-11 weeks old mature leaves using Trizol reagent (Sigma-Aldrich) based on the manufacturer's instruction. The DNase treatment was performed using DNase I Promega kit before the cDNA synthesis to eliminate any genomic DNA contamination. The quantity and purity of the mRNA were analyzed using Nanodrop^{TM} 1000 spectrophotometer at the absorbance ration of $A_{260}/A_{280.}$ Whereas, the mRNA quality was determined using agarose gel electrophoresis on 1 % (w/v) agarose in 1 X TAE buffer stained with SYBR safe (Invitrogen). Using the GoScript^{TM} reverse transcription system (Promega), the cDNA was synthesized by using 5 µg of poly $(A)^+$ mRNA, Oligo$(dT)_{18}$ primer and the reverse transcription enzyme according to the manufacturer's instructions. The cDNA was stored at -20 °C prior to subsequent experiment.

2.3. Reverse Transcription PCR Amplification of RFT1 Gene and Sequencing

The complete cDNA was used as a template for the reverse transcription PCR amplification of the full-length *RFT1* gene from the Malaysian upland rice (designated as Mu-*RFT1*) through gene-specific primers, as outlined in Table 1. The amplification was performed at 94°C for a four minute-pre-denaturation, followed by thirty cycles of denaturation for thirty seconds at 94°C. Annealing was implemented for forty seconds at 55°C, sequential extensions for one minute and twenty seconds at 72°C, and the final extension for five minutes at 72°C before cooling to 4°C. The amplicons were subjected to electrophoresis on 1% (w/v) agarose gel stained with SYBR safe (Invitrogen).

The gel bands of the PCR products were purified using Wizard SV gel and the PCR clean-up system (Promega) according to the manufacturer's instructions. Subsequently, the purified sample was sent to the First Base Sdn Bhd Malaysia for sequencing prior to the cloning experiment. The sequences were analyzed using bioinformatics tools (BLASTn and BLASTp) that are available in NCBI (Jin *et al.*, 2013) for identification and similarity respectively. The resulted nucleotide to amino acid translation was predicted using a tool provided by ExPASy as reported earlier by Jin *et al*. (2013) and Xu *et al*. (2012).

Table 1. Primers used for the *RFT1* gene amplification and molecular cloning

Primer name	Primer Sequence 5' - 3'
RFT1-EX1 (F)	TGGCTAGCTTAACCTTCCTG
RFT1-EX1 (R)	GTCTACCATCACCTGTAGGT
RFT1-EX4 (F)	CGGAGGGAGTATCTATTTTG
RFT1-EX4 (R)	CACACTTAAGAGCCTGCATG
RFT1-S1 (F)	GCTCGTGAAGGCAGGAGATA
RFT1-S1 (R)	TTTTTACATGGCGAGGCCGG
EX1-C (F)	TAAGCAGTCGACTGGCTAGCTTAACCTTCCTG
EX1-C (R)	GTCTACCATCACCTGTAGGTCTGCAGTGCTTA

NB: EX1-C is the cloning primer containing *SalI* and *PstI* restriction sites as underlined.

2.4. Construction of the RFT1 Gene and Bacterium Transformation

The cloning vector pGEM-T was constructed using the specific primers (EX1-C in Table 1) with *SalI* and *PstI* restriction sites designed to amplify the full-length Mu-*RFT1* gene. The pGEM-T plasmid (vector) and the purified gene fragment (insert) from the PCR product were double digested separately earlier using the *SalI* and *PstI* restriction enzymes (RE), and were ligated together using T4 ligase enzyme according to the manufacturer's instructions (Promega Catalog ID: 9PIM180). The recombinant vector was designated as *pGMT:MuRFT1* (Figure 1), and was transformed into the *Escherichia coli* DH5α strain by a heat-shock method (Sambrook and Russell, 2001). The recombinant bacteria were screened by a blue/white colony on ampicillin (50 µg mL^{-1}) selective plate supplemented with 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). The white colony cells were selected for colony PCR and enzyme digestion analysis, and were then sequenced by First Base Sdn Bhd Malaysia for bioinformatics analysis of the *RFT1* gene insert.

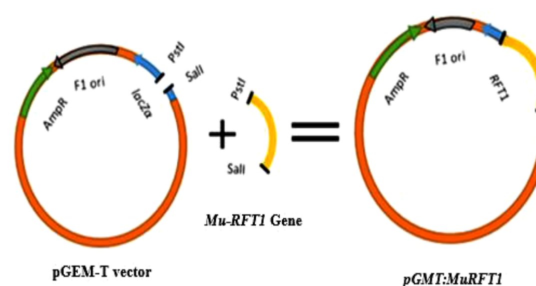

Figure 1. Ligation of pGEM-T vector and the *RFT1* gene at the *SalI* and *PstI* restriction sites designated as *pGMT:MuRFT1*.

2.5. Nucleotide Sequence and Molecular Evolution Analysis

The *RFT1* gene sequence from the Mu-*RFT1* cultivar was analyzed by BLASTn, and BLASTp algorithms (http://www.ncbi.nlm.nih.gov/) for identification and similarity searches. The nucleotides to amino acids translation was predicted using ExPASy (http://www.expasy.org/). Meanwhile, the deduced gene sequence from the Mu-*RFT1* cultivar with ten others from *indica* cultivars, two *japonica* cultivars and one outgroup species were selected for multiple sequence alignment. They included the *RFT1* gene from: Pokkali (BAO03221), Bleiyo (BAJ53916), Kemasin (BAO03216.1), Muha (BAJ53912), Basmati370 (BAH30236), Vandaran (BAO03225.1), Kasalath (BAH30234), Nona Bokra (BAX24675.1), Deng Pao Zhai (BAJ53911) and Tadukan (BAO03183.1) of *indica* sub-species, Nipponbare (BAB78480) and Dianyu 1 (BAO03202) from the *japonica* sub-species and *Oryza glumipatula* (BAH56284.1) as the outgroup gene extracted from the GenBank for the multi-sequence alignment. The sequence alignment was performed with ClustalX (Xu *et al.*, 2012). Subsequently, a phylogenetic tree of the sequences was constructed using Molecular Evolutionary Genetics Analysis (version 7.0, MEGA7) (Kumar *et al.*, 2016). The MEGA7 tree was generated by the maximum-likelihood method (Li *et al.*, 2017).

3. Results

3.1. Isolation and Characterization of the Mu-RFT1 Gene

Optimum concentration at 1,079.2±11.73 and purity 2.04±0.09 of the isolated mRNA were obtained by the spectrophotometric analysis, while the quality was validated on 1 % agarose which showed clear bands at 18S and 28S rRNA. The integrity bands analyses involving concentration, purity and rRNA bands determination indicated that the mRNA is contamination-free, and can be used for further experiment. Subsequently, the mRNA was used for downstream applications to amplify the *RFT1* gene from the Wai cultivar of Malaysia upland rice (Mu-*RFT1*).

The Mu-*RFT1* gene fragment with the size of 645 bp (Figure 2) was amplified using the EX1 and EX1-C primers. It was found that the deduced sequence from the methionine start codon to the stop codon was exactly 178 amino acids which corresponds to the findings of Ebana *et al.* (2011) and Ogiso-Tanaka *et al.* (2013). The authors reported that the *RFT1* gene has 178 amino acids or the equivalent to over 534 nucleotides. The Mu-*RFT1* gene sequence obtained in the present study has an additional nucleotide from the 5' and 3' sites (before the start codon and few after the stop codon) similar to the reports by Chen *et al.* (2004) and Zhu *et al.* (2017). This Mu-*RFT1* gene sequence residues were confirmed to be the *RFT1* gene after the BLAST analyses.

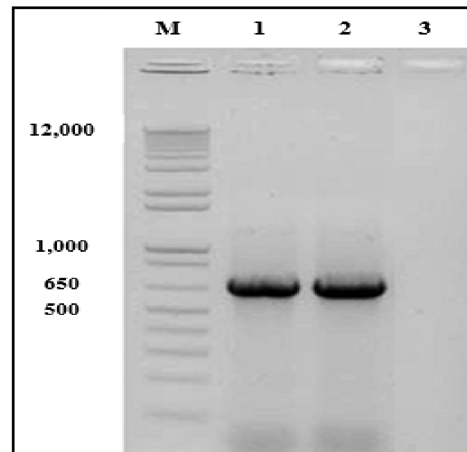

Figure 2. RT-PCR amplification of the full-length Mu-*RFT1* gene from the mature leaves of cultivar Wai. M; 1kb Plus DNA marker, Lane 1-2; Mu-*RFT1* gene, and Lane 3; negative control.

Figure 3 showed the sequencing result of the Mu-*RFT1* gene after the RT-PCR amplification. The outcome revealed a full-length isolation with the nucleotide composition of 99 %, and the amino acid residues showed an 80 % similarity to the *Oryza sativa* cultivars including Nona Bokra, Vandaran, and Kemasin. Further sequence analysis of the Mu-*RFT1* gene sequence obtained from the present study indicated some nucleotide diversity in comparison with the *RFT1* gene from the different candidate cultivars. This corresponds to the findings of Hagiwara *et al.* (2009) and Ogiso-Tanaka *et al.* (2013), which revealed changes in sixteen amino acids in the *RFT1* gene of *indica* cultivars. Previous gene analyses and transformations were conducted on *japonica* (Komiya *et al.*, 2009; Zhao *et al.*, 2015) or wetland *indica* (Ogiso-Tanaka *et al.*, 2013). Accordingly, the present study is the first report on the full-length *RFT1* gene isolation and characterization from the upland rice of *indica* sub-species.

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atggcgggcagcggccgcgatgatccgctggtggtgggccgcattgtgggcgatgtgctg
 M  A  G  S  G  R  D  D  P  L  V  V  G  R  I  V  G  D  V  L
gatccgtttgtgcgcattaccaacctgagcgcgagctatggcgcgcgcattgtgagcaac
 D  P  F  V  R  I  T  N  L  S  A  S  Y  G  A  R  I  V  S  N
ggctgcgaactgaaaccgagcatggtgacccagcagccgcgcgtggtggtgggcggcaac
 G  C  E  L  K  P  S  M  V  T  Q  Q  P  R  V  V  V  G  G  N
gatatgcgcaccttttataccctggtgcgcattattagctggatgcagcgcccgaccaac
 D  M  R  T  F  Y  T  L  V  R  I  I  S  W  M  Q  R  P  T  N
ccgaacctgcgcgaatatctgcattggctggtgaccgatattccgggcaccaccggcgcg
 P  N  L  R  E  Y  L  H  W  L  V  T  D  I  P  G  T  T  G  A
acctttggccagaaagtgatgtgctatcgcagctatctgattacctttattattcatcgc
 T  F  G  Q  K  V  M  C  Y  R  S  Y  L  I  T  F  I  I  H  R
ctgaactatcataaaacccgcctgattatttttttacctggatgcatgtgaactttgtg
 L  N  Y  H  K  T  R  L  I  I  F  F  T  W  M  H  V  N  F  V
tattttcatctgaccaaagattttgcggaactgtataacctgggcagcccggtggcggcg
 Y  F  H  L  T  K  D  F  A  E  L  Y  N  L  G  S  P  V  A  A
gtgtattttaactgccagcgcgaagcgggcagcggcggccgccgcgtgtatccgtag
 V  Y  F  N  C  Q  R  E  A  G  S  G  G  R  R  V  Y  P  *
```

Figure 3. Nucleotide and the deduced amino acid sequence of the Mu-*RFT1* gene. Start codon is shown in bold and stop codon is indicated by an asterisk and bold font. Both nucleotide and amino acid equivalent was predicted using the ExPASy tool.

3.2. RFT1 Gene Construction into the Cloning Vector

The construct *pGMT:MuRFT1* was successfully transformed into the *E. coli* DH5α. The positive transformants screening involves growing the cells on ampicillin (50 µg mL^{-1}) plates which were differentiated by white and blue colonies. From the plate, multiple white and light blue cell colonies were obtained. Thus, only the

dense white colonies were selected and used for subsequent experiments in this study. Furthermore, the success of gene cloning was examined by colony PCR analysis of the constructed vector (*pGMT:MuRFT1*) as shown in Figure 4. The results revealed full-length Mu-*RFT1* gene isolation from the recombinant vector which demonstrates the efficiency of the amplification process.

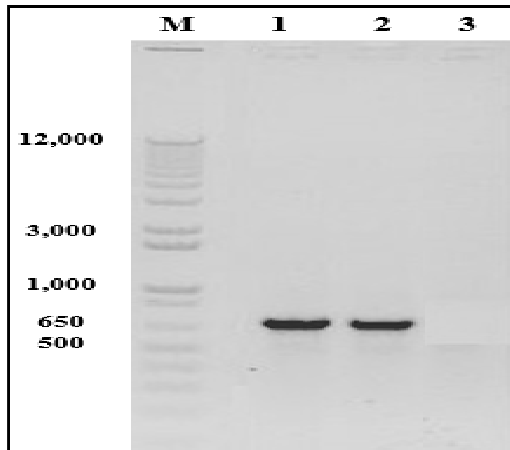

Figure 4. Colony PCR of the recombinant cells using EX1-C primers: M; 1kb Plus DNA marker, Lane 1, 2; Mu-*RFT1* gene from the *pGMT:MuRFT1* construct and Lane 3; negative control.

Double digestion of the *pGMT:MuRFT1* construct isolated from the recombinant cells using the *SalI* and *PstI* enzymes revealed two separate bands on the gel which depicts the vector and insert as shown in Figure 5. Therefore, the digestion analysis further confirmed the presence of the insert gene in the constructed vector. The *RFT1* gene band from the construct was excised, purified, and sequenced. The nucleotide and amino acid sequences were analyzed for further confirmation. The results revealed a 99 % nucleotide similarity to Keiboba, Kemasin, Nona Bokra, Tadukan and Vandaran (with 99 % query cover and an E-value of 0.0).

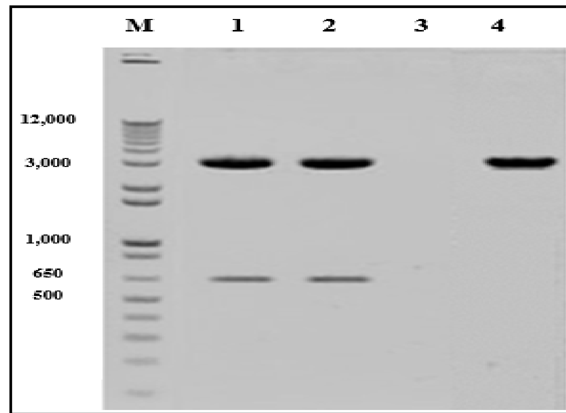

Figure 5. RE digestion of the *pGMT:MuRFT1* recombinant vector using *SalI* and *PstI* enzymes. M; 1kb plus marker, Lane 1-2; vector (at up 300 bp) and insert (at down 645 bp) separation, Lane 3; negative control and Lane 4; control vector.

3.3. Molecular Evolutionary Relationship Analysis of the RFT1 Gene

The multiple sequence alignment of the *RFT1* gene was examined through ClustalX tool. Figure 6 shows the *RFT1* amino acid sequence with high conservation at more than 90 % of the coding sequence. On the amino acids 31, 146 and 160, Basmati370 (*indica*), Nipponbare (*japonica*) and Dianyu1 (*japonica*) showed a unique amino acid (1) similarity which indicated their close evolutionary relationship, as denoted in black. A phylogenetic tree was constructed through the maximum likelihood method by the MEGA7 software. This was to identify and investigate the evolutionary relationships between the Mu-*RFT1* gene sequences from diverse *Oryza sativa* cultivars and a single *Oryza glumipatula* as the outgroup. The relationship of all the rice cultivars is represented in the phylogram in Figure 7.

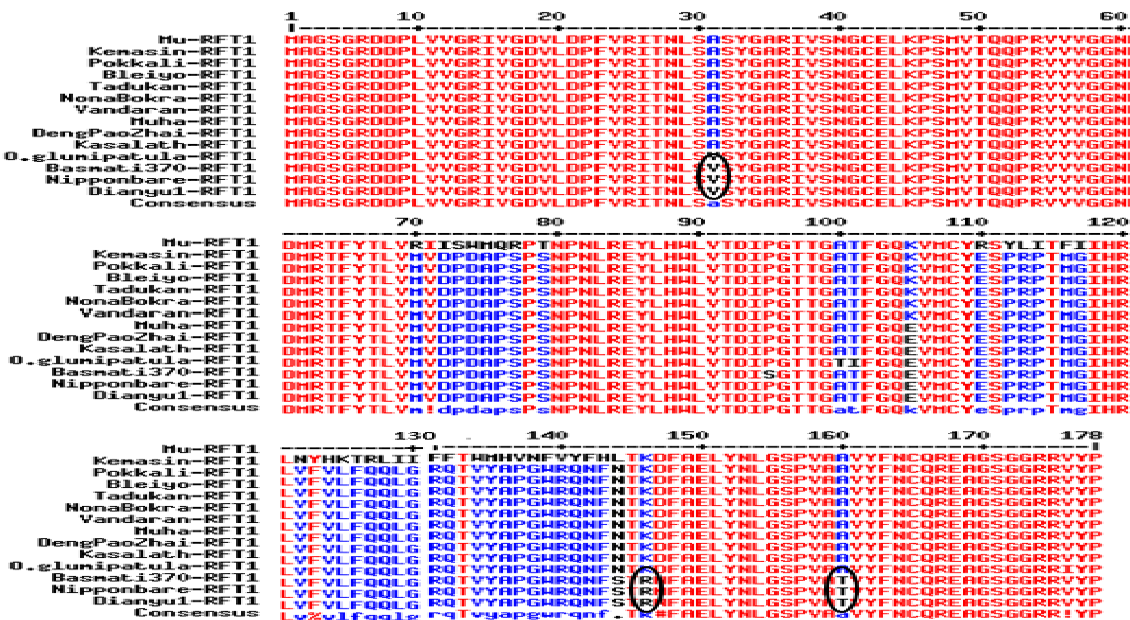

Figure 6. Multisequence alignment of the deduced Mu-*RFT1* gene amino acids obtained from the present study with that of the other rice cultivars using MEGA7. The other cultivars include Kemasin (BAO03216.1), Pokkali (BAO03221), Bleiyo (BAJ53916), Tadukan (BAO03183.1), Nona Bokra (BAX24675.1), Vandaran (BAO03225.1), Muha (BAJ53912), Deng Pao Zhai (BAJ53911), Kasalath (BAH30234), Basmati370 (BAH30236), Nipponbare (BAB78480), Dianyu 1 (BAO03202) and *Oryza glumipatula* (BAH56284.1).

In the phylogenetic tree, the cultivars were classified into two main groups. The first group comprises all the *Oryza sativa* sub-species and their cultivars, while the second group only contained *Oryza glumipatula*. The *Oryza sativa* collection was further categorized into two more sub-groups and clustered. The phylogenetic tree revealed that the Mu-*RFT1* gene was clustered with Kemasin, and are closely related to the *indica* cultivars including Pokkali, Bleiyo, Muha, Deng Pao Zhai, Kasalath, Nona Bokra and Vandaran (Figure 7). Of all the *Oryza sativa* cultivars, Tadukan exists independently in one sub-clade. The *japonica* cultivars; Nipponbare and Dianyu1 existed in the same cluster but formed a sub-clade with Basmati370 (Figure 7).

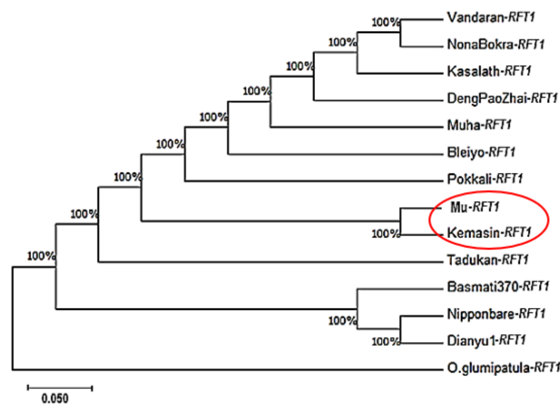

Figure 7. A phylogenetic tree derived from multiple sequence alignment using MEGA7; illustrating the relationships between the Mu-*RFT1* and corresponding genes from other cultivars including Vandaran (BAO03225.1), Nona Bokra (BAX24675.1), Kasalath (BAH30234), Deng Pao Zhai (BAJ53911), Muha (BAJ 53912), Bleiyo (BAJ53916), Pokkali (BAO03221), Kemasin (BAO03216.1), Tadukan (BAO03183.1), Basmati370 (BAH 30236), Nipponbare (BAB78480), Dianyu 1 (BAO03202) and *Oryza glumipatula* (BAH56284.1).

4. Discussion

4.1. RFT1 Florigen and its Cloning Construction

The most important prerequisite in the nucleic acid downstream application is the isolation of high-quality RNA (Zhang *et al.*, 2012). This step serves as a key evidential factor for further analyses through cDNA synthesis, reverse transcription PCR, microarray or cDNA library construction. As reported by Portillo *et al.* (2006), Trizol is the best reagent for RNA isolation from plant tissues compared to other methods as also confirmed by this study. Therefore, this is evidence for producing pure and high concentrated RNA from the matured leaves of the Malaysian upland rice, cultivar Wai of *indica* sub-species and subsequence *RFT1* gene downstream amplification. Research on the *RFT1* gene has recently attracted attention as it plays a crucial reproductive role by promoting flowering in rice at LD conditions, although the rice is an SD plant (Komiya *et al.,* 2008; Komiya *et al.*, 2009).

The *RFT1* gene is the second rice florigen and has been hypothesized as a hormone-like molecule for promoting flowering processes under LD conditions. This gene which is located on rice chromosome 6, is an ortholog to *FT* in *Arabidopsis thaliana* (Komiya *et al*., 2009). The gene is normally produced in the leaves, but interacts with the transcriptional factor *flowering locus D* (*FD*). Furthermore, it acts as a mobile signal in SAM buds and growing tips to control floral transition mainly by dual regulation of the *Early heading date 1* (*Ehd1*) (Komiya *et al.*, 2009; Sun *et al.*, 2016; Zhao *et al.*, 2015). Under LD conditions, rice flowering is regulated by the *Ehd1* promoter which is the B-type response regulator of *FT*-like genes that also up-regulates the positive regulator *RFT1*. This can also act as a constitutive activator and flowering inducer. The *Ehd1* and *Hd1* (*heading date 1* that promotes and activates heading) concurrently promote floral transition preferentially under SD and are antagonistic at LD conditions. This revealed the double function of *Ehd1* under both conditions (Chen *et al.*, 2018).

Evidence indicates that overexpression of the *RFT1* gene with the vascular-specific or constitutive promoter results in an early-flowering phenotype under LD conditions. However, its suppression by RNA-interference (RNAi) delays occurrence of the flowering (Komiya *et al.*, 2009). Moreover, the nucleotide diversity of the *indica* rice *RFT1* genes are closely related to the cultivar's genotype and regional distribution (Zhao *et al.*, 2015). This suggest that the functional constraint was relaxed in the *RFT1* gene after its duplication, which also demonstrates the haplotype diversity of the *RFT1* gene in the cultivated rice. The *RFT1* haplotype number is larger in the entire gene region, but smaller in the coding region (Ogiso-Tanaka *et al.*, 2013). The gene plays a crucial role in the reproductive cycle of rice cultivars in the LD environment. Hence, full-length isolation of Mu-*RFT1* gene and cloning are crucial for future genetic transformation.

The *pGMT:MuRFT1* was successfully constructed and introduced into the *E. coli* DH5α bacterium cell. The hand-pick screening of the transformed cells indicated the victory of the cloning and transformation analysis. Furthermore, the gene construction, recombinant DNA transformation and screening of transformants are important in molecular biology (Padmanabhan *et al.*, 2011). The presence of ampicillin plus X-gal and IPTG in the growth selective medium really simplifies the process of identifying the positive cloned from the negative transformants. In addition, the gene amplification via colony PCR and RE digestion were performed. The two separate analysis and victory obtained further demonstrated the success of the construction analysis. The colony PCR simply determined the presence of insert in the recombinant cells as well as sensitivity of the primers used for amplification, whereas the RE digestion excised the insert from the recombinant vector. The colony PCR screening techniques are cost-effective, fast, simple, and require no additional steps compared to sonication, mechanical high-speed cell disruption or the use of toxic chemicals (Mirhendi *et al.*, 2007). Additionally, restriction enzyme digestion is considered as the most reliable and accurate technique for verifying the plasmid constructs in the gene isolation and cloning experiments (Glover, 2013).

4.2. The Phylogeny Relationship of the RFT1 Genes

Construction of the phylogram tree is important in evolutionary studies, vital for inferring and clarifying the biological evolution and relationships between species or cultivars of the same origin. The relationship between thirteen amino acid sequences of the *RFT1* genes in the datasets obtained from Wai cultivar in this study was

analyzed with all gaps and missing data elimination. The phylogram shows the consensus phylogenetic relation amongst the thirteen *Oryza sativa* cultivars belonging to two sub-species and the *Oryza glumipatula* as an outgroup. This is based on a consistency index (CI) of 1 and retention index (RI) of 1, which indicated a steadily complete phylogenic character without homoplasy (Nei and Kumar, 2000; Arif *et al.*, 2016). The analysis confirmed the Mu-*RFT1* cultivar originates from the *indica* sub-species. However, the Basmati370 *RFT1* gene indicated some differences in nucleotides and amino acids compared to the other *indica* cultivars. This finding corresponds with the previous findings of Kovach *et al.* (2009) which revealed that Basmati370 shares a close evolutionary relationship with the *japonica* varieties based on its fragrant characteristic.

The discoveries presented in this study provide novel insights into the relationship between the aligned cultivars. Furthermore, the phylogeny analysis illustrates the evolutionary relationship between the rice cultivars and the outgroup species. Moreover, it inferred classification of the diverse *indica* cultivars in the study. This finding is supported by the distinct phenotypic characteristics of rice cultivars. For example, in terms of appearance and size, *indica* varieties possess slender and long grains, while *japonica* has stumpy and short grains. Historically, *indica* cultivars originated from India, Thailand and Indonesia, whereas *japonica* cultivars are from China, Japan, Laos, Taiwan and Vietnam. Therefore, there is a high degree of similarity in the gene sequences of the verified cultivars as was discovered in the present study. This corresponds with the suggestion by Hagiwara *et al.* (2009) who revealed that the *RFT1* gene had a high variability in Asia based on its phylogenetic relations.

5. Conclusion

Isolation of the qualified RNA and full-length Mu-*RFT1* gene were performed in the current study. The gene was subsequently constructed into the cloning vector pGEM-T and transformed into the *E. coli* DH5α strain, followed by characterization and molecular evolutionary relationship analysis of the gene sequences. The phylogenetic tree and amino acid sequence analyses demonstrated that the Mu-*RFT1* gene was closely related to Kemasin as the *indica* rice cultivar which grows in LD environments. Similarly, the multiple sequence alignment of the *RFT1* proteins from the *indica* and *japonica* cultivars and the out-group species showed that the florigen is well-conserved in the genus *Oryza*. This is a milestone in the molecular analysis, gene construction, and phylogeny characterization of the *indica* sub-species of upland rice. Consequently, a better understanding of the *RFT1* gene has been scrutinized to provide a better understanding of flower development at the molecular level. Furthermore, the data generated from this study provides an important understanding of the rice flowering system under LD conditions. The gene examined in this study is a potential target for genetic transformation and is required for producing a transgenic rice variety with improved reproductive phases, particularly, the flowering development. However, it is noteworthy to state that this part of the current research only presented data on the gene isolation, sequencing, phylogenic relation, and cloning vector construction of the *RFT1* gene in an upland rice, Malaysian cultivar. Therefore, this will be followed by a study on the expression construct development, plant genetic transformation of the gene to test its potentiality toward enhancing early flowering in rice. The novel discovery in this study is an indication of the prospects of expression construct, genetic transformation, and efforts to improve the flowering process of the upland rice cultivars.

Disclosure Statement

No competing financial interests or any conflict exist between the authors.

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