Postprint: Cloning, Phylogenetic Tree, and Expression Analysis of GL2 Homolog in *Rosa roxburghii*

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Abstract

The spines of Rosa roxburghii cause inconvenience for fruit harvesting and food processing. This study cloned the gene RrGL2 associated with spine development and formation from Rosa roxburghii fruits, providing a theoretical basis for investigating the molecular mechanisms underlying spine formation and development. The cytological development process of fruit spines in Rosa roxburghii was observed through paraffin sectioning. Using cDNA from Rosa roxburghii 'Guinong 5' as a template, the RrGL2 gene, a homolog of GL2 involved in trichome formation in Arabidopsis thaliana, was cloned via RACE, followed by bioinformatic and expression analyses of this gene. During the early stage of flower bud formation, cells at the base of the spine structure divide continuously and develop outward; cells in the middle region become thinner and elongated to form a "needle" -like structure, while cells at the apex gradually lignify, hardening the spine to form the fruit spine. RACE amplification yielded a full-length RrGL2 cDNA of 2,292 bp, encoding 763 amino acids. RrGL2 possesses a Homeodomain and a StAR-related lipid transfer (START) domain. RrGL2 exhibits high amino acid homology with GL2 proteins encoded by other species, and phylogenetic tree analysis revealed that Rosa roxburghii RrGL2 is closely related to GL2 from Fragaria subspecies. Finally, qRT-PCR analysis demonstrated that RrGL2 expression levels were higher in stems and fruits than in other tissues, with the highest expression observed in fruit spines at 7 weeks post-anthesis, being 7.87-fold and 2.10-fold higher than in spines at 3 weeks and 5 weeks, respectively. The function of RrGL2 is closely associated with spine formation and development, and this study provides a theoretical foundation for understanding the molecular mechanisms of spine formation and for breeding in Rosa roxburghii.

Full Text

Preamble

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Abstract

The prickles of *Rosa roxburghii* cause significant inconvenience during fruit harvesting and food processing. This study cloned the *RrGL2* gene from *R. roxburghii* fruit, which is associated with prickle development and formation, providing a theoretical foundation for investigating the molecular mechanisms underlying prickle formation and development. The cytological development of prickles was observed through paraffin sectioning. Using cDNA from *R. roxburghii* 'Guinong 5' as a template, the *RrGL2* homolog of *Arabidopsis thaliana GLABRA2* (*GL2*) was cloned via RACE. Bioinformatics and expression analyses were subsequently performed. During early flower bud formation, cells at the base of the prickle structure divided continuously and developed outward. Middle cells became elongated and slender, forming a "needle-like" structure, while apical cells gradually lignified, hardening the prickles. The full-length *RrGL2* cDNA obtained through RACE amplification was 2,292 bp, encoding 763 amino acids. RrGL2 possesses a homeodomain and a StAR-related lipid transfer (START) domain. Amino acid homology analysis revealed high similarity between RrGL2 and GL2 proteins from other species, with phylogenetic analysis indicating a close relationship between *R. roxburghii* RrGL2 and GL2 from *Fragaria* subspecies. Quantitative real-time PCR (qRT-PCR) analysis demonstrated that *RrGL2* expression levels were higher in stems and fruits than in other tissues, with the highest expression observed in prickles at 7 weeks post-anthesis—7.87-fold and 2.10-fold higher than in prickles at 3 and 5 weeks, respectively. These findings suggest that *RrGL2* function is closely associated with prickle formation and development, providing both theoretical insights into the molecular mechanisms of prickle formation in *R. roxburghii* and a genetic resource for breeding programs.

Keywords: *Rosa roxburghii*, trichomes, prickles, *RrGL2*, gene expression

Introduction

Rosa roxburghii, a member of the Rosaceae family, is widely cultivated in southwestern China, particularly in Guizhou Province, where its favorable flavor and high nutritional value have made it popular among consumers. The fruit contains various phenolic compounds and antioxidants (Van et al., 2008), enabling its use as a radioprotective and tumor-inhibiting agent (Xu et al., 2014; Liu et al., 2012). Additionally, *R. roxburghii* serves as an important raw material for juice and dried fruit products.

However, the dense covering of prickles on *R. roxburghii* fruits creates substantial difficulties for harvesting, food processing, and orchard management. In recent years, with the development of horticulture, plant prickles have attracted increasing research attention.

Prickles are sharp outgrowths derived from epidermal tissue that are widespread across many plant species (Kellogg et al., 2011), occurring on leaves, stems, fruits, and other organs (Feng et al., 2015). Their presence increases epidermal thickness, reduces heat and water loss, and protects against insect and pathogen attack or mechanical damage (Gomes et al., 2012). Current research indicates that prickles in raspberry and rose originate from modified glandular trichomes that continue growing and eventually harden into their final prickle morphology as epidermal outgrowths. Thus, prickles represent a deformation of trichomes together with a few cortical cells.

Trichomes are specialized unicellular structures on plant organ surfaces. The spatiotemporal expression of genes required for trichome development is coordinated by a ternary activation complex (An et al., 2011). Significant progress has been made in understanding trichome development regulation, particularly in *Arabidopsis thaliana*. This process involves transcription factors including MYB proteins [GLABRA 1 (GL1), WEREWOLF, CAPRICE, TRIPTYCHON], WD40-type proteins [TRANSPARENT TESTA GLABRA 1 (TTG1)], bHLH proteins [GLABRA 3 (GL3) and ENHANCER OF GLABRA 3 (EGL3)], HD-Zip proteins [GLABRA2 (GL2)], and WRKY transcription factors [TRANSPARENT TESTA GLABRA2 (TTG2)] (Zhao et al., 2008; Gan et al., 2011). Yeast two-hybrid experiments have shown that GL1 and TTG1 bind to different regions of GL3 and EGL3, indicating that the MYB-bHLH-WD40 complex forms a trimeric transcriptional activation component that regulates downstream genes and morphogenesis during trichome development (Pesch et al., 2015; Ramsay & Glover, 2015). GL2 can switch the function of the MYB-bHLH-WD40 complex through feedback mechanisms that activate the TTG1 complex involved in trichome maturation. During trichome and root hair formation, two adjacent cells can compete for expression regulatory factors such as GL2/TTG1 (Pu et al., 2003).

GL2 plays a crucial role in trichome morphogenesis, including cell branching, ex-

pansion, and cell wall maturation (Szymanski et al., 1998). GL2 is a homeobox gene encoding an HD-Zip transcription factor containing a StAR (steroidogenic acute regulatory protein) domain. Such homeobox proteins coordinate target gene expression during various plant developmental processes (Rerie et al., 1994; Di & Al, 1996). GL2 is continuously expressed in mature trichomes and is required for non-hair cell differentiation and trichome differentiation during early morphogenesis (Fyvie et al., 2015). Recent studies show that GL2 is necessary for regulating root hair development and is preferentially expressed in non-hair epidermal cells within the root meristem and elongation zones (Masucci et al., 1996). Both *gl2* mutants and *gl2/gl3* double mutants affect trichome morphogenesis, causing degradation and reduction of trichomes on leaves, while *gl2* mutants also exhibit phenotypes including mucilage deficiency in seed coats and ectopic root hair formation (Gao et al., 2008; Shi et al., 2012). Additionally, *gl2* mutants cannot form hair-like subsidiary cells surrounding trichomes. Genetic experiments have demonstrated that GL2 functions downstream of GL1 and TTG1 (Pesch & Hülskamp, 2011).

This study isolated and cloned *RrGL2* associated with prickle development from the widely cultivated 'Guinong 5' variety in Guizhou Province. Through bioinformatics analysis and spatiotemporal expression detection of the *RrGL2* gene, we provide both genetic resources and a theoretical foundation for further investigation into the molecular mechanisms of prickle formation and development in *R. roxburghii*, as well as for engineering thornless varieties through genetic modification.

Materials and Methods

1.1 Plant Materials and Cytological Analysis

Leaves and fruits of *R. roxburghii* 'Guinong 5' were collected. One portion was immediately frozen in liquid nitrogen and stored at -80°C. Leaves, young fruits, and mature fruits were selected, and prickles were collected at 3, 5, and 7 weeks after flowering (WAF) to detect *RrGL2* expression levels. Another portion was observed and photographed under a stereomicroscope (SZX7, OLYMPUS, Japan).

Young buds at different developmental stages were fixed in FAA fixative solution. Samples were placed in 3 mL fixative in centrifuge tubes, sealed with parafilm, punctured with dissecting needles, and vacuum infiltrated. After vacuum treatment, 2 mL additional fixative was added (sample-to-fixative ratio approximately 1:20). For long-term storage, fixative was replaced with 70% ethanol and stored at 4°C. On day 1, samples were treated with 70% ethanol overnight. On day 2, samples underwent sequential dehydration: 85% ethanol, 95% ethanol, absolute ethanol (twice), 1/5 xylene (removing 1/5 volume and adding 1/5 xylene), 2/5 xylene, 3/5 xylene, 4/5 xylene, pure xylene (twice), followed by paraffin infiltration (adding crushed paraffin and incubating at 36°C

for 3 days). Fixed and trimmed paraffin blocks were mounted on sample holders and sectioned at 8–15 m thickness using a microtome. Paraffin ribbons were flattened in boxes for slide mounting. Sections were observed under a microscope (BX53, Olympus, Japan) and photographed using a SPOT FLEX™ CCD camera (Diagnostic Instrument, USA).

1.2 RNA Extraction and Purification

Total RNA was extracted from stems, leaves, flower buds, seeds, and prickle tissues at 3, 5, and 7 WAF using Trizol reagent (TaKaRa, Japan) according to the manufacturer' s protocol. RNA samples were treated with DNase (TaKaRa, Japan) and reverse-transcribed using an oligo dT-adapter primer RT-PCR Kit (TaKaRa, Japan) following the kit instructions.

1.3 Isolation of GL2 cDNA

For 3'RACE, first-strand cDNA was synthesized from leaf RNA using a 3'RACE Kit (TaKaRa, Japan). Primers were designed based on GL2 homologous sequences from other species reported in NCBI (https://www.ncbi.nlm.nih.gov/) that are associated with prickle development. Two rounds of PCR were performed to amplify the 3' end of the gene (Table 1). The first-round PCR program consisted of initial denaturation at 94°C for 3 min, followed by 20 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 2 min) and final extension at 72°C for 10 min. The first-round PCR product served as template for the second-round PCR, which was run for 35 cycles under identical conditions.

For 5' RACE, first-strand cDNA was synthesized from leaf total RNA using the SMARTer™ RACE cDNA Amplification Kit (No. 634923, Clontech). Gene-specific primers were designed based on the 3' end sequence (Table 1) for Touchdown PCR: 5 cycles of (94°C for 30 s, 72°C for 90 s), followed by 5 cycles of (94°C for 30 s, 70°C for 30 s, 72°C for 1 min), and finally 30 cycles of (94°C for 30 s, 55°C for 2 min, 72°C for 2 min).

1.4 Cloning and Sequencing

PCR products were detected on 1% agarose gels, excised, and purified using an Agarose Gel DNA Purification Kit (DV805A, TaKaRa, Japan). Purified fragments were cloned into the pMD18-T vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5 competent cells (Trans, China) via heat shock. Positive clones were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

1.5 Sequence Analysis

The open reading frame of the amplified GL2 fragment was analyzed using NCBI ORF Finder. Homologous proteins were identified via NCBI BLASTp. Protein

molecular weight, isoelectric point, and amino acid composition were analyzed using ProtParam. Hydrophilicity/hydrophobicity was predicted using ProtScale (Kyte & Doolittle method). Transmembrane domains were predicted using the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/). Protein structural domains were analyzed using SMART online tools. Secondary structure was predicted using ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA 6.0 software.

1.6 Gene Expression Analysis

Quantitative real-time PCR (qRT-PCR) for *RrGL2* was performed using the FastStart DNA Master SYBR Green I Kit on a LightCycler 480 instrument (Roche, Switzerland). -actin served as the internal reference gene. Primers were: *RrGL2* Forward (5 -3): CGAGGCAGTGACAGTGAAGG; Reverse (5 -3): GGCAGACTCAACAGACTCCATAG. -actin Forward (5 -3): CCGCCATGTATGTTGCCATCC; Reverse (5 -3): AGCCAGGTCAAGACGCAGAAT. The qRT-PCR program followed the SYBR Green protocol: 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Relative expression levels were calculated using the 2^(-ΔΔCt) method (ΔΔCt = sample ΔCt –control ΔCt, where ΔCt = *RrGL2* Ct – -actin Ct).

1.7 Statistical Analysis

All data represent the mean of three biological replicates with corresponding standard deviations. Statistical significance was determined using one-way ANOVA in SPSS software.

Results

2.1 Morphological Observation and Early Cytological Studies of Different *R. roxburghii* Tissues

Various tissues of *R. roxburghii* were examined, including stems [Figure 1: see original paper]A, leaves [Figure 1: see original paper]B, flower buds [Figure 1: see original paper]C, fruits [Figure 1: see original paper]D, and seeds [Figure 1: see original paper]E. Hard prickles were observed on stem and fruit surfaces (stem prickles and fruit prickles), with a few prickle-like structures present on the outermost parts of flower buds. No hard prickles were found on leaf or seed surfaces.

Prickle number and length were quantified at different fruit developmental stages . Fruits at 3, 5, and 7 WAF had diameters of 1.5, 2.3, and 3.4 cm, respectively. Fruits were divided into upper, middle, and lower regions for analysis. Prickles were predominantly concentrated in the middle region across all

developmental stages, with fewer prickles in the upper and lower regions. Prickle length increased significantly in all three regions as fruits developed.

After a period of vegetative growth, *R. roxburghii* transitions to reproductive growth when environmental conditions become favorable, which is essential for fruit formation. During flower development, prickle cells on the calyx begin to differentiate continuously. This dynamic process was observed initially as a few formed prickle structures on the calyx, which increased in number as the flower developed [Figure 2: see original paper]A. As floral structures expanded outward, stamen primordia at the petal primordium base developed into thick, short, elliptical structures. Ground meristem cells within the prickle primordia underwent tangential division, increasing in number, while epidermal cells also divided to accommodate outward prickle extension [Figure 2: see original paper]B. Rapid cell division at the base expanded the prickle base, while middle cells increased in number and changed morphology, with elliptical cells becoming slender and elongated to form a needle-like tip, establishing the overall prickle shape [Figure 2: see original paper]C.

2.2 Sequence Analysis of *R. roxburghii* RrGL2

Using RNA from 'Guinong 5' as template, the full-length *RrGL2* cDNA was obtained via RACE. *RrGL2* (GenBank accession: MG386498) spans 2,292 bp and encodes 763 amino acids. BLAST analysis revealed high homology between RrGL2 and GL2 proteins from strawberry (*Fragaria vesca* subsp. *vesca*, 96% similarity), peach (*Prunus persica*, 88%), apple (*Malus domestica*, 86%), physic nut (*Jatropha curcas*, 78%), and mulberry (*Morus notabilis*, 80%).

ProtParam analysis indicated a molecular weight of 84.9 kDa and an isoelectric point of 5.73 for RrGL2. The protein composition includes 13.76% acidic amino acids, 13.63% basic amino acids, 38.79% hydrophobic amino acids, 27.39% charged amino acids, and 61.07% polar amino acids. ProtScale analysis showed that hydrophilic amino acids predominate over hydrophobic residues, with an average hydrophilicity value of -0.525, indicating that RrGL2 encodes a soluble protein. TMHMM 2.0 prediction revealed no transmembrane domains in RrGL2.

2.3 Homologous Protein and Phylogenetic Analysis of RrGL2

Comparative analysis of GL2 proteins from different species [Figure 3: see original paper]A showed that RrGL2 shares high sequence similarity with strawberry (98%), peach (92%), apple (90%), physic nut (83%), and mulberry (82%). Based on the RrGL2 three-dimensional structure (PDB: 2Z9Z), ESPript 3.0 was used to predict secondary and tertiary structures. -turns constitute the major secondary structure element [Figure 3: see original paper]A, and multi-sequence alignment revealed conserved secondary structural domains across species. SMART analysis identified two main domains: a homeobox (HOX) domain at amino acids 107–169 and a START (StAR-related lipid transfer) domain

at amino acids 276–500 [Figure 3: see original paper]B. The tertiary structure [Figure 3: see original paper]C shows DNA/RNA-binding helical structures and orthogonal folds, suggesting RrGL2 can interact with DNA to control gene expression. Phylogenetic analysis using MEGA 6.0 revealed that RrGL2 is most closely related to strawberry GL2, with close relationships to other Rosaceae species including peach, cherry, plum, and apple [Figure 4: see original paper].

2.4 Expression Patterns of *RrGL2* in Different Tissues and During Fruit Development

Real-time PCR analysis revealed *RrGL2* expression levels across different tissues [Figure 5: see original paper]A. Expression was lowest in seeds, with stem and fruit showing 59.95-fold and 33.52-fold higher expression, respectively. Leaf and flower expression was 7.99-fold and 15.62-fold higher than in seeds, respectively.

Expression in prickles at 3, 5, and 7 WAF [Figure 5: see original paper]B showed that *RrGL2* expression increased progressively with fruit development and prickle growth, reaching its highest level at 7 WAF—7.87-fold and 2.10-fold higher than at 3 and 5 WAF, respectively. These results suggest that *RrGL2* plays a role in prickle formation.

Discussion

Morphological observations revealed hard prickles on *R. roxburghii* stems and dense fruit prickles on fruit surfaces. Cytological analysis demonstrated that fruit prickles initiate during the flower bud stage. The elevated *RrGL2* expression in stems and fruits compared to other tissues suggests that RrGL2 may play an important role in prickle and trichome morphogenesis. Our results show *RrGL2* expression in early-stage fruit prickles, indicating its function during the fruit budding stage (Vernoud et al., 2009; Ohashi et al., 2002). As fruits matured, both prickle number and length increased, correlating with enhanced *RrGL2* expression levels. These findings imply that *RrGL2* may be closely associated with prickle initiation in *R. roxburghii* (Rerie et al., 1994). The peak *RrGL2* expression in fruit prickles at 7 WAF suggests that prickle development-related genes can enhance prickle formation. Transcription factors EGL3 and TTG1 participate in *GL2* expression regulation, ultimately leading to trichome formation (Song et al., 2015). MYB proteins such as GL1 and WER can regulate the spatial expression of *GL2* homeobox genes through interaction with bHLH proteins, while GL2 expression is also repressed by CAPRICE MYB (Lee & Schiefelbein, 1999). Further functional analysis is required to determine the precise role of *RrGL2* as a key gene coordinating with other regulators in prickle development.

This study cloned *RrGL2* from *R. roxburghii* using RACE technology. BLAST searches in the NCBI database identified highly similar GL2-like protein members across species, suggesting that *RrGL2* may share similar functions in reg-

ulating prickle initiation, morphogenesis, and development (Wang et al., 1999). Domain analysis revealed that RrGL2 is a homeodomain protein. Homeodomain proteins can bind DNA, regulate transcription, and participate in multi-protein complexes that control developmental gene expression (Foronda et al., 2009). The RrGL2 tertiary structure contains DNA/RNA-binding helical structures, suggesting its capacity to interact with DNA and control gene expression. Additionally, the interaction between GL1 and GL3 transcription factors activates *GL2* expression (Wang & Chen, 2008). Previous reports indicate that *GL2* regulates root hair development in a cell position-dependent manner and affects seed oil content (Shen et al., 2006; Masucci et al., 1996).

Through bioinformatics analysis, we identified *RrGL2* as an ortholog of GL2 proteins from other species, demonstrating the potential for positive evolutionary selection in GL2. Spatiotemporal expression analysis revealed the relative expression levels of *RrGL2* across different tissues and prickle developmental stages. Therefore, this study provides a molecular foundation for understanding the genes involved in *R. roxburghii* prickle development and the underlying molecular mechanisms, offering a theoretical basis for engineering *R. roxburghii* varieties with reduced or no prickles through genetic modification.

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Note: Figure translations are in progress. See original paper for figures.