The Mulberry SPL Gene Family and the Response of MnSPL7 to Silkworm Herbivory through Activating the Transcription of MnTT2L2 in the Catechin Biosynthesis Pathway

Hongshun Li, Bi Ma, Yiwei Luo, Wuqi Wei, Jianglian Yuan, Changxin Zhai and Ningjia He *

State Key Laboratory of Silkworm Genome Biology, Southwest University, Beibei, Chongqing 400715, China; hong_shun_li@163.com (H.L.); mbzlz@swu.edu.cn (B.M.); luoyiweil28@swu.edu.cn (Y.L.); swuwuqi@email.swu.edu.cn (W.W.); yuanjiangl@swu.edu.cn (J.Y.); zhaichangxin1996@163.com (C.Z.)

* Correspondence: hejia@swu.edu.cn; Tel.: +86-23-6825-0797; Fax: +86-23-6825-1128

Abstract: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes, as unique plant transcription factors, play important roles in plant developmental regulation and stress response adaptation. Although mulberry is a commercially valuable tree species, there have been few systematic studies on SPL genes. In this work, we identified 15 full-length SPL genes in the mulberry genome, which were distributed on 4 Morus notabilis chromosomes. Phylogenetic analysis clustered the SPL genes from five plants (Malus × domestica Borkh, Populus trichocarpa, M. notabilis, Arabidopsis thaliana, and Oryza sativa) into five groups. Two zinc fingers (Zn1 and Zn2) were found in the conserved SBP domain in all of the MnSPLs. Comparative analyses of gene structures and conserved motifs revealed the conservation of MnSPLs within a group, whereas there were significant structure differences among groups. Gene quantitative analysis showed that the expression of MnSPLs had tissue specificity, and MnSPLs had much higher expression levels in older mulberry leaves. Furthermore, transcriptome data showed that the expression levels of MnSPL7 and MnSPL14 were significantly increased under silkworm herbivory. Molecular experiments revealed that MnSPL7 responded to herbivory treatment through promoting the transcription of MnTT2L2 and further upregulating the expression levels of catechin synthesis genes (F3H, DFR, and LAR).

Keywords: mulberry; SPLs; phylogenetic analysis; silkworm herbivory; MnSPL7/MnTT2L2 module

1. Introduction

SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes encode plant-specific transcription factors and are typified by highly conserved SQUAMOSA promoter-binding (SBP) domains. SBP domains contain 75–79 amino acid residues with two Zn²⁺–binding sites (Cys–Cys–His–Cys and Cys–Cys–Cys–His) and a nuclear location signal (NLS) which is involved in DNA binding and nuclear localization [1,2]. SPL genes were first identified in the floral organs of Antirrhinum majus while screening the nuclear protein interactions within the promoter of the SQUAMOSA gene by electrophoretic mobility shift assay (EMSA) [3].

Importantly, SPL genes are distributed in a vast majority of green plants, including single-celled algae, mosses, gymnosperms, and angiosperms [4–6]. To date, since the completion of genome sequencing and the thorough development of the functional genome, 17, 19, 56, 16, and 14 SPL genes have been systematically identified in A. thaliana, O. sativa, Triticum aestivum, Paeonia suffruticosa, and Paeonia suffruticosa, respectively [7–11].

As unique plant transcription factors, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes play important roles in plant vegetative phase transition [12–14], flowering regulation [15,16], leaf morphogenesis [17], and root development [18–20]. Moreover, SPL genes can respond to various biotic and abiotic stresses by regulating the abundances of anthocyanin [21,22], abscisic acid [23], and jasmonate [24]. SPL genes have a higher
expression level in relatively more mature tissues. In Antirrhinum, SPL1 and SPL2 were detected in inflorescences but not in the tissues of juvenile plants [25]. In Arabidopsis, the expression profiles of SPL genes increased as plants aged and were regulated by sequence-conserved microRNAs (miR156/157) [12,15,26,27]. AtSPL9 and AtSPL10, which were directly repressed by miR156/157, controlled the juvenile-to-adult phase transition [12]. AtSPL3/4/5 were also directly suppressed by miR156/157 and positively regulated floral meristem identity and influenced the trichome distribution [16,28]. AtSPL9 and AtSPL15 affected leaf shape [12], while AtSPL3, AtSPL9, and AtSPL10 were involved in the repression of lateral root growth, and AtSPL10 played a dominant role in the primary root meristem activity regulation [18–20]. In addition, the expression levels of SPL genes fluctuated significantly under adverse conditions including salt stress, drought stress, heat stress, nitrogen starvation, and viral defense [29–31]. The overexpression of miR156 repressed the expression of SPL genes and further increased anthocyanin accumulation in the stems of Arabidopsis and the stem apex of poplar. The increased expression of miR156 also improved the accumulation of flavonoids in poplar [21,22,32]. In Arabidopsis, high levels of SPL9 under salt and drought stress treatment suppressed anthocyanin accumulation by directly repressing the expression of anthocyanin biosynthetic genes, such as ANS, F3′H, DFR, and UGT775C1, through interfering with the integrity of the MYB-bHLH-WD40 transcriptional activation complex [21,22].

Mulberry, also known as Morus alba, is widely distrusted around the world [33]. In addition to its use for breeding silkworms, mulberry is also a traditional fruit tree and Chinese herbal medicine that has significant economic value in food and medicine production [34–36]. Abundant flavonoids have been detected in mulberry leaves and fruits, which indicates the medicinal properties and high stress resistance of mulberry [37,38]. Research has shown that flavonoid (anthocyanins, proanthocyanidins, flavones, and flavonols) accumulation is regulated by the MYB-bHLH-WD40 ternary activation complex in plants [39]. In mulberry, the TT2L1 and TT2L2 proteins interact with bHLH3 or GL3 to promote proanthocyanidin (the multimer of catechin or epicatechin) accumulation [40]. Furthermore, mulberry genome sequencing has been completed [33,41], and our previous study also verified that nine mulberry SPL genes are directly regulated by miR156 [42]. However, to date, systematic research on mulberry SPL genes is still lacking; consequently, the specific molecular biological functions of SBP-box transcription factor genes in development and stress responses remain unclear.

In this work, we identified 15 SPL genes in the mulberry genome and named these genes according to their evolutionary relationship with AtSPL genes. The MnSPL gene family was characterized through comprehensive analyses of gene structures, phylogenetic relationships, chromosomal locations, conserved motifs, and expression patterns. A dual-luciferase assay was carried out to verify the interaction between the MnSPL7 gene and catechin synthesis-regulated genes (TT2L2) under silkworm herbivory in mulberry. In summary, our works have provided basic information for elucidating the molecular biological functions of SPL genes in mulberry.

2. Results

2.1. Identification and Analysis of the SPL Gene Family in Mulberry

A total of 15 full-length SPL genes were identified in the mulberry genome (MorusDB, https://morus.swu.edu.cn/, accessed on 3 March 2020) [43] using BLASTAN and HMMER with SPL genes from A. thaliana as the query sequences (Table 1). Mapping SPLs to the M. notabilis genome showed that 15 SPLs were unevenly distributed on 4 chromosomes, with 5 on Chr1 (SPL1, SPL3, SPL4, SPL8, and SPL10) and Chr2 (SPL2, SPL6, SPL7, SPL14, and SPL16B), 3 on Chr4 (SPL5, SPL15, and SPL16A), and 2 on Chr6 (SPL12 and SPL13) (Figure 1).
Table 1. Gene ID and gene structures of SPLs in mulberry. The gene ID of MnSPLs were obtained from the Morus Genome Database (MorusDB) (https://morus.swu.edu.cn/, accessed on 3 March 2020).

| Gene Name | Gene ID    | mRNA Length | CDS Length | Exon Number | Strand | miR156 Target Site                  |
|-----------|------------|-------------|------------|-------------|--------|-------------------------------------|
| MnSPL1    | Morus013868| 5282        | 3081       | 10          | -      | GUGUCUCUCUCUCUCUCUCUCUGUCAA          |
| MnSPL2    | Morus015493| 2478        | 1503       | 4           | -      | /                                   |
| MnSPL3    | Morus009607| 10,219      | 480        | 2           | +      | GUGCUCUCUCUCUCUCUCUCUCUGUCAA        |
| MnSPL4    | Morus014488| 1855        | 513        | 2           | -      | UUGCUCUCUCUCUCUCUCUCUCUGUCAA        |
| MnSPL5    | Morus010322| 1381        | 630        | 2           | -      | /                                   |
| MnSPL6    | Morus026457| 3240        | 1491       | 3           | -      | GUGCUCUCUCUCUCUCUCUCUCUCUGUCAA      |
| MnSPL7    | Morus011281| 5842        | 2535       | 12          | +      | GAUGUCUCUUUCUCUCUCUCUGAG             |
| MnSPL8    | Morus021788| 2020        | 1095       | 3           | -      | /                                   |
| MnSPL10   | Morus021787| 2565        | 1302       | 3           | +      | /                                   |
| MnSPL12   | Morus025152| 6389        | 3072       | 10          | -      | /                                   |
| MnSPL13   | Morus010123| 2469        | 1200       | 3           | -      | GUGCUCUCUCUCUCUCUCUCUCUCUGUCAA      |
| MnSPL14   | Morus024784| 5168        | 3129       | 10          | -      | UUGCUCACGUCUCUCUCUCUCUGA             |
| MnSPL15   | Morus018032| 2947        | 1110       | 3           | +      | GUGCUCUCUCUCUCUCUCUCUCUCUCUCAC      |
| MnSPL16A  | Morus010792| 1816        | 1239       | 3           | -      | GUGCUCUCUCUCUCUCUCUCUCUCUGUCAA      |
| MnSPL16B  | Morus017456| 1515        | 1041       | 3           | -      | GUGCUCUCUCUCUCUCUCUCUCUCUGUCAA      |

Figure 1. Distribution of SPL genes in the mulberry genome. The scale is presented on the left.

To investigate the evolutionary relationships of SPL genes in plants, we collected a dataset of 104 putative SPL protein sequences, including 18 from rice, 16 from Arabidopsis, 28 from poplar, 27 from apple, and 15 from mulberry, for a phylogenetic analysis with a neighbor-joining (NJ) phylogenetic tree. The result of phylogenetic analysis showed that these 104 SPL genes were relatively evenly clustered into 5 groups (G1–G5) and each group contained at least 1 SPL protein from these 5 species. However, MnSPLs had proximate relationships to MdSPLs and outermost relationships to SPLs from rice (Figure 2). These results suggest that plant SPL genes may have originated from common ancestral genes, but some SPL genes may have been differentiated separately between monocots and eudicots. Nine MnSPL genes have been verified as the target genes of miR156 in mulberry [42]. Here,
we found that most of the miR156-target MnSPLs clustered with miR156-target AtSPLs, except for MnSPL7 and MnSPL14 (Figure 2).

Figure 2. Phylogenetic tree of the SPL family based on the amino acid sequences of SBP domains. Posterior probability values of nodes are shown near the nodes. Different shapes represent different plant species (Md: Malus × domestica Borkh; Pt: P. trichocarpa; Mn: M. notabilis; At: A. thaliana; Os: O. sativa).

Gene structure analysis revealed that all the MnSPL genes contained the SBP domain, and the number of exons in 15 MnSPL genes varied from 2 to 12. The number of MnSPLs with 2, 3, 4, 10, and 12 exons were 3 (MnSPL3, MnSPL4, and MnSPL5), 7 (MnSPL6, MnSPL8, MnSPL10, MnSPL13, MnSPL15, MnSPL16A, and MnSPL16B), 1 (MnSPL2), 3 (MnSPL1, MnSPL12, and MnSPL14), and 1 (MnSPL7), respectively. There were 9 miR156-target SPL genes in mulberry, 7 of which had miR156 recognition sites in the exon region in addition to 1 in the intron region and 1 in the 5′-UTR region (Figure 3a and Table 1). The SBP domains in MnSPLs had 75 amino acid residues. Sequence analysis of the SBP domains in MnSPLs revealed that the conserved zinc binding sites, the zinc fingers Zn1 and Zn2, also existed in the SBP domains. In addition to zinc binding sites, the SBP domains also contained a conserved NLS in the C-terminus of the SBP domains (Figure 3b). Furthermore, other conserved motifs were searched using the online tool MEME with the default parameters. The results showed that 20 conserved motifs were identified in 15 MnSPLs, and genes in the same group had highly similar motif distribution (Figure 4).
2.2. Temporal-Spatial Expression Profile Analysis of SPL Genes in Mulberry

To uncover the potential biological functions of MnSPL genes, we gathered the read data of these genes in five different mulberry tissues (roots, branch bark, winter buds, male flowers, and leaves) from the MorusDB (https://morus.swu.edu.cn/, accessed on
Statistical results showed that these 15 MnSPL genes had tissue-preferential expression in 5 samples. Three SPL genes (MnSPL1, MnSPL13, and MnSPL14) were significantly highly expressed in roots. MnSPL12 was expressed mainly in branch bark, MnSPL4, and MnSPL5 were expressed mainly in leaf tissues, 6 genes (MnSPL3, MnSPL6, MnSPL8, MnSPL10, MnSPL15, and MnSPL16A) had significantly high expression levels in winter buds, and 3 genes (MnSPL2, MnSPL7, and MnSPL16B) were prominently highly expressed in male flowers (Figure 5 and Table S1). Then, we tested the expression profiles of MnSPL genes in the mulberry root, bark, and leaf tissues at the juvenile and mature phases. We found that all the MnSPL genes had higher expression levels in mature leaf tissue than in juvenile leaf tissue, except for MnSPL2. In mulberry roots, five SPL genes (MnSPL1, MnSPL4, MnSPL7, MnSPL14, and MnSPL16A) showed no difference in expression between juvenile and mature samples. The expression level of three genes (MnSPL6, MnSPL12, and MnSPL16B) decreased with age, and two genes (MnSPL13 and MnSPL15) had a higher expression level in the older root tissue. Most of the MnSPL genes showed no difference in expression between juvenile and mature mulberry bark, except for two genes (MnSPL4 and MnSPL13) that were more highly expressed in mature bark and two genes (MnSPL16A and MnSPL16B) that had lower expression in mature bark (Figure 6). The tissue-specificity expression profile implied that MnSPL genes had functional diversity. The expression levels of MnSPLs were increased with age, which suggested that the biological functions of MnSPLs were regulated by age in mulberry leaves.

**Figure 5.** Tissue-specific expression patterns of MnSPL genes. Transcriptome data were obtained from the Morus Genome Database (MorusDB) (https://morus.swu.edu.cn/, accessed on 3 March 2020). Blue indicates that the expression levels of SPL genes are low, while red indicates that the levels are high.

### 2.3. Silkworm Herbivory Influenced the Expression Profile of MnSPL Genes

Plants balance their energy assignment between development and stress responses to ensure their survival using the miR156-SPL module [24,44]. The co-evolution between mulberry and silkworm was influenced by artificial selection for thousands of years. We were curious to learn whether mulberry SPLs responded to silkworm herbivory. Transcriptome data showed that the expression levels of SPL7 and SPL14 significantly increased, and five SPL genes (SPL2, SPL12, SPL16A, SPL5, and SPL15) prominently decreased under herbivory in wild mulberry leaves (Chuansang, *M. notabilis*) (Figure 7c and Table S3). The results of RT-qPCR showed that except for SPL12 and SPL14, all SPL genes were significantly less expressed under herbivory treatment in cultivated mulberry leaves (*Guisangyou 62, M. atropurpurea cv. Guisangyou 62*) (Figure 7d and Table S3). Further quantitative analysis showed that the expression levels of miR156 in both cultivated and wild mulberry leaves significantly increased after herbivory treatment (Figure 7a,b). These results indicated that
the miR156-SPL module responded to silkworm herbivory in both cultivated and wild mulberry; however, SPL7 and SPL14 were more highly expressed, independent of the miR156-SPL module, in the wild mulberry leaves under herbivory.

Figure 6. The expression profiles of MnSPL genes in the juvenile and mature phases of 3 mulberry tissues. (a) Leaves, J-ML: mature leaves at the juvenile phase of mulberry, M-ML: mature leaves at the mature phase of mulberry. (b) Roots, J-R: roots at the juvenile phase of mulberry, M-R: roots at the mature phase of mulberry. (c) Bark, J-B: bark at the juvenile phase of mulberry, M-B: bark at the mature phase of mulberry. Values represent the mean ± SD of 3 biological replicates and were statistically analyzed (independent samples t-test): * p < 0.05.
Figure 7. The expression pattern analysis of SPL genes in mulberry leaves under herbivory treatment. (a) Mulberry leaves with 1 h of herbivory treatment. CS-CK: the control group of Chuansang. CS-H1h: Chuansang leaves after herbivory treatment for 1 h. GY62-CK: the control group of Guisangyou 62. GY62-H1h: Guisangyou 62 leaves after herbivory for 1 h. (b) The expression profiles of mulberry miR156 after herbivory treatment. Mn: M. notabilis. Ma: M. alba. (c) The expression profiles of SPL genes in mulberry (M. notabilis) leaves after herbivory treatment. (d) The expression profiles of SPL genes in mulberry (M. alba) leaves after herbivory treatment. H_L: Leaves under herbivory treatment. CK_L: Control mulberry leaves with no herbivory treatment.

In identify the molecular mechanism of SPL in mulberry leaves under silkworm herbivory treatment, we analyzed the transcriptome data of wild type and herbivory treatment leaves from Chuansang. The results revealed that genes (TT2L2, bHLH, TTG1, F3’H, DFR, and LAR) associated with catechin (the monomeric procyanidine) synthesis were significantly more highly expressed in leaves under herbivory (Figure 8a,b). However, the results of RT-qPCR showed that the expression levels of F3’H, DFR, and LAR in Guisangyou 62 leaves decreased under herbivory treatment (Figure 8c). Promoter analysis of genes (TT2L2, bHLH, TTG1, F3’H, DFR, and LAR) associated with catechin synthesis revealed that there was a predicted ggaCGTACa cis-acting element on the promoter of the TT2L2 gene in M. notabilis, which could be recognized by the SBP domain of SPL genes (data not shown). The dual-luciferase assay verified that SPL7 could combine with the promoter of TT2L2 and promoted its transcription (Figure 9). Taken together, these results suggested that SPL7 responded to herbivory treatment through promoting the transcription of TT2L2 in wild mulberry (Chuansang), and this interaction was not detected in cultivated mulberry (Guisangyou 62).
Figure 8. The expression profiles of genes involved in the mulberry flavonoid synthesis pathway after silkworm herbivory treatment. (a) The expression profiles of genes involved in the Chuansang flavonoid synthesis pathway. (b) The expression trend of TT2L2 and LAR in Chuansang. (c) The expression pattern of genes involved in the Guisangyou 62 flavonoid synthesis pathway.

Figure 9. Dual-luciferase assays identified the interaction between SPL genes and the promoter of MnTT2L2. CK: Control group. OE: overexpressed mulberry SPL genes. Values represent the mean ± SD of 3 biological replicates and were statistically analyzed (independent samples t-test): * \( p < 0.05 \).

3. Discussion

3.1. The Evolutionary Conservation and Functional Diversity of SPL Genes in Mulberry

Based on phylogenetic analysis, 107 SPL proteins from *Malus × domestica* Borkh, *P. trichocarpa*, *M. notabilis*, *A. thaliana*, and *O. sativa* were clustered into 5 groups. Each group contained at least one sequence from all five species, even though there were visible
differences in gene number among these five plants, which indicated that the ancestral gene of SPLs already existed before the speciation between monocot and dicot plants (Figure 2). Moreover, the NJ phylogenetic tree showed that most mulberry SPLs were classed together with MdSPLs, and mulberry SPLs had the farthest genetic distance to OsSPLs (Figure 2). SPL genes encoded SPL proteins with a highly conserved DNA-binding domain named the SBP domain [1]. Systematic comparative analysis of mulberry SPL genes revealed that the highly conserved SBP domain had about 75 amino acid residues (Figures 3 and 4), which indicated the evolutionary conservation of mulberry SPL genes.

In Arabidopsis, SPL2, SPL9, SPL10, SPL11, SPL13 and SPL15 exhibit functional redundancy in both the vegetative phase transition and the vegetative-to-reproductive transition [26]. This study found that mulberry SPL2, SPL10, and SPL15 were clustered into group 5 with AtSPL2, AtSPL9, AtSPL10, AtSPL11 and AtSPL15 in the phylogenetic tree (Figure 2) and were significantly expressed in the reproductive organs (winter buds and male flowers) in mulberry (Figure 5). Based on the above data, we inferred that the SPL2, SPL10 and SPL15 genes also contributed to reproductive growth in mulberry. The tissue differential expression profiles of mulberry SPL genes among winter buds, male flowers, roots, branch bark, and leaves were also detected in this work, just as PtSPLs [7], AtSPLs [26], and OsSPLs [45], which implied the functional diversity of SPL genes in mulberry (Figure 5).

The expression levels of AtSPL genes increased with Arabidopsis aging [12,15]. Similarly, we determined that SPL genes had a higher expression level in the older mulberry leaves (Figure 6a), which indicated that the function of mulberry SPL genes was also influenced by age.

3.2. The MnSPL7/MnTT2L2 Module Responds to Silkworm Herbivory through Regulating Catechin Synthesis Gene Expression in Wild Mulberry (Chuansang)

In Arabidopsis, AtSPL9 took part in responses to drought and salt stress by influencing anthocyanin metabolism [22]. We also found that the expression levels of mulberry SPL genes fluctuated after silkworm herbivory treatment. SPL7 and SPL14 were significantly more highly expressed, while SPL2, SPL5, SPL12, SPL15, and SPL16A were prominently less expressed in Chuansang leaves, and the expression levels of SPL genes in Guisangyou 62 all decreased to varying degrees (Figure 7c,d and Table S3). We inferred that the long period of artificial selection caused the different expression patterns of SPL7 and SPL14 genes between Chuansang and Guisangyou 62 under silkworm herbivory treatment. In addition, it was also found that a series of genes (TT2L2, GL3, bHLH, TTG1, FNS, F3′H, DFR, and LAR) involved in flavonoid biosynthesis were more highly expressed after herbivory treatment in Chuansang (Figure 8a,b). In mulberry, the TT2L1/bHLH/TTG1 ternary complex regulates the transcription of genes associated with catechin synthesis [40]. Promoter analysis and dual-luciferase assay verified that SPL7 could promote the transcription of TT2L2 in Chuansang (Figure 9). However, similar experimental results were not found in Guisangyou 62 (Figure 8c). Moreover, it was also observed that miR156 had higher expression after herbivory treatment in Chuansang (Figure 7b) and had the same expression profile as miR156-targeted SPL genes (SPL7 and SPL14). In conclusion, this study had found that SPL genes in Chuansang and Guisangyou 62 responded differently to silkworm herbivory, and verified that SPL7, independent of the miR156/SPL module, promoted the transcription of TT2L2 and further increased the expression levels of catechin synthesis genes (F3′H, DFR, and LAR) in response to silkworm herbivory in Chuansang.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Guisangyou 62, Chuansang, and tobacco seeds (Nicotiana tabacum L.) were planted in sterilized soil and left at 4 °C for 2 d before transfer to a climate chamber at 25 °C under long-day conditions (16 h light/8 h dark), as were mulberry seedlings. Mature leaves from
the juvenile phase of Guisangyou 62 (J-ML) and mature leaves from the mature phase of Guisangyou 62 (M-ML) were used to investigate the expression levels of SPL genes.

The 3-month-old Guisangyou 62 and Chuansang leaves were treated with second-stage silkworm herbivory (Bombyx mori cv. Dazao) for 1 h until obvious damage was caused to mulberry leaves.

4.2. Bioinformatics Analysis of SPL Genes in Mulberry

BLAST and HMMER searches of MnSPLs against the MorusDB (https://morus.swu.edu.cn/, accessed on 3 March 2020) were conducted using AtSPL as the query sequences. The SBP domain of SPLs was identified using the CD-search online tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 3 March 2020) (Table S4).

Neighbor-joining phylogenetic trees of SPL proteins were constructed using MEGA5.1 with the best JTT + G model. Branching reliability was assessed by the bootstrap resampling method using 500 bootstrap replicates.

Chromosomal locations of SPL genes in mulberry were determined by BLAST analysis of SPLs against the mulberry genome (https://www.ncbi.nlm.nih.gov/genome/?term=Morus+alba, accessed on 3 March 2020). The structures of SPL genes were predicted with the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/chinese.php, accessed on 3 March 2020). Sequence logos of SBP domains were generated by the Weblogo platform (http://weblogo.berkeley.edu/, accessed on 3 March 2020). Potential protein motifs were predicted using the MEME online tool (http://meme.sdsc.edu/meme/, accessed on 3 March 2020). Promoter analysis of gene was processed by the PlantPAN software (http://plantpan.itsp.ncku.edu.tw/promoter.php, accessed on 3 March 2020), and the specific transcription factor binding motifs from P. trichocarpa and A. thaliana were selected as the query motifs.

4.3. Expression Analysis of miR156 and SPL Genes in Mulberry

The transcriptome data used in SPL gene expression profiling in five mulberry tissues was obtained from MorusDB (https://morus.swu.edu.cn/, accessed on 3 March 2020). The transcriptome data of SPL genes in mulberry leaves under silkworm herbivory are shown in Table S3. The RPKM values of SPL genes were normalized through a min-max normalization algorithm ($x^* = (x - x_{\text{mean}})/(x_{\text{max}} - x_{\text{min}})$), and analyzed using TBtools software [46].

Small RNAs (sequence lengths < 200 bp) and the total RNA of mulberry tissues were both extracted using the miRcute Plant miRNA Isolation Kit purchased from TIANGEN (DP504, Beijing, China), the OD260/280 values of all RNA samples were detected by NanoDrop2000 (Thermo Scientific, Waltham, USA), and agarose gel electrophoresis (AGE) was performed to verify the integrity of RNA samples (data not shown). Small RNA reverse transcriptions were performed using the miRNA First Strand cDNA Synthesis Kit (tailing reaction) (B532451, Sangon Biotech, Shanghai, China). The levels of mno-miR156 were quantified by the MicroRNA qPCR Kit (SYBR Green method) (B532461, Sangon Biotech, Shanghai, China). For reference genes, mno-miR166b and MnU6 were selected to calibrate data in small RNA RT-qPCR. The relative expression of miR156 was defined using the $2^{-[Ct(\text{target gene}) - Ct(\text{control gene})]}$ algorithm. The cDNA of total RNA was synthesized according to the instructions of Primer Script RT reagent kit (RR047A, Takara, Japan). For the RT-qPCR, a reaction was performed according to the manufacturer’s instructions for the SYBR Premix Ex Taq II (RR820A, Takara, Japan) and processed using a Step One Plus Real-Time PCR System (Applied Biosystems, Singapore, Singapore). The mulberry ribosomal protein L15 (RPL15, Morus024083) gene was used as a control for expression normalization, and the relative expression of genes was defined using the $2^{-[Ct(\text{target gene}) - Ct(\text{control gene})]}$ algorithm. Gene-specific primers used for RT-qPCR are listed in Table S2.
4.4. Dual-Luciferase Reporter Assay

MnSPL genes were cloned into the GreenII 62-SK vector. A 2000-bp promoter sequence of TT2L2 predicted by the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/, accessed on 31 May 2020) was amplified and cloned into the pGreenII 0800-LUC vector. Agrobacterium-mediated co-transformation of the pGreenII 0800-LUC and GreenII 62-SK vectors into tobacco leaves was performed as described previously [40]. After infiltration for 2 days, the ratio of LUC/REN activity was measured using the Dual-Luciferase Reporter Gene Assay Kit (11402ES80, Yeasen, Shanghai, China) on a configurable multi-mode microplate reader (Synergy™ H1, BioTek, Beijing, China). All the primers and probes used in this work are listed in Table S2.

5. Conclusions

In this study, a total of 15 full-length SPLs were identified in the mulberry genome. The evolutionary conservation and functional diversity of mulberry SPL genes were inferred through a comprehensive analysis of chromosomal locations, phylogenetic relationships, gene structures, conserved motifs, and spatial and temporal expression profiles. In addition, we found that SPL genes in cultivated mulberry (M. atropurpurea cv. Guisangyou 62) and wild mulberry (M. notabilis) were differentially expressed after silkworm herbivory and verified that the SPL7/T2L2 module increased the expression levels of catechin synthesis genes (F3′H, DFR, and LAR) to response to silkworm herbivory in Chuansang. Our work provides useful information to elucidate the functions of SPLs in mulberry.

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