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Cloning and Bioinformatics Analysis of *Rosa rugosa* β-1,3-Glucanase Gene (*RrGlu*)

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Abstract

In order to reveal which role the callose played in *R. rugosa* pollination incompatibility, the full-length cDNA sequence of *β*-1,3-glucanase gene was cloned for the first time from the stylus of *Rosa rugosa* "Tanghong" with RT-PCR and RACE methods and named as *RrGlu*. The full-length cDNA is 1380 bp with an open reading frame of 1041 bp, encoding 346 amino acids. The derived protein has a molecular weight of 37.85 kD, a calculated pI of 9.12, a pfam00332 conserved domain at position 36 - 345, and belongs to glycosyl hydrolase family 17. The derived protein is a hydrophilic protein secreted into the vacuole. There is a signal peptide cleavage site at position 34 - 35, a transmembrane domain at position 13 - 32, six Ser phosphorylation sites, three Thr phosphorylation sites, three Tyr phosphorylation sites, one N-glycosylation site, and five O-glycosylation sites. There are 31.50% *α*-helixes, 30.92% random coil, 25.14% extended peptide chain, and 12.43% *β*-corner structure. This protein and the *Glu* protein from eight other species, including *Prunus persica*, share a sequence homology of greater than 72%; all of the proteins contain a pfam00332 conserved domain and a *β*-1,3-glucanase active center sequence (LIVM)-X-(LIVMFYW)3-(STAG)-E-(ST)-G-W-P-(ST)-X-G. Furthermore, their phylogenetic relationships are consistent with their traditional classifications. These results were meaningful to reveal the molecular mechanism of *R. rugosa* pollination incompatibility and improve the theory and techniques of breeding ornamental *R. rugosa.*

Keywords

***Rosa rugosa*, *β*-1,3-Glucanase Gene, Clone, Bioinformatics**

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1. Introduction

The *Rosa rugosa* is a famous traditional Chinese flower. It is fragrant as well as resistant to cold, drought, pest, disease, salt, and alkali [1]-[3]. However, due to its short bloom phase and uniform color and pattern, it has not been widely used in landscaping. In contrast, the *Rosa hybrid* belongs to the same species but comes in many varieties, has a year-round bloom, rich color, and ornate patterns. The hybridization of the traditional rose with the *R. hybrid* resulted in new varieties of roses that were aromatic, highly resistant, and highly ornamental. Nevertheless, the incompatibility between the two plants seriously hindered the breeding process [4]-[6]. In addition, the rose fruit is highly ornamental as well. However, its complete gametophytic self-incompatibility causes it to be very inconvenient for landscaping [7]. Thus far, the self-incompatibility mechanism has not been reported. Xiaoyan Yu *et al.* found that both the interspecific hybridization incompatibility between the *R. rugosa* and the *R. hybrid* as well as the self-incompatibility of the *R. rugosa* caused the pollen tube in the style to stop growing. In addition, the large amount of callose deposition at the top of the pollen tube and the intercellular space of the style channel may be the critical factors hindering the growth of the pollen tube [8] [9]. Furthermore, it is important to note that a similar phenomenon appeared in the outcrossing pollination of the *Rhododendron*, *Solanum*, *Actinidia*, and other *Gramineae* plants as well as the self-pollination of the *Rosaceae*, *Solanaceae*, and, *Theaceae* plants [10]-[16]. Therefore, callose may play a very important role in plant pollination incompatibility.

The hydrolysis and synthesis of callose are catalyzed by β-1,3-glucanase (*Glu*) and β-1,3-glucan synthase (*Cals*) [17] [18], respectively. *Glu* is an important class of pathogenesis-related proteins that can be induced in pathological or related conditions, and *Glu* is involved in the development of a variety of plant growth processes, such as cell division, microsporogenesis, fruit ripening, and seed germination [19]-[23]. According to previous studies, we speculate that the *Glu* gene is likely involved in regulating the affinity of plant pollination. However, at the present, *Glu* gene research primarily focuses on the disease resistance genetic engineering of plants [24]-[28]. Its regulation of pollination affinity has not been reported nor has any study of the *R. rugosa Glu* gene. Therefore, the aim of this study is to clone the *R. rugosa Glu* gene from *R. rugosa* styles and perform a bioinformatics analysis to establish a foundation for further exploring the role of callose in *R. rugosa* pollination incompatibility from the molecular point of view.

2. Material and Methods

2.1. Plant Material

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

2.2. Methods

2.2.1. Pollination Drawn

Between May 2015 and June 2015, the robust "Tanghong" buds were parchment isolated at 5:00-6:00 pm the day before blooming. At 6:00-7:00 am the next morning, self-pollination was performed, and the buds were parchment isolated when the anthers started to release pollen. Twelve hours after pollination, the styles were collected and 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 the total RNA from the *R. rugosa* style tissue. Agarose gel electrophoresis and spectrophotometer were used to determine the quality and concentration of the 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 *Glu* sequences of *Prunus persica*, *Prunus mume*, *Malus domestica*, *Malus hupehensis*, and *Pyrus bretschneideri*, the degenerate primers F1 (5'-TACATYGCBGTWGGAAAYGAA-3') and R1 (5'-GGCCAACCRSTYTCSGATA-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 MiniBEST 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'-GCGCTGCTCGATCCCATTATACGCT-3') and MG2 (5'-CGATGCCATGTTGGACGCTGTGTAT-3') and the 5' RACE specific primers GSP1 (5'-CAGACTTGAAGGAACC-3'), GSP2 (5'-GTGTCGATGGCTGTGGAAAC-3'), and GSP3 (5'-CAGCATTGGAAATTGCGGTT-3') were all designed with Primer Premier 5.0. Nested PCR was conducted using MG-1, MG-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'-terminal 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 Premier 5 as follows: F2 (5'-GCTCTAGAATGTCTAAATGCAATTCTTCAG-3') and R2 (5'-CGGGATCCATTGAAATTGATAGGGTATTTTGG-3'). The spliced sequence was amplified using the reverse 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 properties. 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 4.0 Server were used to predict potential protein glycosylation sites. ExPaSy-SOPMA was used to predict protein secondary 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 Glu* Gene

The cloned middle fragment is 560 bp (**Figure 1(a)**), the cloned 3'-terminal fragment is 515 bp (**Figure 1(b)**), and the cloned 5'-terminal fragment is 591 bp (**Figure 1(c)**). These three fragments were spliced together with DNAstar in order to obtain a 1380 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 *Glu* gene. Thus, the obtained gene is the *R. rugosa* β-1,3-glucanase gene, which was named *RrGlu* (GenBank accession number: KU144821).

3.2. Bioinformatics Analysis of the *RrGlu* Gene

The *RrGlu* gene has a full length of 1380 bp, an open reading frame of 1041 bp, a 5' UTR of 131 bp, and a 3' UTR of 208 bp, encoding 346 amino acids. The derived protein (the *RrGlu* protein) has a molecular weight of 37.85 kD, an isoelectric point of 9.12, a pfam00332 conserved domain at position 36 - 345, and a conserved β-1,3-glucanase motif LEIVVSDSGWPTAG in the activity center at position 264 - 277. Thus *RrGlu* protein

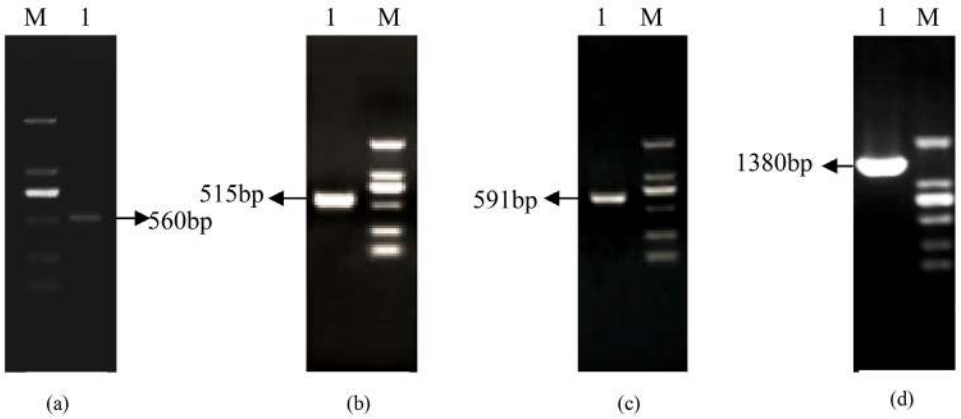

Figure 1. PCR amplification of *β*-1,3-glucanase cDNA. (a) Intermediate fragment; (b) 3'-RACE; (c) 5'-RACE; (d) Full-length fragment.

belongs to the glycosyl hydrolase family 17. Furthermore, the subcellular localization prediction result indicated that the protein is a secreted protein and is probably located at the vacuole. The hydrophilicity analysis further showed that the overall average hydrophobic index is −0.191, thus indicating a hydrophilic protein. The signal peptide prediction result demonstrated that a signal peptide cleavage site (ADA-QI) exists at position 34 - 35. The transmembrane domain analysis showed that a transmembrane domain exists at position 13 - 32. The phosphorylation site prediction results demonstrated that there are six Ser phosphorylation sites, three 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 N-glycosylation site and five O-glycosylation sites. The secondary structure prediction result demonstrated that there is 31.50% α-helix, 30.92% random coil, 25.14% extended peptide chain, and 12.43% *β*-corner. The BLAST results showed that the protein shares 72% - 82% homology with the *Glu* amino acid sequences of *Prunus persica* (AAL30426.1), *Prunus mume* (XP 008240769.1), *Malus hupehensis* (ADR71671.1), *Malus domestica* (XP 008351633.1), *Pyrus bretschneideri* (XP 009363234.1), *Morus notabilis* (XP 010090235.1), *Eucalyptus grandis* (XP 010056683.1), and *Citrus sinensis* (CAA03908.1). The multiple sequence alignment result demonstrated that the *RrGlu* protein and the above plant *Glu* amino acid sequences all have a pfam00332 conserved domain and a conserved *β*-1,3-glucanase enzyme active site sequence (LIVM)-X-(LIVMFYW)3-(STAG)-E-(ST)-GWP-(ST)-XG (**Figure 2**). The above results further confirm that *RrGlu* indeed belongs to *β*-1,3-glucanase. Furthermore, the constructed phylogenetic tree revealed that *RrGlu* is closely related to *Glu* from the same family member *Malus domestica*, *Malus hupehensis*, and *Pyrus bretschneideri*, whereas it is relatively distant from *Eucalyptus grandis* and *Citrus sinensis*, which are from different families, consistent with the traditional classification results (**Figure 3**).

4. Discussion

4.1. Classification of *β*-1,3-Glucanase Gene

Nearly all higher plants contain *β*-1,3-glucanase genes, and they belong to a large gene family. At present, the *Glu* genes of dozens of plants have been cloned, and different species have different *Glu* genes [29]-[32]. Thus far, *Glu* genes can be divided into four types according to their isoelectric point, positioning, mRNA expression patterns, and sequence homology: type I *Glu* is a basic protein that is primarily located in the vacuole; type II *Glu* is an acidic protein that is primarily located in the intercellular space; type III *Glu* is also an acidic protein that is primarily located in the extracellular space and is an inducer releasing *β*-1,3-glucanase; and type IV *Glu* is an acidic protein as well that is primarily located in the extracellular space but is a non-inducible *β*-1,3-glucanase [33]-[36]. Our study revealed that the *RrGlu* gene encodes a derived protein (*RrGlu*) with an isoelectric point of 9.12 and is located in the vacuole. Therefore, the *RrGlu* may belong to type I *Glu*. However, it is necessary to further investigate the specific function of the *RrGlu* gene by transgenic experiments. This study has laid a foundation for the further exploration of the function of the *RrGlu* gene.

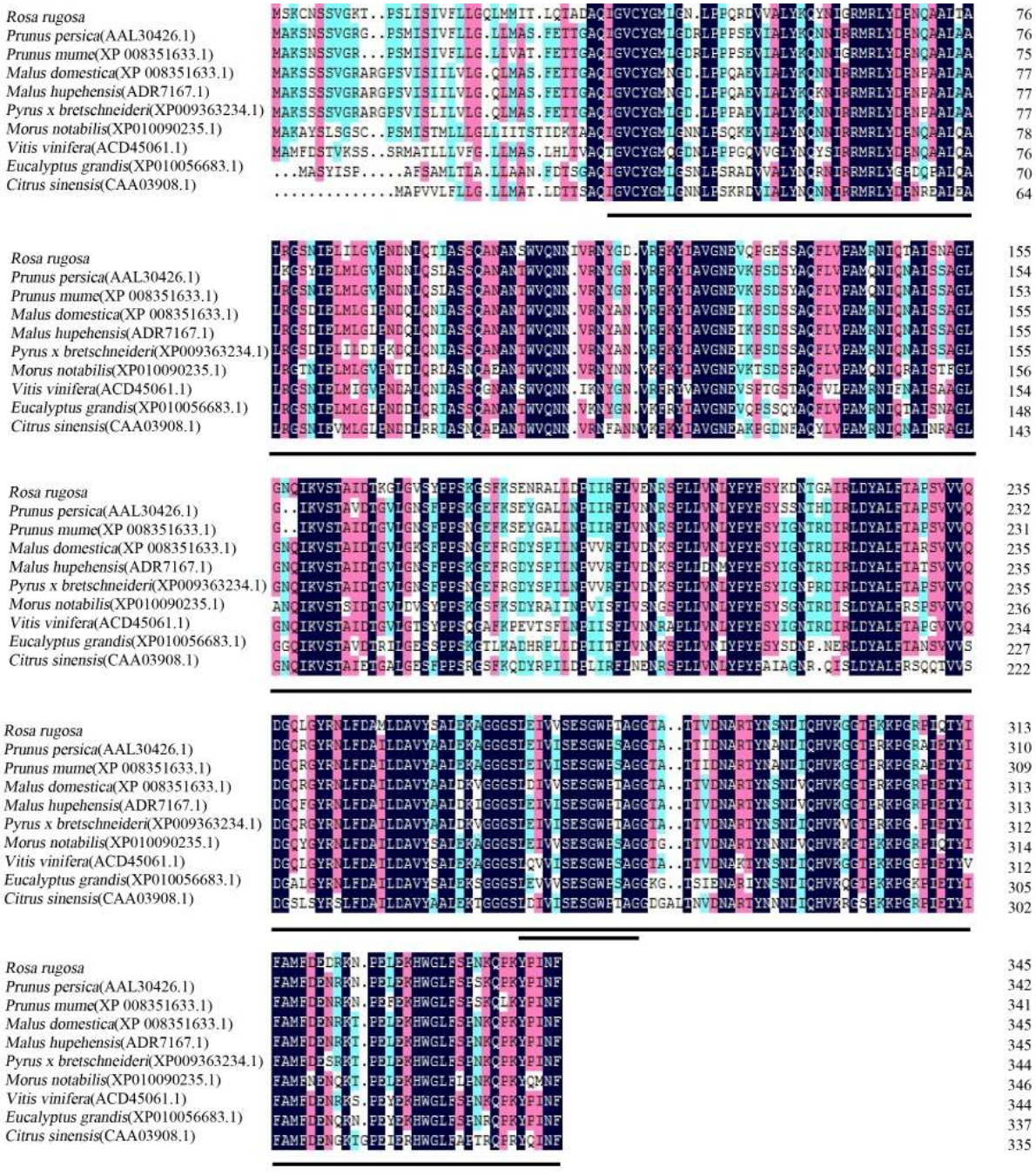

Figure 2. Multiple alignment of the *RrGlu* with other *Glus*. Notes: The underlined line is a conserved domain of *RrGlu*, and the double line is the common sequence of the enzyme active center of *Glus*.

4.2. The Function of *β*-1,3-Glucanase Gene

β-1,3-glucanase gene is an important class of pathogenesis-related proteins that can be induced in pathological or related conditions. Previous studies showed that the plants of *β*-1,3-glucanase gene expression level is very low in normal circumstances. The gene expression increased, activity was significantly enhanced when it is induced by abiotic factors such as ethylene, cytokinins, mechanical damage and metal ions as well as subjected to biological factors, for example, pathogens, insect feeding and so on [37]. At the same time, *β*-1,3-glucanase gene is involved in the development of a variety of plant growth processes, such as cell division, microsporogenesis,

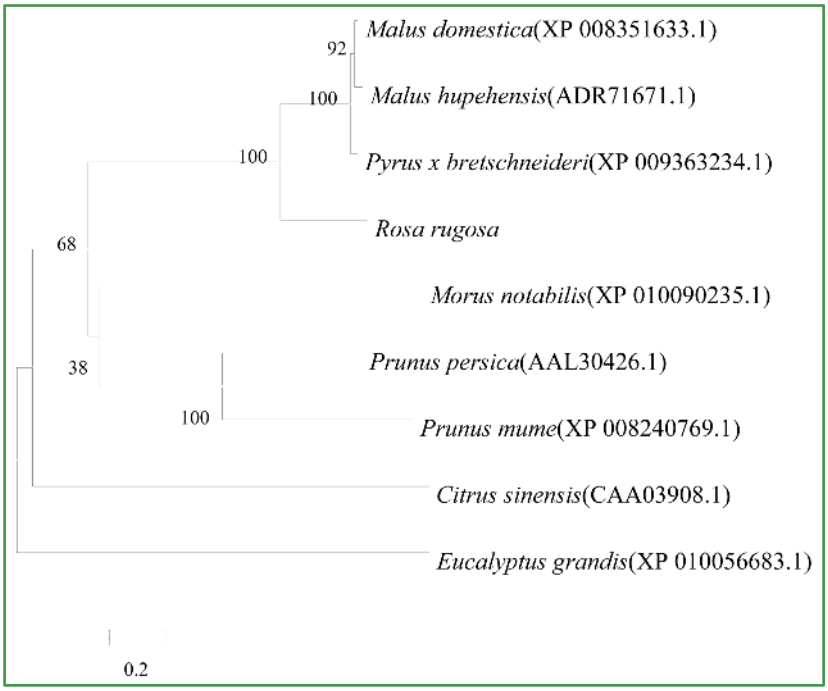

Figure 3. The phylogenetic tree derived from the alignment of amino acid secquences of *RrGlu* and other *Glus.*

fruit ripening, and seed germination. For example, *Arabidopsis* anther specifically expressed *β*-1,3-glucanase gene expressed in advance can cause pre-dissolution of the microsporocyte callose wall at anther development process, which affected the microspores further to develop into mature pollen grains, result in male sterility [33]. Previous studies in our laboratory have found that the gene is likely to be involved in the regulation of the pollination affinity of *R. rugosa*. Therefore, we cloned the gene from the stylus of *R. rugosa*, and the gene was analyzed by bioinformatics. Next, we will further through the transgenic to verify whether the gene has pollination compatibility regulation function.

4.3. Evolution of *β*-1,3-Glucanase Gene

In addition, previous studies on the *Glu* phylogenetic relationships of bananas, cotton, tobacco, grapes, and other plants have demonstrated highly conserved *Glu* sequences in evolution and consistency between the *Glu* evolution and the kinship of the plant sources. This study also found that *RrGlu* shares 72% sequence homology with nine other species, including *Prunus persica*. Furthermore, *RrGlu* is closely related to *Glu* from the same family *Malus domestica*, *Malus hupehensis*, and *Pyrus bretschneideri*, whereas it is relatively distant from *Eucalyptus grandis* and *Citrus sinensis*, which are from different families. This indicates that the results of this study are consistent with previous studies.

In this study, the full-length cDNA sequence of *β*-1,3-glucanase gene was cloned successfully from *R. rugosa* and named as *RrGlu*. The bioinformatics characteristics of the *RrGlu* gene were analyzed, which provided the basis for further research on the function of *RrGlu* gene.

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