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

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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 *SIRBR* 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 *SIRBR* cDNAs were cloned into the pBS plasmid and sequenced. Both 5′ and 3′-RACE were generated and sequenced. FlcDNA of the *SIRBR* 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 *SIRBR* 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

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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 animals³ 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
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. Furthermore, RB plays a critical role in chromosome condensation, centromeric function, and chromosome stability.

The RB protein contains a number of functional domains, two of which, denoted as A and B, are conserved in humans and plants. 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. 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.

Arabidopsis thaliana contains only a single RBR gene, whereas RB has two more relatives (p107 and p130) in animals. Although many plants appear to possess only one RBR gene, maize and related cereals have at least two distinct types, RBR1 and RBR3.

Plant RBR proteins control not only cell cycle arrest/progression but also development and cellular differentiation in the endosperm, leaf, and root. 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. The loss of the RB protein also prevents or delays cell determination during plant male gametogenesis. 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 the homeostasis of stem cells and organ production in every stem cell niche.

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. 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.

Sabelli et al. 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 programmed cell death.

RESULTS

To isolate the cDNA encoding the SIRBR gene, PCR reactions were performed using a primer set targeting the SIRBR 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 EcoRV restriction enzyme, and transformed into Escherichia coli DH5α. The SIRBR 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 SIRBR cDNA contig, 5′- and 3′-RACE gene-specific primers were designed and used to amplify the ends of the SIRBR 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 SIRBR flcDNA contig. Computer analysis using the BLASTn algorithm confirmed that the obtained sequence corresponded to the SIRBR gene.

The constructed SIRBR 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 SIRBR 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.
Cloning and expression profiling of RBR from tomato

**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^−ΔΔCT 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

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**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     | Intron01   | 659     |
| Exon01   | 1 . . 89   | 89      | Intron02   | 100     |
| Exon02   | 90 . . 197 | 108     | Intron03   | 82      |
| Exon03   | 198 . . 338| 141     | Intron04   | 240     |
| Exon04   | 339 . . 435| 97      | Intron05   | 119     |
| Exon05   | 436 . . 483| 48      | Intron06   | 503     |
| Exon06   | 484 . . 680| 197     | Intron07   | 123     |
| Exon07   | 681 . . 748| 68      | Intron08   | 73      |
| Exon08   | 749 . . 923| 175     | Intron09   | 93      |
| Exon09   | 924 . . 1,118| 195    | Intron10   | 129     |
| Exon10   | 1,119 . . 2,004| 886   | Intron11   | 99      |
| Exon11   | 2,005 . . 2,164| 160   | Intron12   | 443     |
| Exon12   | 2,165 . . 2,237| 73    | Intron13   | 86      |
| Exon13   | 2,238 . . 2,414| 177   | Intron14   | 85      |
| Exon14   | 2,415 . . 2,535| 121   | Intron15   | 128     |
| Exon15   | 2,536 . . 2,674| 139   | Intron16   | 151     |
| Exon16   | 2,675 . . 2,786| 112   | Intron17   | 74      |
| Exon17   | 2,787 . . 2,903| 117   |            |         |
| Exon18   | 2,904 . . 3,054| 151   |            |         |
| 3'UTR    | 3,055 . . 3,414| 360   |            |         |
The 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. 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. 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* and *N. tabacum*, 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 that is primarily known as a negative regulator of cell cycle progression, controlling the G1/S transition in plants and animals. 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. 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

![Figure 1](https://example.com/figure1.jpg)  
**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.
two cereal RBR proteins indicates gene duplication, which is prevalent in plants and may lead to evolutionary innovation.\textsuperscript{23}

The \textit{RBR} gene was not only identified in animals (Metazoa) but also in plants (monocots, dicots, lycophytes, mosses, and green algae), Heteroconota (oomycetes, diatoms, and brown algae), Alveolata, red algae (Rhodophyta), Heterolobosphaera, Haptophyta, and Amoebozoa. Only fungi appear to be an exception because only the most primitive phylum retained an \textit{RBR}, whereas the more advanced phyla evolved a functionally similar key regulator instead.\textsuperscript{36,37} \textit{RBR} genes have been identified not only in the multicellular species \textit{V. carteri}\textsuperscript{38,39} but also in the unicellular organism \textit{Chlamydomonas reinhardtii}.\textsuperscript{40} This distribution of \textit{RBR} genes suggests that \textit{RBR} genes have an ancient origin. Within the bryophytes, the nonvascular embryophyte \textit{Physcomitrella patens} has at least three \textit{RBR} genes in its genome (\textit{PpRBL1}, \textit{PpRBL2}, and \textit{PpRBR}).\textsuperscript{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 \textit{SlRBR} gene were generally observed in the flower and closed floral bud tissues, whereas lower expression levels were observed in the unripe fruit tissues. \textit{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.\textsuperscript{24,25,27,41} Similarly, the \textit{RBR} gene of \textit{N. benthamiana} is expressed in all plant tissues, including the roots, stems, open flowers, flower buds, and young and mature leaves.\textsuperscript{34} We found that \textit{SI-RBR} gene expression was abundant in closed floral bud

Figure 2. Multiple sequence alignments of the conserved domains sequences of the SIRBR 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 SIRBR protein using NCBI CDD. (A) Conserved domains of the SIRBR protein; (B) RB A domain from amino acid 426 to 616; (C) CYCLIN domain from amino acid 738 to 868.
tissues, pro-weak in unripe fruit tissues, and between these levels in flower tissues. Our observation of the **SIRBR** gene expression patterns supports the expression of the **SIRBR** gene in reproductive tissues, where **RBR** mRNA was detected in developing ovules and anthers. This pattern suggests that the **SIRBR** 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.

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 **SIRBR** 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 **SIRBR** 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 from various tissues with TRI reagent (Sigma, Bloomington, MN, USA), and cDNA was synthesized from the total RNA using SuperScript® III reverse transcriptase (Invitrogen, Grand Island, NY, USA) using oligo(dT) primers. The synthesized cDNA...
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 EcoRV 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. The transformation was performed using the heat-shock protocol. The cells were spread onto LB plates (Display Systems Biotech, Vista, CA, USA) containing 100 µg/mL ampicillin prepared in H₂O, 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’-GAAGCGGCTGAATACATGTTGCGG-3’), and the SlRBR-specific primer 2 was used for 3’-end amplification (5’-GCTATATGCTTGTGGGAGA-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 deoxyribonucleotide method. 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.phytozone.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 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 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; orange Citrus sinensis, orange1.1g001701m; soybean Glycine max, Glyma04g36700; rice O. sativa, LOC_Os08g42600; poplar Populus trichocarpa, POPTR_0001s06870; peach Prunus persica, ppa000710m; castor oil Ricinus communis, 30040.t000007; grape Vitis vinifera, GSVIVG0103560001; and V. carteri f. nagariensis, Vocr20003526 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 and maximum likelihood, with the help of the PHYML-3.68 package. V. carteri f. nagariensis was used as an outgroup. The domains were predicted in the deposited SlRBR protein using NCBI CDD from the website http://www.ncbi.nlm.nih.gov/structure/cdd/.

**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

| 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 |
|---|---|---|
| SIRBRF01 | 5‘-GCT TAT CAG AGC CCC TCA AA | 3‘ |
| SIRBRF02 | 5‘-GAA GAG AAC GAG CAT CTT TG | 3‘ |
| SIRBRF03 | 5‘-GGC ATG ATT GAG CGG CTA CA | 3‘ |
| SIRBRF04 | 5‘-ATG CAC ATT ACAA CCT TTG GGA | 3‘ |
| SIRBRF05 | 5‘-GGG CAA AGA ACC TGG ATG ATA TCG ACG C | 3‘ |
| SIRBRF06 | 5‘-TCC CAA AGT TGG GTT AAT GTG CAT | 3‘ |
| SIRBRF07 | 5‘-TGT AGC CGC TCA ATC ATG CC | 3‘ |
| SIRBRF08 | 5‘-AGA ATG AAG ATG ATT CCT TCT TG | 3‘ |
| SIRBRF09 | 5‘-GCC ATG ATT GAG CGG CTA CA | 3‘ |
| SIRBRF10 | 5‘-AAC AGC TAA AAG ATG ATT CCT CTC CC | 3‘ |
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'-TGAAGGCTGACCTC-CAGACAAG-3' and reverse: 5'-GCCAAAGACCTG-GACAATA-3') were used. The Actin 2/7 gene was used as a constitutively expressed gene control with the forward primer 5'-GGACTGTGGTATGTTGTTAG-3' and reverse primer 5'-CCAAGCAGTACTGAGGTTG-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 H2O. 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. 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^(-ΔΔCt) was calculated.

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.

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