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Research Article

Isolation and molecular phylogeny of *RFT1* gene from upland rice leaves cultivar

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Abstract

Rice is a modern short-day plant but equally flower during non-inductive long-day condition due to the possession of second florigen, *Rice Flowering Locus T1* (*RFT1*). *RFT1* is an ortholog of *Arabidopsis FT* gene which triggers flowering induction under long-day (LD) condition. The gene isolation from Malaysian upland rice *cv.* Wai that induces flowering were reported. Nucleic acids isolation and amplification of the *RFT1* were observed, then phylogenetic relationship among other 5 *indica* and 1 *japonica* rice were inferred by comparing the gene amino acids sequence data sets using multiple sequence alignment databases. The amplicon quality result indicated that the gene was fully amplified at above 0.5kb, whereas the sequence alignment revealed that the *RFT1* gene was partially amplified based on the gaps identified. Among the 6 *indica* cultivars sequence aligned, they were almost all conserved except on only 12 amino acids. While the cladogram tree result classified the cultivars into three major groups and include Wai under clade that contains both *indica* and *japonica* cultivars. This finding concluded that the cultivars are of high evolutionary relationship and well established their molecular relationship. The cultivars relationship provides necessary information for better understanding of molecular evolution and designing scientific breeding system for generation of new rice cultivar.

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1. Introduction

Rice or its scientific name *Oryza sativa* L.; the second major cereal crop cultivated across the globe, is a suitable source of food and model plant for studies of genome organization, gene expression as well as transgenes behaviuor (Bajaj and Mohanty, 2005; Manimaran et al., 2013). It is a facultative short-day (SD) plant as it shows early flowering at SD environment, but

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equally flower during non-inductive long-day (LD) condition due to the possession of second florigen, *Rice Flowering Locus T1* (*RFT1*), as described by Komiya et al. (2008). Previous analyses point-out the correlation between early flowering under LD condition and high *RFT1* mRNA level, which suggest that *RFT1* is the main activator to induce flowering under LD condition in *japonica* rice sub-species (Mohammed et al., 2019).

RFT1 is a homolog of rice *Hd3a* and ortholog of *Arabidopsis FT* gene which triggers floral induction processes under LD environment. *RFT1* regulates flowering period through a complex genetic network by translocation from leaf to shoot apical meristem (SAM) (Komiya et al., 2008; Osugi et al., 2011; Tsuji et al., 2011; Itoh and Izawa, 2013). The rice flower normally evolved from vegetative structures i.e. SAM and then later transit to reproductive stage that produces flower and fruits/seeds (Komiya et al., 2008). Flowering time in *Oryza sativa* is intensively regulated by both florigen and range of environmental signals (photoperiod) which provides suitable sign for heading season. Such signals must integrate into single decision-to flower (Komiya et al., 2008; Albani and Coupland, 2010; Xiang et al., 2013).

However, to date, there is no report on the *RFT1* gene isolation, molecular activity and transformation from Malaysian upland rice, sub-species *indica*. Understanding of the gene function in such rice cultivars grown under LD condition countries is still unclear. Though Malaysia has many upland rice cultivars as reported by Sohrabi et al. (2012), Sohrabi et al. (2013); generally upland rice cultivars comprises almost 80% of the world cultivated rice, but contribute only 12% of the global rice production. Because of such shortages in production; molecular isolation, characterization and transformation of *RFT1* gene responsible for promoting flowering under LD condition is of significant interest. It will necessitate the development of transgenic rice with early flower production. Here we reported on *RFT1* gene isolation from Malaysian upland rice cultivar Wai which could later be used for genetic transformation to induce early flowering.

2. Materials and Methods

2.1. Genomic DNA Extraction and PCR Amplification of RFT1 Gene from Malaysian Upland Rice Cultivar Wai

Genomic DNA (gDNA) was extracted as described by Edwards et al. (1991) with little modification from leaves of upland Malaysian rice (*indica* subspecies) cultivar Wai between 8 – 11 weeks old of growing period. The gDNA quantity was analysed using NanoDrop1000 spectrophotometer and later stored at -20^{o}C prior to further analysis. *RFT1* gene was amplified from the gDNA using different gene-specific primers: *EX1*-F (5'TGGCTAGCTTAACCTTCCTG3'), *EX1*-R (5'GTCTACCATCACCTGTAGGT3'), *EX4*-F (5'CGGAGGGAGTATCTATTTTG3'), *EX4*-R (5'CACACTTAAGAGCCTGCATG3'), *RS1*-F (5'GCTCGTGAAGGCAGGAGATA3') and *RS1*-R (5'TTTTTACATGGCGAGGCCG G3').

The thermo-cycling conditions were; initial denaturation for 4 min at 94^{o}C, followed by 30 cycles of denaturation for 30 sec at 94^{o}C, annealing for 40 sec at 53^{o}C and extension for 1 min 20 sec at 72^{o}C, then final extension for 5 min at 72^{o}C and cooling at 4^{o}C. The quality of the amplicon was analyzed by electrophoresis (74 V, 425 A and 45 min) on 1 % (w/v) agarose gel stained with SYBR safe. The gel bands were excised, purified using Wizard SV gel and PCR clean-up system as according to manufacturer's instruction (Promega) and sent for sequencing to Medigene Sdn Bhd and 1st Base Sdn Bhd Company.

2.2. RNA Extraction, cDNA Synthesis and PCR Amplification of RFT1 Gene

Total RNA was also isolated from the leaves at same age for gDNA extraction using Trizol reagent (Sigma-Aldrich) according to the Manufacturer's instruction. The isolated RNA was treated with DNase prior to reverse transcriptase PCR (RT-PCR) for cDNA synthesis. cDNA was synthesized from the DNase-treated RNA using Superscript II Reverse transcriptase kit (Invitrogen) as described by the manufacturer. PCR amplification of *RFT1* was performed using cDNA as template and *EX1* primers at the same thermo-cycling condition as above. The amplicon was run on 1 % (w/v) agarose. The gel bands were excised, purified and sent for sequencing as described above.

2.3. Bioinformatics Analysis of RFT1 Gene Sequences

RFT1 amino acids sequences were analyzed using protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. Meanwhile, the *RFT1* sequences from five different *indica* rice cultivars which include Basmati 370 (BAH30236), Pokkali (BAO03221), Bleiyo (BAJ53916), Muha (BAJ53912) and Kemasin (AB838579.1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) for multiple sequence alignment (MSA) with the current sequence. All the sequences (from Wai and 5 other cultivars) were aligned in FASTA format and analyzed using clustalX and T-Coffee softwares. Phylogenic tree analysis of the six cultivars sequence plus Nipponbare [(BAB78480) a *japonica* sub-species] were also observed.

3. Results and Discussion

3.1. Molecular Amplification and Sequence Analysis of RFT1 Gene from Malaysian Upland Rice

The study tried to isolate and amplify the gene from Malaysian upland rice cultivar Wai using different primer sets. From all the primers used in this study, *EX1* primer set gave the best amplification result. The agarose gel electrophoresis outcome indicated that the actual expected size of the gene was found using the primer set as shown in Figure 1. The band size on the gel was above 0.5 kb which correspond with the finding of Ebana et al. (2011) which shows that the gene has 178 amino acids, approximately 534 bp nucleotides. *RFT1* gene is the second rice florigen and has been hypothesized as a hormone-like molecule for promoting flowering processes under LD condition (Komiya et al., 2008; Tsuji et al., 2011; Itoh and Izawa, 2013). The gene is normally produced in the leaves and act as mobile signal in the SAM of buds as well as growing tips. Evidences indicated that over-expression 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 et al. (2008) and Komiya et al. (2009). This implies that flowering under LD condition in rice is basically a result of expression of this highly conserved florigen, *RFT1* (Tamaki et al., 2007; Tsuji et al., 2008).

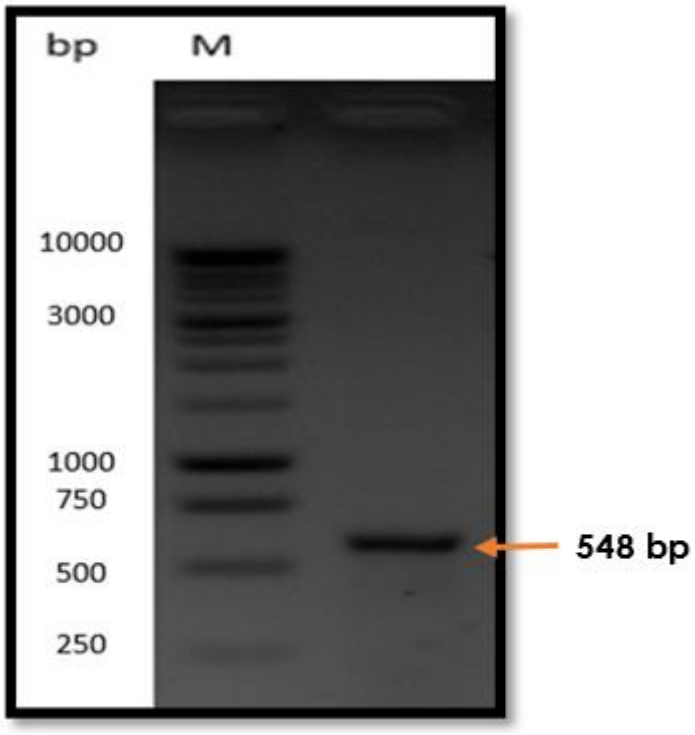

Figure 1. *EX1* primer; separation of PCR product on agarose gel following amplification

The result of the sequencing analysis indicated that all the six sequences are highly conserved from the region beginning at the start of the *RFT1* until amino acid in position 69 with 99 % degree of similarity (Figure 2). The highly conserved region among the different cultivars may be due to their close evolutionary trend (Kojima et al., 2002, Komiya et al., 2008). However, the sequence alignment revealed that *RFT1* gene from the present research was partially amplified due to presence of few gaps after the identified conserved regions. Moreover, the target sequence was 182 amino acid sequence longer than the database template sequences. Uniquely, the target sequence had some amino acids at the beginning of the sequence, which is not in the other *RFT1* sequences.

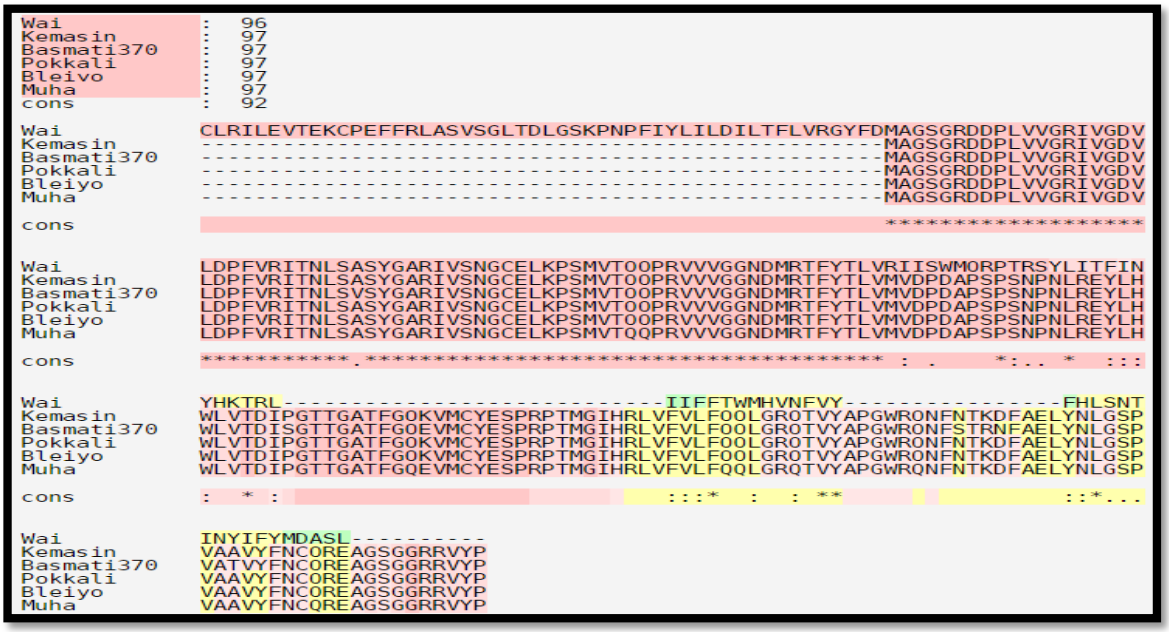

Figure 2. Multiple sequence alignment of six indica rice cultivars. Pink colour indicates highly conserved region (*); Yellow colour indicates average conserved region (:); Green colour indicates less conserved region

Additionally, the sequence alignment between Wai cultivar and other five cultivars from NCBI revealed that the amino acids diversity towards the end of the sequences is quite enormous. This corresponds with the finding of Hagiwara et al. (2009) and Ogiso-Tanaka et al. (2013) which indicated that defective *RFT1* gene is found in *indica* cultivars and higher diversity of nucleotides. They reported that about 16 amino acids changes in *RFT1* was found and 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.

There were almost 12 amino acid differences observed at closely loci as specified by regions with blank or single dot below (cons) (Figure 2). These amino acid differences seem to be lesser than the one reported by Ogiso-Tanaka et al. (2013) which communicated that about 16 amino acid changes in *RFT1*, but still they shared higher uniformity. Equally, all the *indica* cultivars with the exception of Basmati 370 were virtually 100 % conserved, while amino acid substitution observed in Basmati 370 may contribute to the diversity of the cultivar.

3.3. Molecular Phylogeny of RFT1 Gene from Malaysian Upland Rice

In evolutionary study, cladogram tree construction is of paramount importance as it functions in inferring and clarifying the evolutionary relationship among species being studied (Mohammed et al., 2019). As for this study, the relationship of amino acid sequences from different rice cultivars (five *indica* and one *japonica*) obtained from the NCBI database and with the one obtained from this study were analyzed with all positions. The phylogenetic relationship of all rice cultivars was presented in a cladogram (Figure 3). The cladogram shows the consensus phylogenetic tree of the 6 *indica* varieties and single *japonica* variety with a consistency index (CI) of 1 and retention index (RI) of 1 which indicates that there is no homoplasy and the character is totally steady with phylogeny (Drummond and Strimmer, 2001; Norulaini et al., 2001). As shown in the consensus tree, the cultivars were classified into three major groups and group one was categorized into sub-clades. The first group comprises of Wai, Basmati370, Nipponbare, Muha and Kemasin. The second group consists of only Pokkali, while third group has only Bleiyo.

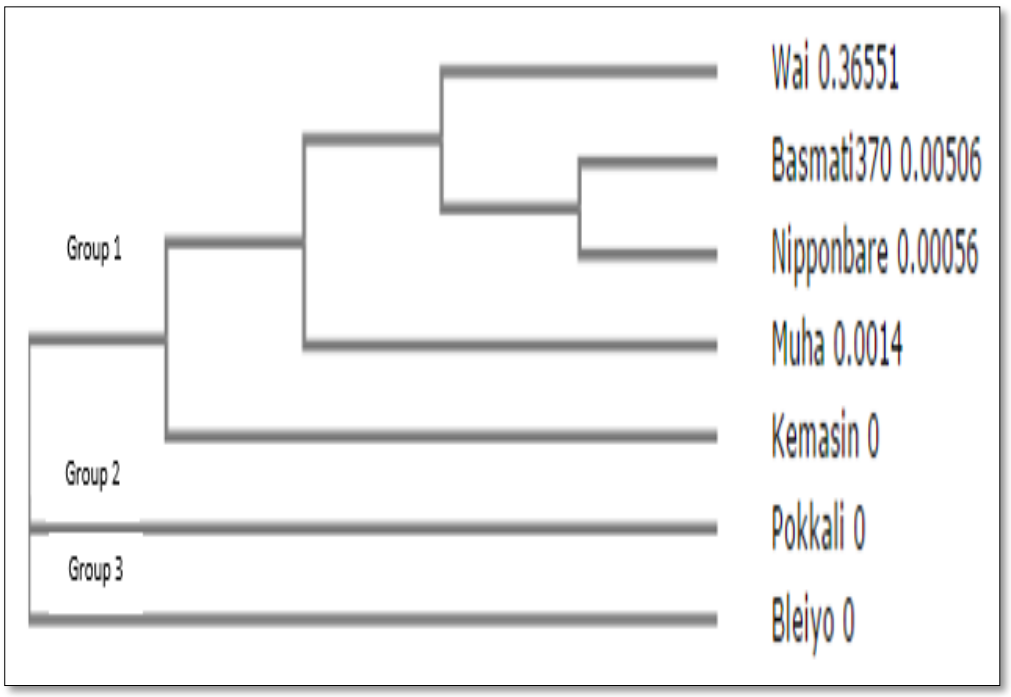

Figure 3. The consensus tree derived from the multiple sequence analysis of *RFT1* gene of *indica* rice varieties

From the cladogram tree, Basmati 370 had some nucleotides as well as amino acids differences from the other *indica* cultivars but showed close relation with Nipponbare

(*japonica*). These correspond to the previous finding of Kovach et al. (2009), which revealed that Basmati 370 has close evolutionary relationship with *japonica* varieties that is based on its fragrance characteristic. Similarly, this analysis inferred classification of diverse *indica* into three major groups and suggested that single *indica* cultivar from the five obtained from database and Wai had some amino acids difference and relationship with *japonica*, even though they originate from different areas. This actually gave better insight with regards to the relationship among the aligned cultivars. However, upon construction of cladogram, Wai cultivar indicated similarity to the *japonica* close related cultivar and *japonica* cultivar. Though it forms the same sub-clade with Muha, a typical *indica* rice.

As well, these findings were supported by distinct phenotypic characteristic of the rice cultivars. For example; *indica* varieties in terms of appearance and size they possess slender and long grains, while *japonica* possess stumpy and short grains (Khush, 2000). Additionally, most *indica* cultivars originated from many countries like India, Thailand and Indonesia, whereas *japonica* cultivars originated from countries like China, Japan, Laos, Taiwan and Vietnam (Sasaki et al., 2010). Therefore, there is a degree of similarity and diversity of *RFT1* gene sequence among the verified cultivars from these two rice subspecies and thus, suggest that *RFT1* have high variability in Asia, which was recommended based on the *RFT1* gene phylogenetic analysis as described by Hagiwara et al. (2009).

4. Conclusion

The sequence alignment outcome showed that *RFT1* was partially isolated, even though EX1 primer amplified the gene-based on its expected size. The analysis indicated that some amino acids are missing at the middle or not amplified by the primers, but shown some amino acids at the beginning. Similarly, the phylogenetic analysis inferred classification of diverse *indica* into three major groups and, then, suggested that Wai and Basmati had some amino acids differences and high similarity even though they originate from different areas. This actually gave better insight with regards to the relationship among the aligned cultivars. Hence, this implies that *RFT1* gene could function as a potential biomarker for molecular characterization of rice cultivars. Also, provides important information for better understanding of molecular evolution as well as developing good breeding programs that would lead to development of new cultivar with early flower production and good grain quality. Furthermore, better understanding of *RFT1*

gene function and regulation will make rice a powerful model plant for understanding the flower development at molecular level.

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