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Cloning and Bioinformatics Analysis of *Rosa rugosa* TFL1 Gene (*RrTFL*1)

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Abstract

In order to determine if the *TFL*1 is related with the continuous flowering phenotype of wild *Rosa rugosa* from Muping, the full-length cDNA sequence of *TFL*1 *Gene* was cloned for the first time from the flower buds of wild *Rosa rugosa* from Muping with RT-PCR and RACE methods and named as *RrTFL*1. The full-length cDNA is 973 bp with an open reading frame of 519 bp, encoding 172 amino acids. The derived protein has a molecular weight of 19.48 kD, a calculated pI of 9.13, a c100227 conserved domain at position 1-172, and belongs to PEBP family. The derived protein is a Hydrophilic protein secreted into the cytoplasmic. There is no transmembrane domain and no signal peptide cleavage site, five Ser phosphorylation sites, seven Thr phosphorylation sites, three Tyr phosphorylation sites, one O-glycosylation site, and no N-glycosylation sites. There are 24.42% α-helixes, 36.63% random coil, 27.91% extended peptide chain, and 11.05% β-corner structure. This protein and the *TFL*1 protein from Rosaceae plants, including *Rosa chinensis*, share a sequence homology of 87% - 96%. All of the proteins contain a c100227 conserved domain, two highly conserved modules D-P-D-x-P, G-x-H-R, and two functional sites His, Asp. Furthermore, their phylogenetic relationships are consistent with their traditional classifications. These results not only laid a foundation for further researching the expression and function of *RrTFL*1, but also cultivating new varieties of *R. rugosa* which can flower continuously by gene engineering.

Keywords

Rosa rugosa, *TFL*1 Gene, Clone, Biological Analysis

*These authors contribute equally.

1. Introduction

Obtaining plants that flower over a long period is the goal of many gardeners, so as to be able to achieve year-round flower production. The flowering period can be lengthened by exploiting the blossoming duration or number of flowering cycles. Most plants have a single annual flowering period. Some perennial plants have the ability to flower again during the year: they can flower continuously during the favourable season or they may only have a second bloom later in the season, which may be occasional. Some Rosaceae plants, such as *Rosa hybrid*, have the ability of flowering more than once or recurrent flowering in a year, which is an important horticultural and economical characteristics, but their molecular background is not clear [1] [2] [3] [4].

There are three gene classes such as inflorescence meristem genes, floral meristem genes and flower organ identity genes involved or interacted in flower development. The inflorescence meristem genes control the transition of shoot apical meristem from vegetative to reproductive (inflorescence), forcing or delaying the flowering time. The TERMINAL FLOWER 1 (*TFL*1) is an inflorescence meristem identity gene, and encodes phosphatidylethanolamine binding protein (PEBP) belongs to the TFL/CENTRORADIALIS (CEN) family. *TFL*1 was first identified from *Arabidopsis thaliana* mutants, followed by cloning of its homologous genes in *Antirrhinum majus*, *Nicotianin tobacco*, *Rose chinensis*, *Malus domestica*, *Fragaria* × *ananassa*, *Pyrus pyrifolia*, *Pyrus communis*, *Prunus armeniaca* and other plants [5] [6] [7] [8] [9] ruminate rather than indeterminate, and early flowering occurs due to the shortened vegetative growth. In recent years, several researchers believed that the *TFL*1 may be a regulator of continuous flowering in roses. Lina Wang *et al*. (2012) examined the relationship between the recurrent flowering character and the expression patterns of *TFL*1 homologs in *Rosa multiflora*, *R. rugosa*, *R. chinensis*, six other rose species and nine rose cultivars [10]. *TFL*1 was expressed at high levels at all four flowering stages in non-recurrent flowering species, such as *R. multiflora* and *R. rugosa*, whereas it was barely detected at any stage in recurrent flowering species, such as *R. chinensis*. These results suggest that the recurrent flowering habit in roses results from lower expression of *TFL*1, which may be related to recurrent flowering character in roses. Hikaru Iwata *et al*. (2012) found a similar phenomenon in roses and strawberries [11]. Therefore, *TFL*1 probably demonstrates a new role of modifying flowering seasonality in perennial plants. But, it still needs more experimental evidence.

The *Rosa rugosa* is a famous traditional Chinese flower. It is fragrant as well as resistant to cold, drought, pest, disease, salt, and alkali [12] [13] [14]. It is not only a kind of fine plant material in landscaping, but also one of the oldest natural spice plants, which is the raw material of perfume, cosmetics and other chemical products, food and precious Chinese herbal medicines. Therefore, the *R. rugosa* is a collection of ornamental, medicinal, edible, beauty in a beautiful flowers and trees. But the vast majority of *R. rugosa* varieties only once a year to spend flowers, flowering are in April-May, and the flowering period is only

about 2 weeks, which severely limits the application of *R. rugosa*. After years of observation, it was found that the wild *Rosa rugosa* which was introduced from Muping by our laboratory had a unique recurrent flowering trait, but their molecular background is not clear.

In order to determine if the *TFL*1 is related with the continuous flowering phenotype of wild *Rosa rugosa* from Muping, we isolated the *TFL*1 homolog from wild *Rosa rugosa* from Muping, and comparatively analyzed its bioinformatics characteristics.

2. Material and Methods

2.1. Plant Material

The plant material, Chinese wild *Rosa rugosa* from Muping, was from the *R. rugosa* germplasm resources garden at Shandong Agricultural College. The wild *Rosa rugosa* from Muping is the most representative traditional *Rosa rugosa* in China.

2.2. Methods

2.2.1. Flower Buds Collection

Between March 2016 and May 2016, the flower buds in different developmental stages (Vegetative growth period; Growth cone transformation period; Receptacle development period; Pistil and stamen morphogenesis period) of wild *Rosa rugosa* from Muping were collected, flash frozen with liquid nitrogen and then stored in a −80˚C freezer.

2.2.2. Total RNA Extraction and cDNA Synthesis

An EASYspin plant RNA Rapid Extraction Kit from Adlai Biotechnology Co., Ltd. was used to extract and mix the wild *R. rugosa* from Muping at different stages of flower bud RNA. Agarose gel electrophoresis and spectrophotometer were used to determine the quality and concentration of the mixed total RNA. An EasyScript First-Strand cDNA Synthesis SuperMix Kit from Beijing TransGen Biotech Co., Ltd. was used to synthesize the first-strand cDNA.

2.2.3. Cloning of the Middle Fragment

According to the reported *TFL*1 sequences of Rosaceae, the degenerate primers F1 (5'-CCTCTTGTKGTTGGRAGAGT-3') and R1 (5'-AGRTADGGRTCACTRGGDCCBGG-3') were designed with Primer Premier 5.0 PCR amplification was conducted using the synthesized cDNA in Section 2.2.2 as a template and F1 and R1 as the primers. The reaction system included 1 μL cDNA, 1 μL F1 primer (10 μmol/L), 1 μL R1 primer (10 μmol/L), and 12.5 μL PCR MIX, with ddH_2O added to a total volume of 25 μL. The reaction conditions were: 94˚C for 3 min; 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s for a total of 36 cycles; and then extension at 72˚C for 10 min. Next, 1% agarose gel electrophoresis was used to detect the PCR products. The target PCR fragment was recovered with the MiniB-EST Agarose Gel DNA Extraction Kit Ver. 3.0 (TaKaRa). The recovered fragment was ligated to the pMD18-T vector and then transformed into *E. coli*

DH5a. The positive clones were selected and sent to BGI for sequencing.

2.2.4. 3' RACE and 5' RACE

The 3'RACE specific primers MG1 (5'-TGTCACTTACAACACCAAACTCG-TCTTC-3') and MG2 (5'-CACCGCCAAACCTAGAGTTGAGATT-3') and the 5'RACE specific primers GSP1 (5'-TAGGTTTGGCGGTGAC-3'), GSP2 (5'-AT-GTCCATTGAAGACGAGTTTG-3') and GSP3 (5'-CAATCATTTTAGTAGTT-GGGGT-3') were all designed with Primer Premier 5.0. Nested PCR was conducted using 3'1022-1, 3'1022-2, and the SMARTer™ RACE cDNA Amplification Kit (Clontech) in order to obtain the 3'-terminal sequence of the target gene. Nested PCR was also conducted using GSP1, GSP2, GSP3, and the 5' RACE System for Rapid Amplification of cDNA Ends (Version 2.0, Invitrogen) in order to obtain the 5'-ter-minal sequence of the target gene.

2.2.5. Full-Length Gene Sequence Splicing and Verification

DNAstar software was used to splice the middle fragment, the 5'-terminal sequence, and the 3'-terminal sequence in order to obtain the full-length cDNA sequence of the gene. The 5'- and 3'-primers for the spliced sequence were designed with Primer Premier5 as follows: F2 (5'-ATGGACATGTCGGAACC-3') and R2 (5'-CTAGCGTCTTCTTGCTGCC-3'). The spliced sequence was amplified using the re-verse transcription product of cDNA as a template, and then, it was further validated and verified.

2.2.6. Bioinformatics Analysis of Gene

BLASTX (NCBI) was used to study the homology of the nucleotide sequence and the deduced amino acid sequence. The ORF finder (NCBI) was used to search for an open reading frame, and the Conserved Domains database (NCBI) was used to analyze the conserved domains. The ProtParam Tool was used to analyze protein physical and chemical properties. Post Prediction, WOLF PSORT, and SubLocv were used to predict protein sub-cellular localization. Furthermore, ProtScale was used to predict hydrophilic or hydrophobic protein proper-ties. The SignalP 4.0 Server was used to predict the protein signal peptide. The TMHMM Server v2.0 was used to predict the protein transmembrane domain. The NetPhos 2.0 Server was used to predict potential protein phosphorylation sites, and the NetNGlyc 1.0 Server and NetOGlyc 3.1 Server were used to predict potential protein glycosylation sites. ExPaSy-SOPMA was used to predict protein secondary structure. SWISS-MODEL was used to predict protein tertiary structure. DNAMAN5.2.2 was used to conduct multiple sequence alignment. The Neighbor-Joining method from Mega5 was used to create the phylogenetic tree.

3. Results and Analysis

3.1. Cloning of the *Rosa rugosa TFL*1 Gene

The cloned middle fragment is 200 bp (**Figure 1(a)**), the cloned 3'-terminal fragment is 650 bp (**Figure 1(b)**), and the cloned 5'-terminal fragment is 278 bp

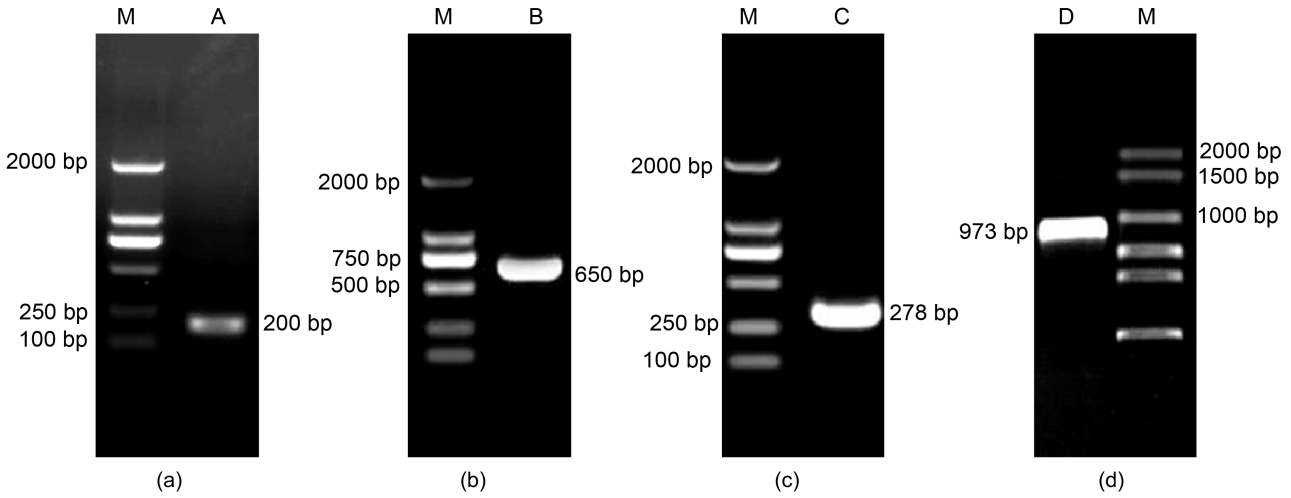

Figure 1. PCR amplification of *RrTFL*1 cDNA. (a) Intermediate fragment; (b) 3'-RACE; (c) 5'-RACE; (d) Full-length fragment.

(Figure 1(c)). These three fragments were spliced together with DNAstar in order to obtain a 973 bp cDNA sequence. The spliced sequence was then validated by PCR amplification (Figure 1(d)). In addition, the Blast analysis confirmed that all its homologous genes are the *TFL*1 gene and named *RrTFL*1 (GenBank accession number: KY928070).

3.2. Bioinformatics Analysis of the *RrTFL*1 Gene

The *RrTFL*1 gene has a full length of 973 bp, an open reading frame of 519 bp, a 5' UTR of 153 bp, and a 3' UTR of 301 bp, encoding 172 amino acids. The derived protein has a molecular weight of 19.48 kD, an isoelectric point of 9.13, a (c100227) conserved domain at position1-172. Thus *RrTFL*1 protein belongs to the PEBP family. Furthermore, the subcellular localization prediction result indicated that the protein is probably located at the cytoplasm. The hydrophilicity analysis further showed that the overall average hydrophobic index is −0.302, thus indicating a hydrophilic protein. The signal peptide prediction result demonstrated that no signal peptide cleavage site, thus indicating a non-secretory protein. The transmembrane domain analysis showed that no transmembrane domain exists. The phosphorylation site prediction results demonstrated that there are five Ser phosphorylation sites, seven Thr phosphorylation sites, and three Tyr phosphorylation sites, thereby providing a reference for the future study of the regulation of gene expression and protein modification. The glycosylation site prediction results showed that there is one O-glycosylation site and no N-glycosylation sites. The secondary structure prediction results demonstrated that there is 24.42% α-helix, 36.63% random coil, 27.91% extended peptide chain, and 11.05% β-corner. The tertiary structure prediction indicated that *RrTFL*1 and other homologous *TFL*1 gene proteins are composed of a large number of random coils, no spiral coil, the overall structure is similar. The BLAST results showed that the protein shares 96% homology with the *TFL*1 amino acid sequences of Rosaceae Plants including *Rosa chinensis* (ADO64261.1), *Fragaria* × *ananassa* (AFA42328.1), *Prunus mume* (XP 008241250.1), *Prunus*

armeniaca (ADL62862.1), *Spiraea cantoniensis* (AEO72025.1), *Photinia serratifolia* (AEO72024.1). The multiple sequence alignment result demonstrated that the *RrTFL*1 protein and the above plant *TFL*1 amino acid sequences all have a c100227 conserved domain, two highly conserved modules D-P-D-x-P, G-x-H-R, and two functional sites His, Asp (Figure 2). Furthermore, the constructed phylogenetic tree revealed that *RrTFL*1 is closely related to *TFL*1 from the same family members *Rosa chinensis*, *Fragaria* × *ananassa* and *Prunus mume*, whereas it is relatively distant from *Morusnotabilis* and *Citrus trifoliata*, which are from different families, consistent with the traditional classification results (Figure 3).

4. Discussion

TFL1 inhibits flower development by inhibiting the expression and activity of LFY and AP1 in the central region of the stem to maintain the characteristics of inflorescence meristems. By now, Some homologs of TFL1 have been isolated from many plants, include GMtfl1 in soybean, VvTFL1 in grapevine, Self-Pruning (SP) in tomato, CET in tobacco, CsTFL1 in citrus, MdTFL1 in apple, and PcTFL1 in pear [15]-[20]. In this research, the cDNA sequence of *TFL*1 gene was obtained from the flower buds of wild *Rosa rugosa* from Muping at different

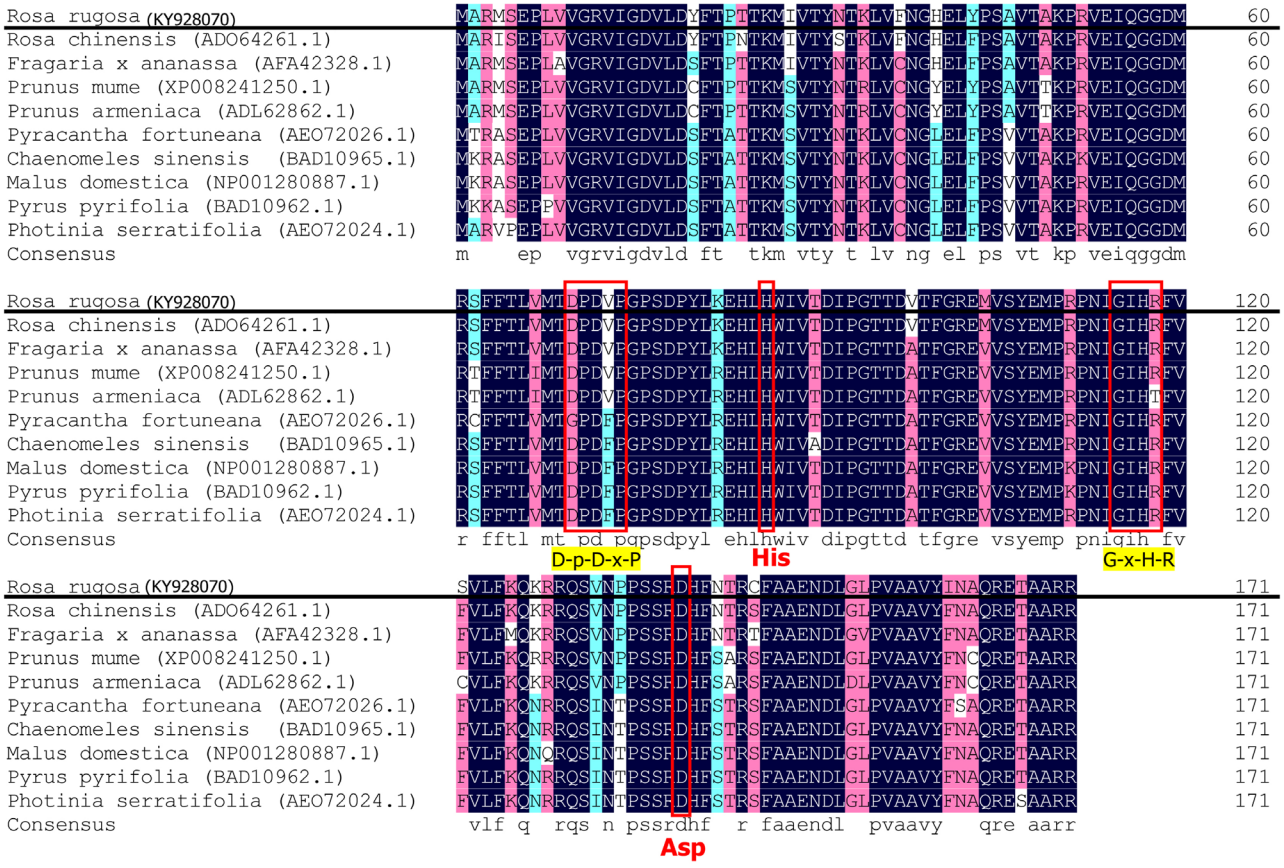

Figure 2. Multiple alignment of the *RrTFL*1 with other *TFL*1. Notes: The black underlined line is the amino acid sequence of *RrTFL*1; The red box area is labeled with two functional modules (D-p-D-x-p, G-x-H-R) and two functional sites (His, Asp) of the *TFL*1 gene.

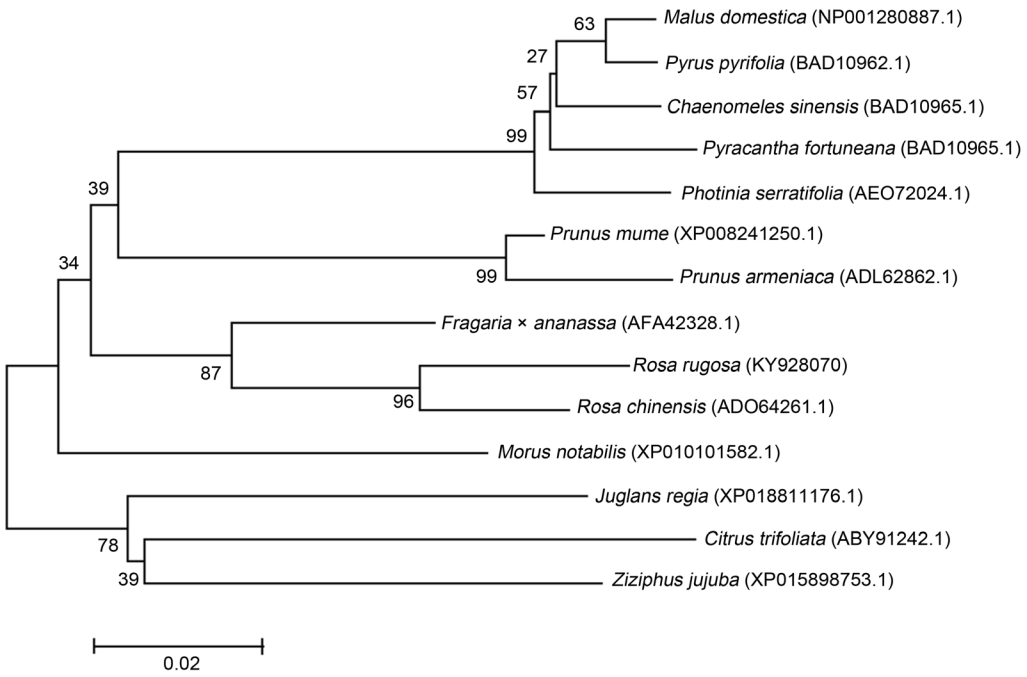

Figure 3. The phylogenetic tree derived from the alignment of amino acid sequences of *RrTFL*1 and other *TFL*1.

developmental stages for the first time, and its biological information was predicted. At present, the report on *TFL*1 gene in *R. rugosa* is very rare. The results of this study laid a foundation for further study of the expression, regulation and flowering patterns of *RrTFL*1, but also cultivating new varieties of *R. rugosa* which can flower continuously by gene engineering.

Previous studies have shown that the different expression pattern of *TFL*1 gene results in the different functions of *TFL*1 gene in different plants. CEN mutant became short, inflorescence became limited inflorescence in *Antirrhinum majus*, indicating that *TFL*1 acts as an inflorescence meristem-specific gene and is capable of maintaining the characteristic of the infinite growth of inflorescence; *Arabidopsis thaliana TFL*1 deletion mutants showed early flowering and multiflorous character, indicating that the *TFL*1 gene was an maintenance gene of inflorescence meristem [21] [22]; Excessive expression of the *TFL*1 gene leads to a flowering delay by prolonging the time of vegetative growth and inflorescence growth in *Antirrhinum majus*, as well as a delayed flowering transition in *Nicotianin tobacco* [23]. These results show that *TFL*1 gene is widely involved in vegetative growth and reproductive tissue development. In recent years, several researchers believed that the *TFL*1 may be a regulator of continuous flowering inroses [24]. But, it still needs more experimental evidence. Previous studies in our laboratory have found that the wild *Rosa rugosa* introduced from Muping had a unique recurrent flowering trait, but their molecular background is not clear. Therefore, we cloned the gene from the wild *Rosa rugosa* from Muping, and the gene was analyzed by bioinformatics. Next, we will further to verify whether the gene has continuous flowering regulation function.

The *TFL*1 gene cloned in this study has a conserved domain (c100227), two highly conserved modules, and two functional sites of His and Asp, which have a PEBP protein family compared with other species such as rose. But the BEBP protein family is divided into two subfamily *TFL*1 gene and *FT* gene, which play the opposite roles in the flowering process. The continuous flowering in *R. rugosa* may also be controlled by multiple genes. This study laid the foundation for further study on the mechanism of continuous flowering of *R. rugosa*. In addition, we further constructed the phylogenetic tree based on the *TFL*1 genes and found that the *RrTFL*1 gene had the smallest phylogenetic distance from the *TFL*1 genes derived from the species of the same family and the longest phylogenetic distance from the *TFL*1 genes derived from the species of different families. This agrees with the conventional plant classification. It is inferred that the evolution of the *TFL*1 gene corresponds with the phylogenetic relationship among the plant species from which the gene is derived.

In this study, the full-length cDNA sequence of *TFL*1 gene was cloned successfully from *R. rugosa* and named as *RrTFL*1. The bioinformatics characteristics of the *RrTFL*1 gene were analyzed, which provided the basis for further research on the function of *RrTFL*1 gene.

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