Cloning and Expression Profiling of the Polycomb Gene, *Retinoblastoma-related* Protein from Tomato *Solanum lycopersicum* L.

Zainab M. Almutairi[1] and Monther T. Sadder[2,3]

[1]Department of Biology, College of Science and Humanities, Salman bin Abdulaziz University, Al-Kharj, Saudi Arabia. [2]Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Saudi Arabia. [3]Plant Biotechnology Lab, Faculty of Agriculture, The University of Jordan, Amman, Jordan.

ABSTRACT: Cell cycle regulation mechanisms appear to be conserved throughout eukaryotic evolution. One of the important proteins involved in the regulation of cell cycle processes is retinoblastoma-related protein (RBR), which is a negative regulator of cell cycle progression, controlling the G1/S transition in plants and animals. In this study, we present the cloning and genomic structure of a putative *SlRBR* gene in the tomato *Solanum lycopersicum* L. by isolating cDNA clones that correspond to the *SlRBR* gene from tomato using primers that were designed from available Solanaceae ESTs based on conserved sequences between the PcG genes in *Arabidopsis thaliana* and tomato. The *SlRBR* cDNAs were cloned into the pBS plasmid and sequenced. Both 5′- and 3′-RACE were generated and sequenced. FlcDNA of the *SlRBR* gene of 3,554 bp was composed of a 5′-UTR of 140 bp, an ORF of 3,054 bp, and a 3′-UTR of 360 bp. The translated ORF encodes a polypeptide of 1,018 amino acids. An alignment of the deduced amino acids indicates that there are highly conserved regions between the tomato SlRBR predicted protein and plant hypothetical *RBR* gene family members. Both of the unrooted phylogenetic trees, which were constructed using maximum parsimony and maximum likelihood methods, indicate a close relationship between the SlRBR predicted protein and the RBR protein of *Nicotiana benthamiana*. QRT-PCR indicates that *SlRBR* gene is expressed in closed floral bud tissues 1.7 times higher than in flower tissues, whereas the expression level in unripe fruit tissue is lower by about three times than in flower tissues.

KEYWORDS: tomato, retinoblastoma-related gene, FlcDNA, phylogenetics

CORRESPONDENCE: z.almutairi@sau.edu.sa

Introduction

The tomato genome was sequenced as the cornerstone of an International Solanaceae Genome Initiative, a project that aims to develop the family Solanaceae as a model for systems biology for understanding plant adaptation and diversification. The tomato genome comprises approximately 950 Mb of DNA, more than 75% of which is heterochromatin and largely devoid of genes.[1] The sequencing of the tomato genome and sequencing of the wild relative were achieved and published in the SGN database (http://solgenomics.net/tomato/). Of the estimated 950 Mb genome size, 760 Mb was assembled into 91 scaffolds that were aligned with the 12 tomato chromosomes.[2]

RBR is a plant homolog of RB, the tumor suppressor gene in animals[3] that is primarily known as a negative regulator of cell cycle progression, controlling the G1/S transition in plants and animals. RBR is part of a cell cycle network involving cyclin-dependent kinases (CDKs) that phosphorylate RBR proteins to regulate the initiation of S phase.[4–6] In the nonphosphorylated state, RBR1 represses the action of the transcription factor E2F, which activates the expression of many genes that are required for DNA replication, such as F-box protein 17, PCNA, and MCM5.[7–9] RBR functions by binding E2F transcription factors, consequently blocking the transcription of cell cycle-related genes.[10,11] The repression of

E2F activity is because of the retinoblastoma protein-recruiting histone deacetylases (HDACs), which are co-repressors of transcription. HDACs remove acetyl groups from histones within DNA resulting in chromatin modification, DNA condensation, and transcription inhibition.[12] Furthermore, RB plays a critical role in chromosome condensation, centromeric function, and chromosome stability.[13]

The RB protein contains a number of functional domains, two of which, denoted as A and B, are conserved in humans and plants.[14] Similar to human RB proteins, plant RBR proteins are composed of an N-terminal region, A and B domains in the pocket region, and a C-terminal domain.[15–17] In the absence of RB-induced repression, E2Fs are eventually capable of inducing the expression of genes that are involved in the G1/S transition of cell cycle progression.[18,19]

Arabidopsis thaliana contains only a single *RBR* gene, whereas RB has two more relatives (p107 and p130) in animals.[20] Although many plants appear to possess only one *RBR* gene, maize and related cereals have at least two distinct types, *RBR*1 and *RBR*3.[21–23]

Plant RBR proteins control not only cell cycle arrest/ progression but also development and cellular differentiation in the endosperm, leaf, and root.[11,24–26] In *A. thaliana*, loss of RBR function is gametophyte lethal because mitotically derived cells from the megaspore fail to differentiate into a functional female gametophyte.[24,27,28] The loss of the RB protein also prevents or delays cell determination during plant male gametogenesis.[29] Furthermore, the loss of RBR function uncouples division and differentiation of meristemoid cells in leaves and disrupts the appropriate division and maintenance of meristem stem cells. Thus, RBR function is necessary during inflorescence and flower development to organize appropriate meristem cell divisions that are required for phyllotactic patterning and floral organ initiation. Together, these findings indicate that RBR plays a critical role in the homeostasis of stem cells and organ production in every stem cell niche.[30]

The *rbr*-2 mutation in *A. thaliana* during meiosis causes a loss of the RBR protein in male meiocytes. *rbr*-2 plants exhibit strongly reduced fertility, while vegetative growth is generally unaffected. The reduced fertility is due to a meiotic defect that results in reduced chiasma formation and subsequent errors in chromosome disjunction. Immunolocalization studies in wild-type meiocytes revealed that RBR is recruited as foci to the chromosomes during early prophase I in a DNA double-stranded break-dependent manner.[31] In addition to the disruption of cell division patterns, the loss of RBR function promotes context-dependent cell proliferation and negatively influences the establishment of cell differentiation in meristematic cells, including the shoot apical meristem, meristemoid mother cells, and procambial cells, which fail to produce appropriately differentiated cells and lateral organ formation.[30]

Sabelli et al.[9] investigated the role of RBR1 during maize endosperm development and found that the downregulation of RBR1 by RNAi resulted in the upregulation of RBR3-type genes, as well as the MCM 2–7 gene family and PCNA, which encode essential DNA replication factors. These results indicate that the RBR1 pathway plays a major role in the regulation of different processes during maize endosperm development by controlling gene expression programs, the mitotic cell cycle, endoreduplication, cell and nuclear sizes, and programed cell death.

FlcDNA clones are fundamental resources for molecular biology experiments of gene functions as well as for the detection of intron–exon structures in genomes. The aim of this study was to isolate and detect the genomic structure of the tomato PcG gene *Solanum lycopersicum* L. *RBR* (*SlRBR*) by cloning and sequencing its flcDNA of mRNA transcripts in tomato tissues. RBR is primarily known as a negative regulator of the cell cycle progression, which plays an epigenetic role in stem cell maintenance, cell specification and differentiation, and, to the best of our knowledge, this is the first gene of retinoblastoma-related gene family to be sequenced and characterized from tomato.

Results

To isolate the cDNA encoding the *SlRBR* gene, PCR reactions were performed using a primer set targeting the *SlRBR* gene with cDNA that was synthesized from total RNA that was extracted from tomato flowers. The amplification products were cloned in the cloning vector pBluescript II SK (+) (pBS), linearized with the *Eco*RV restriction enzyme, and transformed into *Escherichia coli* DH5α. The *SlRBR* cDNA contig was obtained by overlapping eight ESTs. The lengths of the ESTs varied between 453 bp and 1,272 bp, with an average length of 820 bp. Based on the consensus sequence of the *SlRBR* cDNA contig, 5′- and 3′-RACE gene-specific primers were designed and used to amplify the ends of the *SlRBR* contig. The generated 5′-and 3′-RACE fragments were 767 bp and 162 bp in length, respectively. Both the 5′- and 3′-RACE fragments were sequenced and assembled using the obtained ESTs to construct the *SlRBR* flcDNA contig. Computer analysis using the BLASTn algorithm confirmed that the obtained sequence corresponded to the *SlRBR* gene.

The constructed *SlRBR* flcDNA was located on tomato chromosome 9 in a genomic region of approximately 6,739 bp. Eighteen exons were determined using a flcDNA and BLASTn search in the SGN database. The exon/intron sizes are illustrated in Table 1. The constructed *SlRBR* flcDNA comprised 3,554 bp, containing 140 bp in the 5′-UTR, 3,054 bp in the ORF, and 360 bp in the 3′-UTR without a poly (A) tail. The ORF encodes a polypeptide of 1,018 amino acids, spanning from nucleotide position (np) 141, where the first ATG codon is located, to np 3,194, adjacent to a termination codon (TAA) (Fig. 1). The flcDNA and deduced amino acid sequences were submitted to the NCBI GenBank as accession numbers JQ669018 and AFD98848, respectively.

Table 1. Sizes of the exon/intron for the *SlRBR* gene. Eighteen exons were determined by presence within flcDNA using BLASTn search in the SGN database. Complete nucleotide sequence was derived from eight ESTs with 5′-and 3′-RACE fragments.

EXON NO.	COORDINATE	SIZE BP	INTRON NO.	SIZE BP
5'UTR	−140 . . −1	140		
Exon01	1 . . 89	89	Intron01	659
Exon02	90 . . 197	108	Intron02	100
Exon03	198 . . 338	141	Intron03	82
Exon04	339 . . 435	97	Intron04	240
Exon05	436 . . 483	48	Intron05	119
Exon06	484 . . 680	197	Intron06	503
Exon07	681 . . 748	68	Intron07	123
Exon08	749 . . 923	175	Intron08	73
Exon09	924 . . 1,118	195	Intron09	93
Exon10	1,119 . . 2,004	886	Intron10	129
Exon11	2,005 . . 2,164	160	Intron11	99
Exon12	2,165 . . 2,237	73	Intron12	443
Exon13	2,238 . . 2,414	177	Intron13	86
Exon14	2,415 . . 2,535	121	Intron14	85
Exon15	2,536 . . 2,674	139	Intron15	128
Exon16	2,675 . . 2,786	112	Intron16	151
Exon17	2,787 . . 2,903	117	Intron17	74
Exon18	2,904 . . 3,054	151		
3'UTR	3,055 . . 3,414	360		

ORF analysis and protein homology. The 3,054-bp ORF from the *SlRBR* flcDNA encodes 1,018 amino acids. The calculated molecular mass of the SlRBR predicted protein is 113.6 kDa with an estimated isoelectric point of 8.17. Domain prediction using NCBI CDD indicates that the SlRBR protein includes, similar to most other organisms, three conserved domains: a domain of unknown function (DUF3452) from amino acid 88 to 233, a retinoblastoma-associated protein A domain (RB A) from amino acid 426 to 616, and a CYCLIN domain from amino acid 738 to 868. These domains are conserved in homologous proteins of other plant species and appeared as conserved sequences in a multiple sequence alignment (Fig. 2).

A homology analysis for the SlRBR predicted protein against the sequenced genomes of plants revealed strong homology with two solanaceous species, *Nicotiana benthamiana* and *N. tabacum,* which revealed the highest score values (1,748 and 1,652 bits, respectively) and shared 86% and 83% sequence identity with tomato *SlRBR*, respectively. High homology revealed between dicot species and SlRBR predicted protein compared to monocot species. The dicot species shared 69–86% sequence identity with the SlRBR predicted protein, whereas the monocot species shared 61–63% identity with the SlRBR predicted protein for the best 16 hits.

Phylogenetic relationships between SlRBR and homologous proteins. Both of the phylogenetic trees (Figs. 3 and 4) confirmed the close relationship between the RBR predicted proteins of tomato and *N. benthamiana,* both of which belong to the Solanaceae family. These accessions were separated from all accessions as distinct clade, with high bootstrap support in both of the constructed trees (100% in the maximum parsimony tree and 98% in the maximum likelihood tree). The monocot species *Oryza sativa* appeared to be the most distant accession in both of the trees. Other accessions in the phylogenetic analysis revealed the same clustering in both of the trees, with the exception of the outgroup *Volvox carteri* f. nagariensis and *O. sativa*, which have been altered in their position between the trees as first and second outgroups.

Expression profiling for the *SlRBR* gene. The ΔΔCT method was used to compare the expression of the *SlRBR* gene in tomato tissues using leaf tissues as a calibrator. Figure 5 presents the expression levels of the *SlRBR* gene, which differ significantly in various tissues. The calculated $2^{-\Delta\Delta Ct}$ values showed the highest transcript abundance (38.23-fold) in closed floral bud tissues, in which cell division occurs and cell differentiation is not complete. Moderate levels (22.16-fold) were observed in flower tissues, and the lowest levels (7.46-fold) were observed in unripe fruit tissues.

Discussion

Since the completion of the sequencing of the *A. thaliana* genome in 2000, it has become clear that information of the
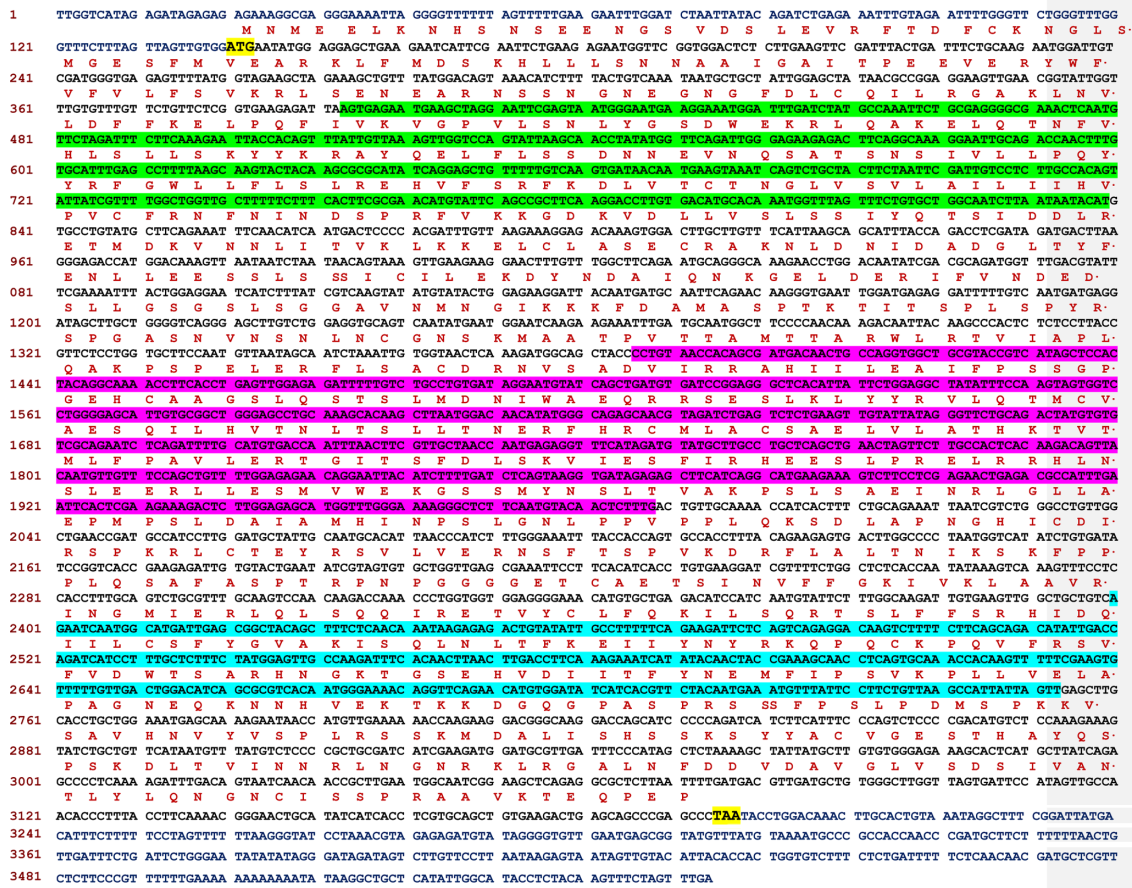
Figure 1. Nucleotide and deduced amino acid sequences of the tomato *SlRBR* gene. The flcDNA nucleotide sequence is composed of ORF (black text) and 5′- and 3′-UTR (blue text). The initiation methionine codon and the stop codon, ATG and TAA, respectively, are shown in yellow. The numbers on the left refer to the nucleotide positions. The amino acid sequence of the tomato SlRBR predicted protein is shown above the ORF sequence (red text). The conserved domains DUF3452, RB A and CYCLIN are shown in green, pink and turquoise, respectively.

genome of a particular plant species can have dramatic benefits in promoting plant molecular genetics. The *A. thaliana* genome sequence provides functional clues of genes from different species.[32] This study focused on the identification and characterization of a tomato gene belonging to the PcG gene family, *SlRBR*, using sequence homology to the orthologous gene in *A. thaliana*. Schubert et al.[33] discussed the epigenetic role of this gene in flowering and floral organ development in *A. thaliana*. To the best of our knowledge, this is the first *RBR* gene to be sequenced and characterized for Solanaceae with the exception of *RBR* genes of the tobacco species, *N. benthamiana*[34] and *N. tabacum*,[35] which have been identified and published in the SGN database under the accession numbers SGN-U519343 and SGN-U432371, respectively. We obtained the complete sequence of the flcDNA of the tomato *SlRBR* gene. The available ESTs sequence in the SGN database covered only 69.22% of the total length that was cloned and presented in this study. The exon/intron regions were determined, the ORF was predicted, and the 5′- and 3′-UTR of flcDNA were determined.

RBR is a plant homolog of RB, the tumor suppressor gene in animals[3] that is primarily known as a negative regulator of cell cycle progression, controlling the G1/S transition in plants and animals.[4] The SlRBR protein contains three conserved domains: DUF3452, RB A, and CYCLIN. These domains, as shown in the multiple sequence alignment, are present in the *RBR* genes of all of the analyzed plant species. A striking degree of conservation exists in terms of the domain organization of the RB family members, including animal and plant species.[15] The conservation of RBR proteins raised the possibility that their function may also be conserved at the molecular level.

A homology analysis of the SlRBR predicted protein against sequenced genomes of plants revealed a high degree of homology with the tobacco species, *N. benthamiana* and *N. tabacum*. The low level of homology with the RBR proteins of monocot species reflected the presence of two distinct classes of *RBR* genes in Graminea species. The *in silico* and functional data highlight considerable differences between dicot and monocot species in the retinoblastoma regulatory pathways and permit an improved classification of the RBR proteins in higher plants. The retinoblastoma functions are shared by two distinct RBR protein subfamilies in the monocot cereal species, whereas dicot plants have only a single RBR protein. The recognition of
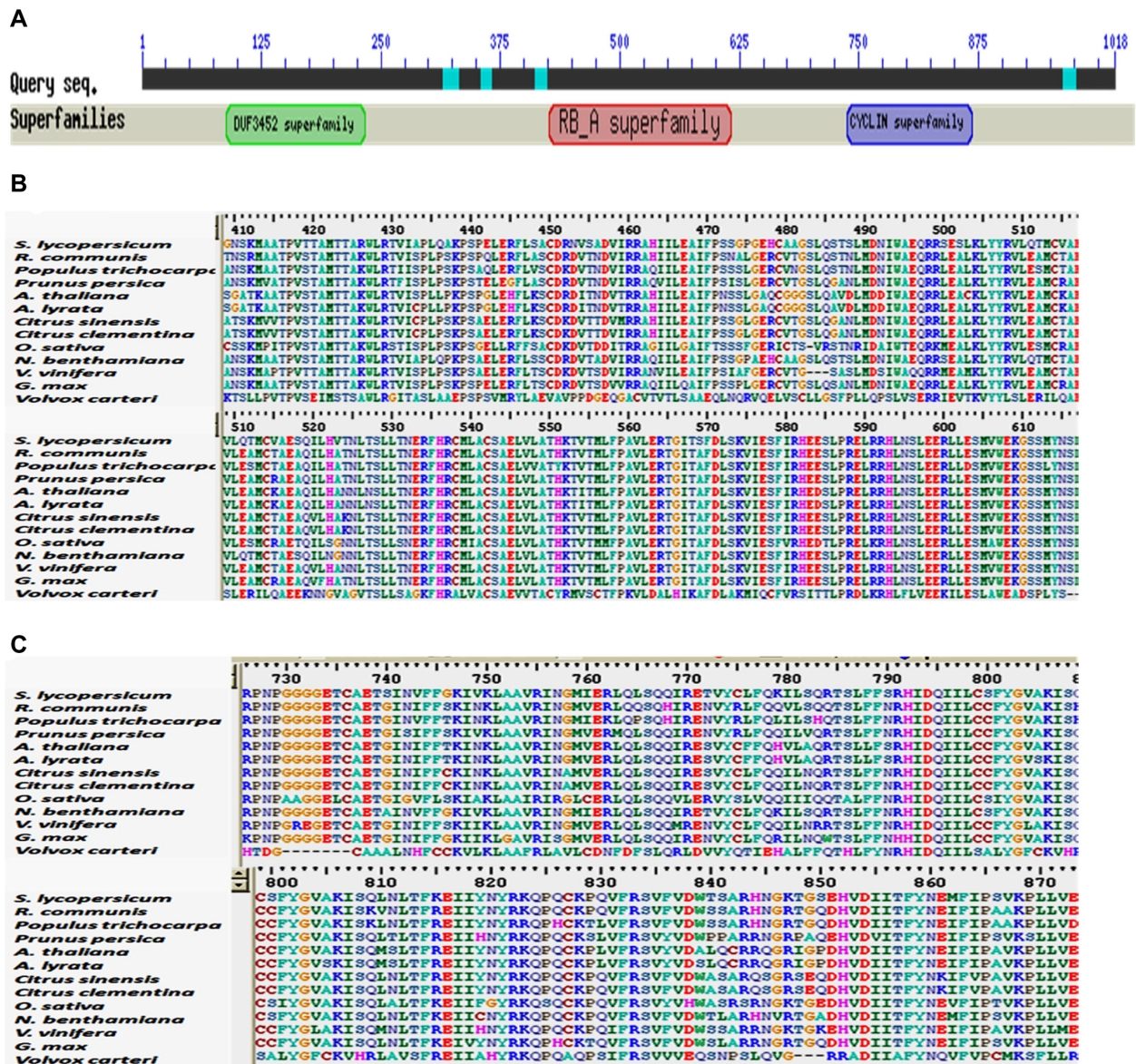
A

B

C

Figure 2. Multiple sequence alignments of the conserved domains sequences of the SlRBR predicted protein and 12 protein sequences of RBR hypothetical gene family members. The alignment was performed using ClustalW 1.83 in the BioEdit software. The conserved domains were identified in the sequence of the SlRBR protein using NCBI CDD. (**A**) Conserved domains of the SlRBR protein; (**B**) RB A domain from amino acid 426 to 616; (**C**) CYCLIN domain from amino acid 738 to 868.

two cereal RBR proteins indicates gene duplication, which is prevalent in plants and may lead to evolutionary innovation.[23]

The *RBR* gene was not only identified in animals (Metazoa) but also in plants (monocots, dicots, lycophytes, mosses, and green algae), Heteroconta (oomycetes, diatoms, and brown algae), Alveolata, red algae (Rhodophyta), Heterolobosea, Haptophyta, and Amoebozoa. Only fungi appear to be an exception because only the most primitive phylum retained an *RBR*, whereas the more advanced phyla evolved a functionally similar key regulator instead.[36,37] *RBR* genes have been identified not only in the multicellular species *V. carteri*[38,39] but also in the unicellular organism *Chlamydomonas reinhardtii*.[40] This distribution of *RBR* genes suggests that *RBR* genes have an ancient origin. Within the bryophytes, the nonvascular

embryophyte *Physcomitrella patens* has at least three *RBR* genes in its genome (*PpRBL*1, *PpRBL*2, and *PpRBR*).[36]

To gain better insight into the dynamic expression pattern of the tomato PcG gene throughout the plant life cycle, we analyzed mRNA accumulation using QRT-PCR. Higher expression levels for the *SlRBR* gene were generally observed in the flower and closed floral bud tissues, whereas lower expression levels were observed in the unripe fruit tissues. *RBR* is an essential cell cycle regulatory gene that is expressed in the embryo, leaves, root and shoot meristems, and the ovule, including the embryo sac.[24,25,27,41] Similarly, the *RBR* gene of *N. benthamiana* is expressed in all plant tissues, including the roots, stems, open flowers, flower buds, and young and mature leaves.[34] We found that *SlRBR* gene expression was abundant in closed floral bud
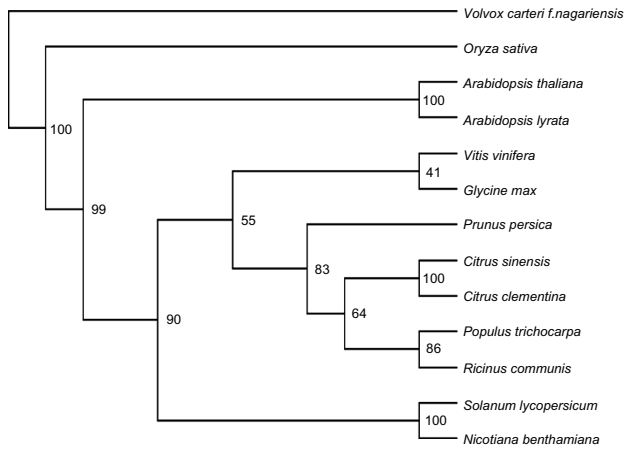
Figure 3. Unrooted phylogenetic tree of the 12 hypothetical RBR protein sequences with the tomato SlRBR predicted protein sequence that was constructed using the maximum parsimony method. *V. carteri f. nagariensis* was used as an outgroup. The numbers on the nodes are bootstrap percentages supporting a given partitioning.

Figure 5. Expression analysis of the tomato PcG gene *SlRBR* in various tomato tissues. The relative transcript levels (*y* axis), which are an average of three replicates, showed high expression levels of the *SlRBR* gene in the closed floral bud tissue (38.23-fold) and a moderate expression level in the flower tissue (22.16-fold), whereas the lowest expression level (7.46-fold) was in the unripe fruits.

tissues, pro-weak in unripe fruit tissues, and between these levels in flower tissues. Our observation of the *SlRBR* gene expression patterns supports the expression of the *SlRBR* gene in reproductive tissues, where *RBR* mRNA was detected in developing ovules and anthers.[42] This pattern suggests that the *SlRBR* gene is more active in the closed floral buds where cell divisions are expected to occur, and cell differentiation is not complete. This observation supports the conclusion that the activity of the *RBR* gene is related to cell division or cell cycle progression.[43,44]

Further studies are needed to identify other PcG genes in tomato and to determine the epigenetic mechanisms and dynamic
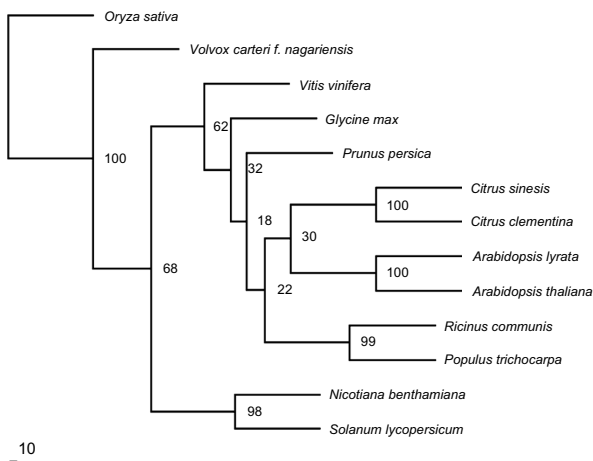
control of cell fate and differentiation in tomato at distinct developmental stages. This will hopefully aid in understanding the role of cell cycle regulators and chromatin complexes that are involved in developmental process in the Solanaceae family.

Experimental Procedures

Sample collection. Plants of the *S. lycopersicum* L., CastleRock variety from the Pacific Seed Company were grown in a greenhouse under standard culture conditions. Various tissues were collected from the plants at different stages, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Primer design. The *A. thaliana* RBR protein available in The *Arabidopsis* Information Resource database (http://www.arabidopsis.org/) under the accession number AT3G12280 was compared with the available tomato proteins in the SGN database. A considerable similarity was found between the *A. thaliana* RBR protein and BAC (C09SLf0089D12.1) and two ESTs (SGN-U570539 and SGN-U582215) in the *S. lycopersicum* L. genome. The predicted *SlRBR* gene in tomato was constructed based on the high similarity between the RBR protein of *A. thaliana* and the mentioned BAC and ESTs using Vector NTI software version 11 (Invitrogen, Grand Island, NY, USA). The forward and reverse primers were designed for the *SlRBR* gene from the predicted exon regions and used to amplify the cDNA clone from tomato flower tissues. The primers that amplify cDNAs are listed in Table 2.

Reverse transcription-PCR. Total RNA was extracted by the method of Chomczynski and Mackey[45] from various tissues with TRI reagent (Sigma, Bloomington, MN, USA), and cDNA was synthesized from the total RNA using Super-Script® III reverse transcriptase (Invitrogen, Grand Island, NY, USA) using oligo(dT) primers. The synthesized cDNA

Figure 4. Unrooted phylogenetic tree of 12 hypothetical RBR protein sequences with the tomato SlRBR predicted protein sequence that was obtained using the maximum likelihood method. *V. carteri f. nagariensis* was used as an outgroup. The numbers on the branches are bootstrap percentages supporting a given partitioning. The scale below the tree measures the distance between the protein sequences where the lengths of branches proportionate to the number of amino acid changes that have occurred along the branch.
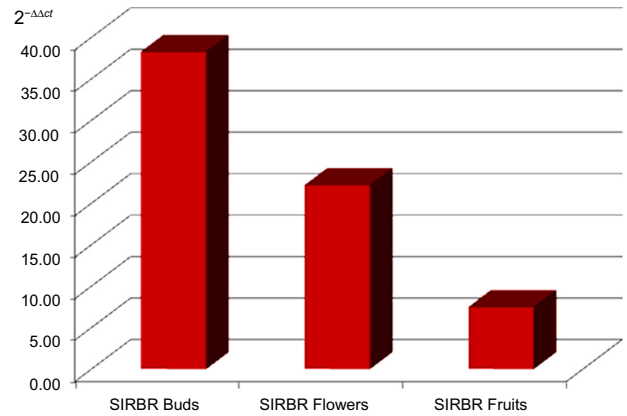
Table 2. The eight primer pairs used to amplify cDNA clones in this study. "F" and "R" in the primer names indicate direction (forward or reverse) of the primer.

PRIMER NAME	PRIMER SEQUENCE
*SlRBR*F01	5'- GCT TAT CAG AGC CCC TCA AA -3'
*SlRBR*R01	5' -GAA GAG AAC GAG CAT CGT TG -3'
*SlRBR*F02	5'- GGT TCA GAA CAT GTG GAT ATC A -3'
*SlRBR*R02	5'- AAG CAT CGG GTT GGT GGC GG -3'
*SlRBR*F03	5'- GGC ATG ATT GAG CGG CTA CA -3'
*SlRBR*R03	5'- TAC CGC TCA TTC AAC ACC CC -3'
*SlRBR*F04	5'- ATG CAC ATT AAC CCA TCT TTG GGA -3'
*SlRBR*R04	5'- AAG AGC AAA GGA TGA TCT GG -3'
*SlRBR*F05	5'- GGG CAA AGA ACC TGG ACA ATA TCG ACG C -3'
*SlRBR*R05	5'- TCC CAA AGA TGG GTT AAT GTG CAT -3'
*SlRBR*F06	5'- GAA CAA GGG TGA ATT GGA TGA GAG G -3'
*SlRBR*R06	5'- TGT AGC CGC TCA ATC ATG CC -3'
*SlRBR*F07	5'- AGA ATG AAG CTA GGA ATT CG -3'
*SlRBR*R07	5'- CCT CTC ATC AAA TTC ACC CTT GTT C -3'
*SlRBR*F08	5'- TGA AGA GAA TGG TTC GGT GG -3'
*SlRBR*R08	5'- ACG ATA AAG ATG ATT CCT CC -3'

was used as a template for the PCR to amplify the tomato *SlRBR* cDNAs with the designed primers using the 2X PCR ready mix (KAPA, Wilmington, MA, USA), 0.4 µM each primer, and 3 µL of cDNA as the template with the following reaction conditions: initial denaturation at 94 °C for 5 minutes and 35 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, and extension at 72 °C for 2 minutes. An extension at 72 °C for 7 minutes was performed after all of the cycles were complete. The cDNA clone was reamplified using Platinum® *Pfx* DNA polymerase (Invitrogen, Grand Island, NY, USA), which produces PCR products with blunt ends for cloning. The obtained blunt-ended fragment was purified by gel extraction using the gel extraction kit from Omega Bio-Tek (Norcross, GA, USA) according to the manufacturer's instruction.

Cloning of the SlRBR cDNA. The cloning vector pBS from Stratagene (La Jolla, CA, USA) was prepared from bacterial cells using the QIAprep Spin Miniprep Kit (Qiagen, USA) according to the manufacturer's instruction, and then digested using *Eco*RV restriction enzyme (Promega, Madison, WI, USA) to produce blunt ends and collected from the gel. The purified cDNA fragments were ligated into the linearized plasmids with T4 DNA ligase (Promega, Madison, WI, USA). The recombinant plasmids were transformed into *E. coli* DH5α host cells that were made competent using the calcium chloride method.[46] The transformation was performed using the heat-shock protocol.[47] The cells were spread onto LB plates (Display Systems Biotech, Vista, CA, USA) containing 100 µg/mL ampicillin prepared in H$_2$O, X-gal

20%, and IPTG 2.4% and incubated overnight at 37 °C. The recombinant plasmids were prepared from the positive clones for use as templates in the sequencing reaction.

5′- and 3′-RACE cDNA amplification. RACE was performed to determine the 5′- and 3′-noncoding region of the tomato PcG gene *SlRBR* using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). The total RNA from the tomato flower tissues was used as template to synthesize first-strand cDNAs for the 5′- and 3′-ends following the manufacturer's instructions. The *SlRBR*-specific primers were designed from the sequence information of the *SlRBR* contig and constructed by overlapping sequenced ESTs; the *SlRBR*-specific primer 1 was used for 5′-end amplification (5′-GAAGCGGCTGAATACATGTTCGCG-3′), and the *SlRBR*-specific primer 2 was used for 3′-end amplification (5′-GCTATTATGCTTGTGTGGGAGA-3′).

Sequence analysis and phylogenetics. The obtained ESTs and generated 5′- and 3′-RACE fragments were used as templates in the sequencing reaction by the dideoxynucleotide method.[48] The sequencing results were assembled using ContigExpress Vector NTI. ORF prediction and translation were performed using the Vector NTI ORF Finder tool. The deduced amino acids were subjected to BLASTp program at the http://www.phytozome.net/database to obtain homologous protein sequences within plants. Hypothetical RBR protein sequences of plant species were aligned by ClustalW 1.83 multiple sequence alignments[49] using the BioEdit software.

To better understand the relationship between the SlRBR protein of tomato and the RBR proteins of other plant species, a phylogenetic analysis was performed using the protein sequences of a hypothetical gene family that were reported in the Phytozome database (code #31805130). The protein sequences for 12 members that are related to the *SlRBR* gene were selected for the phylogenetic analysis. The Phytozome accession numbers for the analyzed protein sequences are as follows: *A. lyrata*, 478590; *A. thaliana*, AT3G12280; clementine *Citrus clementina*, clementine0.9_001232m.g; orange *Citrus sinensis,* orange1.1g001701m.g; soya bean *Glycine max*, Glyma04g36700; rice *O. sativa*, LOC_Os08g42600; poplar *Populus trichocarpa*, POPTR_0001s06870; peach *Prunus persica*, ppa000710m.g; castor oil *Ricinus communis*, 30040.t000007; grape *Vitis vinifera*, GSVIVG01035560001; and *Volvox carteri* f. nagariensis, Vocar20003526 m.g. The *N. benthamiana* ORF sequence is found in the SGN database under accession number SGN-U519343. A phylogenetic analysis was performed using two methods, maximum parsimony[50,51] and maximum likelihood,[52] with the help of the PHYLIP–3.68 package. *V. carteri f. nagariensis* was used as an outgroup. The domains were predicted in the deduced SlRBR protein using NCBI CDD from the website http://www.ncbi.nlm.nih.gov/structure/cdd/.[53–55]

QRT-PCR. The expression level of the tomato PcG gene *SlRBR* was detected using QRT-PCR. First-strand cDNA was synthesized from the total RNA that was extracted from

four tomato tissues, closed floral buds, flowers, fruits, and leaves and was assayed for the *SlRBR* gene expression level. The gene-specific primers (forward: 5′-TGACTGCACCTC-CAGACAAG-3′ and reverse: 5′-GGCCAAGAACCTG-GACAATA-3′) were used. The Actin 2/7 gene was used as a constitutively expressed gene control with the forward primer 5′-GGACTCTGGTGATGGTGTTAG-3′ and reverse primer 5′-CCGTTCAGCAGTAGTGGTG-3′. The QRT-PCR was performed in triplicate as technical replicates. Each reaction (20 µl) consisted of the 2X Hot Start SYBR ready mix (Qiagen, USA), forward and reverse primers (0.125 µM each), 3.5 µL of first-strand cDNA, and 20 µL of H_2O. The reaction conditions were as follows: 50 °C for 2 minutes and 95 °C for 15 minutes for 40 cycles (95 °C for 15 seconds and 60 °C for 30 seconds). The cycles to threshold (Ct) were used to measure the gene expression.[56] The Ct values were calculated (ΔCt) by subtracting the values of the actin 2/7 gene in each replicate. The ΔCt values were calibrated using the *SlVIN3* gene expression in the leaf tissues as a calibrator to obtain ΔΔCt, and then $2^{-\Delta\Delta Ct}$ was calculated.

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{Actin2/7}}$$

$$\Delta\Delta Ct = \Delta Ct \text{ tested tissue} - \Delta Ct \text{ calibrator tissue}$$

$$\text{Ratio} = 2^{-\Delta\Delta Ct}$$

Abbreviations

SlRBR: *Solanum lycopersicum* L. retinoblastoma-related; PcG: Polycomb group; RB: Retinoblastoma; SGN: Solanaceae genomics network database; PCNA: Proliferating cell nuclear antigen; MCM: Minichromosome maintenance protein; ORF: Open reading frame; FlcDNA: Full-length cDNA; EST: Expressed sequence tag; RACE: Rapid amplification of cDNA ends; CDD: Conserved domain database; QRT-PCR: Quantitative real-time PCR.

Conclusion

The molecular machinery that regulates plant flowering and development is conserved throughout the plant kingdom. The *RBR* gene, a negative regulator of cell cycle progression controlling the G1/S transition in plants and animals, plays an important role in the epigenetic regulation of cell cycle genes in the differentiation and development of gametophytes and in reproduction. Here, we isolated and characterized the tomato *SlRBR* gene by cloning and sequencing. The phylogenetic relationships with the homologous proteins that are available in GenBank were investigated. The expression profiling of the *SlRBR* gene reflected its function in cell differentiation and development at different stages of the tomato life cycle.

Acknowledgments

Author Contributions

Conceived and designed the experiments: MTS. Analyzed the data and wrote the first draft of the manuscript: ZMA. Contributed to the writing of the manuscript, agreed with the manuscript results and conclusions, jointly developed the structure and arguments for the paper, and made critical revisions: MTS, ZMA. Both authors reviewed and approved the final manuscript.

Supplementary Data

Supplementary Table 1. The eight primer pairs that were used to amplify the cDNA clones in this study.

REFERENCES

1. SGN (Sol Genomics Network). 2013. Available at http://www.sgn.cornell.edu/index.pl. Retrieved March 8, 2013.
2. The Tomato Genome Consortium. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature*. 2012;485:635–41.
3. Friend SH, Bernards R, Rogelj S, et al. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*. 1986;323:643–46.
4. Gutiérrez C, Ramirez-Parra E, Castellano MM, del Pozo JC. G(1) to S transition: more than a cell cycle engine switch. *Curr Opin Plant Biol*. 2002;5:480–6.
5. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer*. 2008;8:671–82.
6. Nowack MK, Harashima H, Dissmeyer N, et al. Genetic framework of cyclin-dependent kinase function in *Arabidopsis*. *Dev Cell*. 2012;22:1030–40.
7. Gutzat R, Borghi L, Gruissem W. Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. *Trends Plant Sci*. 2012;17(3):139–48.
8. Zhao X, Harashima H, Dissmeyer N, et al. A general G1/S-phase cell-cycle control module in the flowering plant *Arabidopsis thaliana*. *PLoS Genet*. 2012;8:e1002847.
9. Sabelli PA, Liu Y, Dante RY, et al. Control of cell proliferation, endoreduplication, cell size, and cell death by the retinoblastoma-related pathway in maize endosperm. *Proc Natl Acad Sci USA*. 2013;110(19):E1827–36.
10. Brehm A, Kouzarides T. Retinoblastoma protein meets chromatin. *Trends Biochem Sci*. 1999;24:142–5.
11. Magyar Z, Horváth B, Khan S, et al. *Arabidopsis* E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes. *EMBO J*. 2012;31(6):1480–93.
12. Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*. 1998;391:597–601.
13. Sage J, Straight AF. RB's original CIN? *Genes Dev*. 2010;24:1329–33.
14. Harbour JW, Dean DC. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev*. 2000;14:2393–409.
15. Durfee T, Feiler HS, Gruissem W. Retinoblastoma-related proteins in plants: homologues or orthologues of their metazoan counterparts? *Plant Mol Biol*. 2000;43:635–42.
16. Boniotti MB, Gutiérrez C. A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in *Arabidopsis*, a CDKA/cyclin D complex. *Plant J*. 2001;28:341–50.
17. Miskolczi P, Lendvai À, Horváth GV, Pettkó-Szandtner A, Dudits D. Conserved functions of retinoblastoma proteins: from purple retina to green plant cells. *Plant Sci*. 2007;172:671–83.
18. Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S, Dean DC. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature*. 1995;375:812–5.
19. Dewitte W, Riou-Khamlichi C, Scofield S, et al. Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. *Plant Cell*. 2003;15:79–92.
20. De Jager SM, Murray JAH. Retinoblastoma proteins in plants. *Plant Mol Biol*. 1999;41:295–9.
21. Sabelli PA, Dante RA, Leiva-Neto JT, Jung R, Gordon-Kamm WJ, Larkins BA. RBR3, a member of the retinoblastoma-related family from maize, is regulated by the RBR1/E2F pathway. *Proc Natl Acad Sci USA*. 2005;102:13005–12.

22. Sabelli PA, Larkins BA. Grasses like mammals? Redundancy and compensatory regulation within the retinoblastoma protein family. *Cell Cycle*. 2006;5:352–5.

23. Lendvai A, Pettkó-Szandtner A, Csordás-Tóth E, et al. Dicot and monocot plants differ in retinoblastoma-related protein subfamilies. *J Exp Bot*. 2007;58:1663–75.

24. Ebel C, Mariconti L, Gruissem W. Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. *Nature*. 2004;429:776–80.

25. Wildwater M, Campilho A, Perez-Perez JM, et al. The RETINOBLAS-TOMA-RELATED gene regulates stem cell maintenance in *Arabidopsis* roots. *Cell*. 2005;123:1337–49.

26. Desvoyes B, Ramirez-Parra E, Xie Q, Chua NH, Gutiérrez C. Cell type-specific role of the retinoblastoma/E2F pathway during *Arabidopsis* leaf development. *Plant Physiol*. 2006;140:67–80.

27. Johnston AJ, Matveeva E, Kirioukhova O, Grossniklaus U, Gruissem W. A dynamic reciprocal RBR-PRC2 regulatory circuit controls *Arabidopsis* gametophyte development. *Curr Biol*. 2008;18:1680–6.

28. Johnston AJ, Gruissem W. Gametophyte differentiation and imprinting control in plants: cross talk between RBR and chromatin. *Commun Integr Biol*. 2009;2:144–6.

29. Chen Z, Hafidh S, Poh SH, Twell D, Berger F. Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the retinoblastoma protein. *Proc Natl Acad Sci U S A*. 2009;106:7257–62.

30. Borghi L, Gutzat R, Fűtterer J, Laizet Y, Hennig L, Gruissem W. *Arabidopsis* RETINOBLASTOMA-RELATED is required for stem cell maintenance, cell differentiation, and lateral organ production. *Plant Cell*. 2010;22:1792–811.

31. Chen Z, Higgins JD, Hui JT, Li J, Franklin FC, Berger F. Retinoblastoma protein is essential for early meiotic events in *Arabidopsis*. *EMBO J*. 2011;30:744–55.

32. AGI (Arabidopsis Genome Initiative). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*. 2000;408(6814):796–815

33. Schubert D, Clarenz O, Goodrich J. Epigenetic control of plant development by Polycomb-group proteins. *Curr Opin Plant Biol*. 2005;8:553–61.

34. Park JA, Ahn JW, Kim YK, et al. Retinoblastoma protein regulates cell proliferation, differentiation, and endoreduplication in plants. *Plant J*. 2005;42(2):153–63.

35. Nakagami H, Sekine M, Murakami H, Shinmyo A. Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D in vitro. *Plant J*. 1999;18:243–52.

36. Hallmann A. Retinoblastoma-related proteins in lower eukaryotes. *Commun Integr Biol*. 2009;2:538–44.

37. Harashima H, Dissmeyer N, Schnittger A. Cell cycle control across the eukaryotic kingdom. *Trends Cell Biol*. 2013;23(7):345–56.

38. Nematollahi G, Kianianmomeni A, Hallmann A. Quantitative analysis of cell-type specific gene expression in the green alga *Volvox carteri*. *BMC Genomics*. 2006;7:321.

39. Kianianmomeni A, Nematollahi G, Hallmann A. A gender-specific retinoblastoma-related protein in *Volvox carteri* implies a role for the retinoblastoma protein family in sexual development. *Plant Cell*. 2008;20:2399–419.

40. Umen JG, Goodenough UW. Control of cell division by a retinoblastoma protein homolog in Chlamydomonas. *Genes Dev*. 2001;15:1652–61.

41. Ingouff M, Hamamura Y, Gourgues M, Higashiyama T, Berger F. Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. *Curr Biol*. 2007;17:1032–7.

42. Johnston AJ, Kirioukhova O, Barrell PJ, et al. Dosage-sensitive function of retinoblastoma related and convergent epigenetic control are required during the *Arabidopsis* life cycle. *PLoS Genet*. 2010;6:e1000988.

43. Jiang Z, Zacksenhaus E, Gallie BL, Philips RA. The retinoblastoma gene family is differentially expressed during embryogenesis. *Oncogene*. 1997;14:1789–97.

44. Abraham E, Miskolczi P, Ayaydin F, et al. Immunodetection of retinoblastoma-related protein and its phosphorylated form in interphase and mitotic alfalfa cells. *J Exp Bot*. 2011;62:2155–68.

45. Chomczynski P, Mackey K. Short technical report. Modification of the TRIZOL reagent procedure for isolation of RNA from Polysaccharide-and proteoglycan-rich sources. *Biotechniques*. 1995;19(6):942–5.

46. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001:132–50.

47. Froger A, Hall JE. Transformation of plasmid DNA into *E. coli* using the heat shock method. *J Vis Exp*. 2007;6:253.

48. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74(12):5463–7.

49. Thompson JD, Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 1997;25:4876–82.

50. Eck RV, Dayhoff MO. *Atlas of Protein Sequence and Structure* 1966. Silver Spring, MD: National Biomedical Research Foundation; 1966.

51. Fitch WM. Toward defining the course of evolution: minimum change for a specified tree topology. *Syst Zool*. 1971;20:406–16.

52. Felsenstein J, Churchill GA. A hidden Markov model approach to variation among sites in rate of evolution. *Mol Biol Evol*. 1996;13:93–104.

53. Marchler-Bauer A, Bryant SH. CD-search: protein domain annotations on the fly. *Nucleic Acids Res*. 2004;32(W):327–31.

54. Marchler-Bauer A, Anderson JB, Chitsaz F, et al. CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res*. 2009;37(D):205–10.

55. Marchler-Bauer A, Lu S, Anderson JB, et al. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res*. 2011;39(D):225–9.

56. Livak KJ, Schmittgen TD. Analysis of relative gene expression using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*. 2001;25:402–8.