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Characterization of *SPL* Family Genes in Banana Reveals the Critical Role of *MaSPL1* in Regulating Fruit Ripening

Bo Wang ^{1,2}, Tongxin Dou ¹, Ou Sheng ¹ , Weidi He ¹, Guiming Deng ¹ , Fangcheng Bi ¹, Chunyu Li ¹, Tao Dong ¹, Qiaosong Yang ¹, Chunhua Hu ¹, Huijun Gao ¹, Siwen Liu ¹ , Cancan Liu ¹, Jihong Liu ^{2,*} , Ganjun Yi ^{1,*} and Yaoyao Li ^{1,*}

¹ Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Ministry of Agriculture and Rural Affairs Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization, Guangdong Provincial Key Laboratory of Science and Technology Research on Fruit Tree, Guangzhou 510640, China

² College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, China

* Correspondence: liujihong@mail.hzau.edu.cn (J.L.); yiganjun@vip.163.com (G.Y.); lyysky826@163.com (Y.L.)

Abstract: SPLs (SQUAMOSA promoter binding protein-like) are pivotal in regulating plant development and stress responses. Although *SPL* genes have been characterized in a series of plant species, no systematic analysis has been performed on bananas, one of the most consumed tropical fruits with immense economic importance worldwide. Here, 55 putative *MaSPL* genes were identified in *Musa acuminata* ssp. *malaccensis* var. Pahang and classified into seven groups based on phylogenetic analysis. The RNA-seq analysis revealed that the expression of *MaSPLs* presented distinct spatiotemporal patterns in different tissues at different developmental stages, indicating a potential role in banana growth and development. Furthermore, *MaSPL1* was found to be predominantly expressed in banana fruits during the fruit development and the early postharvest stages. Notably, the transient overexpression of *MaSPL1* accelerated the fruit ripening in bananas. In conclusion, this study provides comprehensive information for further investigation of the specific roles of *SPL* genes in banana developmental processes, particularly during fruit development and post-harvest stages, and may implement molecular strategies to regulate maturation and enhance fruit quality in bananas.

Keywords: *Musa acuminata*; squamosa promoter-binding protein-like (SPL); genome-wide analysis; expression profile; functional characterization



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1. Introduction

SQUAMOSA-PROMOTER BINDING PROTEIN (SBP) or SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors, harboring a plant-specific SBP domain, regulate diverse aspects of development and growth across different plant lineages [1]. The SBP domain comprises approximately 76 amino acid residues and contains two specific zinc finger motifs and a nuclear localization signal (NLS) at the C-terminal region [2,3]. The *SPL* genes were first identified in *Antirrhinum majus* as *AmSBP1* and *AmSBP2* with the ability to bind to the promoter of the floral meristem identity gene *SQUAMOSA* [4]. To date, an increasing number of *SPL* members have been identified in different plants, including *Arabidopsis* (*Arabidopsis thaliana*) [5], cotton (*Gossypium* spp.) [6], soybean (*Glycine max*) [7], tomato (*Solanum lycopersicum*) [8], Wheat (*Triticum aestivum*) [9], rice (*Oryza sativa*) [10], and maize (*Zea mays*) [11].

It has been reported that SPL transcription factors are involved in plant development, including, but not limited to, root development [12], stomatal complexes and epidermal hairs [13], floral organ development and fertility [14], vegetative phase transition and flowering [15–17], plant height and architecture [18–24], grain size and yield [25–29], and fruit development and ripening [30]. For example, in maize, *Teosinte glume architecture1* (*TGA1*), a member of the SBP-box gene family of transcriptional regulators, has been identified as the gene conferring naked kernels [31]. In rice, *OsSPL16/GW8* (*Grain Width 8*), the close ortholog of maize *TGA1*, has been identified as an important QTL controlling grain size, shape, and quality [28,29,32]. *SISPL-CNR*, an SPL transcription factor, functions as a multi-functional protein to activate tomato fruit ripening and trigger plant cell death during fruit ripening [33].

Bananas, as a typical climacteric fruit, are susceptible to softening and rotting during transportation and storage at room temperature, which results in significant postharvest losses and is similar to other climacteric fruits [34]. Elucidating the regulatory factors and studying the regulatory mechanism underlying banana fruit ripening is of paramount importance for maintaining the fruit quality, extending the shelf life, and marketing [35–37]. A comprehensive elucidation of the molecular regulatory networks governing ripening and quality formation will contribute to strategies for improving fruit quality and postharvest longevity. To date, several transcription factors (TFs), including MaMYB3 [38], MaWRKY49 [39], MabZIP21 [37], MabHLH28 [40], MaNAC19/029/083/169 [41–44], MaEIL4 [45], and MaNAP1 [46], have been implicated in banana fruit ripening. Notably, recent findings indicate that MaSPL16 regulates the *MaNAC029* transcript level, thereby controlling ethylene biosynthesis and improving fruit quality throughout the entire process of banana fruit ripening [30]. This evidence provides further support for the hypothesis that *SPLs* play a functional role throughout the banana ripening process.

To gain further insight into the banana *SPL* gene family and to identify additional *SPL* genes that regulate fruit ripening, this study has examined the *SPL* gene family in bananas from three principal perspectives: (i) the diversity of gene structure and domain architecture of the banana *SPL* gene family; (ii) the expression profiles of banana *SPL* genes in different tissues; and (iii) the functional analysis of *MaSPL1* in the regulation of banana fruit ripening. In brief, this study lays a solid foundation for further investigating the roles of *MaSPL* genes in developmental processes, particularly during fruit development and postharvest stages, and offers potential targets for molecular strategies to regulate ripening and improve fruit quality in bananas.

2. Materials and Methods

2.1. *SPL* Transcription Factors in *M. acuminata* DH Pahang

To identify members of the banana *SPL* gene family, we downloaded the complete genome sequence data of the banana (*Musa acuminata* ssp. *malaccensis* var. Pahang (*M. acuminata* DH Pahang, AA) from the Banana Genome Hub database (<https://banana-genome-hub.southgreen.fr/>, accessed on 1 April 2024). Furthermore, the complete genome sequences of *Arabidopsis* (*A. thaliana*), rice (*O. sativa*), and maize (*Z. mays*) were obtained from the following sources: the *Arabidopsis* Information Resource (TAIR: <http://www.arabidopsis.org/>, accessed on 1 April 2024), the National Rice Data Center (<https://www.ricedata.cn/>, accessed on 1 April 2024), and the Maize Genetics and Genomics Database (<https://maizegdb.org/>, accessed on 1 April 2024). Subsequently, 66 *SPL* proteins from the *Arabidopsis* (17), rice (19), and maize (30) were employed as target sequences in the *M. acuminata* DH Pahang database for blast comparison with the cutoff (e-value < 1×10^{-1}). Then, all the hits were screened by the HMM (hidden Markov model) profile of the SBP domain (PF03110), which was retrieved from the Pfam database (<http://pfam.janelia.org/>,

accessed on 8 April 2024) [47]. To ensure the accuracy of each *SPL* gene, the candidate protein sequences of the banana *SPL* gene family were further screened and validated by the Conserved Domain Database (CDD) (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 8 April 2024) [48] and the Simple Modular Architecture Research Tool (SMART) database (<http://smart.embl-heidelberg.de/>, accessed on 8 April 2024) [49]. Finally, the protein sequences that exhibited incomplete structural integrity and lacked the conserved structural SBP domain were excluded.

2.2. Gene Structure and Phylogenetic Analysis

The conserved structural domains of *M. acuminata* *SPL* proteins were analyzed using the online toolkit of NCBI CD-Search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 12 April 2024). The conserved motifs of the banana *SPL* amino acid sequence were analyzed using the MEME (<https://meme-suite.org/meme/>, accessed on 12 April 2024) program [50]. The number of motifs was set to 10, and the remaining parameters were set to their default values. The resulting motif results were then visualized in the Visualize MEME Motif Partner of TBtools software [51]. Furthermore, the exon/intron structure of each *MaSPL* gene was generated in the Gene Structure View of the same platform. Moreover, phylogenetic trees were constructed using the maximum likelihood (ML) method, the Poisson correction model, and bootstrap analysis, performed with 1000 replicates in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 [52].

2.3. Synteny Analysis

The chromosomal localization of *MaSPL* genes in *M. acuminata* DH Pahang was conducted via the Show Gene on Chromosome program within the TBtools software (version 2.225). Furthermore, a synteny analysis was performed based on the chromosomal location of *MaSPL* family members and gene annotation information. Subsequently, a One Step MCScanX-Super Fast analysis [53] was conducted in TBtools, which was then visualized using the Advanced Circos program.

2.4. Cis-Acting Element and Collinearity Analysis

A 2000 bp upstream of the coding regions of *MaSPLs* in *M. acuminata* DH Pahang were extracted using the TBtools software, and then the *cis*-acting elements in the *MaSPLs* promoter region (2000 bp) were analyzed by the PlantCARE database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 12 April 2024) [54]. The numbers of *cis*-acting elements were visualized and analyzed using the Basic Biosequence Viewer program in TBtools software. A comparative collinearity analysis was conducted to determine the evolutionary relationship and conservation between the *SPL* proteins of *M. acuminata* DH Pahang, *A. thaliana*, *O. sativa*, and *Z. mays*, which was performed using the Synteny visualization plugin in TBtools software.

2.5. Expression Profiles of *MaSPL* Genes in Different Tissues

Transcriptome data (NCBI BioProject accessions: PRJNA394594) [55] were obtained from *Musa* Cavendish Baxijiao in roots, leaves, and fruits at different stages of development (0, 20, and 80 days after flowering) and ripening (8 and 14 days postharvest). The FPKM (fragments per kb of transcript per million fragments mapped) values were employed to represent the expression abundance of *MaSPL* genes. The expression heatmap of *MaSPLs* was generated using TBtools, based on log₂ (FPKM+1) values.

2.6. Transient Overexpression in Banana Fruits

The coding sequence of *MaSPL1* was subcloned into the pCMABIA1300 vector, which was transferred to *Agrobacterium tumefaciens* strain EHA105, and subsequently employed

for injection of the whole pulp of mature-green banana through the distal end using a syringe. At least twelve fruit fingers were used for each treatment. The transformed fruits were dipped into ethephon solution (200 mg/L) for 1 min on the first day post-inoculation and incubated at 22 °C for six days. Samples were collected to ascertain the gene expression levels. The firmness and hue angle of the fruit were monitored at each predetermined sampling point using a texture analyzer (BosinTech, Shanghai, China) and a color analyzer (Konica Minolta, Tokyo, Japan), respectively. The primers used are listed in Table S4.

2.7. RNA Extraction and RT-qPCR Analysis

Banana samples were collected, frozen in liquid nitrogen, and stored at 80 °C. Total RNA was extracted using the SteadyPure Plant RNA Extraction Kit (Accurate Biotechnology, Changsha, China). Reverse transcription was conducted using 1–2 µg total RNA with the Hifair® III 1st Strand cDNA Synthesis SuperMix (YEASEN Biotechnology, Shanghai, China). A quantitative real-time polymerase chain reaction (RT-qPCR) assay was conducted using the Hieff UNICON® qPCR SYBR Green Master Mix (YEASEN Biotechnology, Shanghai, China) and the LightCycler480 system (Roche, Basel, Switzerland). *MaCAC* was used as a reference gene [56]. The primers used for the RT-qPCR assay are listed in Table S4.

3. Results

3.1. Identification of MaSPL Gene Family Members in *M. acuminata* DH Pahang

To identify the *SPL* genes in *M. acuminata* DH Pahang, we initially constructed a local BLAST library (version 2.16.0) with 66 *SPL* proteins from the *A. thaliana* (17), *O. sativa* (19), and *Z. mays* (30) for comparison. A subsequent screening in combination with HMM, Batch CD-Search, Pfam, and SMART analysis was conducted to eliminate the protein sequences that lacked complete structural integrity and the conserved structural SBP domain. As a result, 55 *SPL* genes were identified in *M. acuminata* DH Pahang (Table S1). Due to the lack of standard annotation designated to the 55 *SPL* genes in *M. acuminata* DH Pahang, we named them *MaSPL1* to *MaSPL55* based on their chromosomal locations.

3.2. Phylogenetic Tree of *M. acuminata* DH Pahang, *A. thaliana*, *O. sativa*, and *Z. mays* *SPL* Proteins

To investigate the evolutionary relationships of *SPL* proteins between eudicots and monocots, we analyzed *SPL* proteins from the diploids *A. thaliana*, *M. acuminata* DH Pahang, *O. sativa*, and *Z. mays*. A phylogenetic tree was constructed using MEGA 7.0 software with the Maximum Likelihood (ML) method and 1000 bootstrap replicates (Figure 1). Clearly, the eudicot and monocot *SPL* genes were present in all seven groups, indicating that the appearance of most *SPL* genes in plants predates the monocot/eudicot divergence. The phylogenetic analysis also showed that *SPL* gene members from different plant species were not evenly distributed among the groups. Each group contained at least one *MaSPL* gene, and some *SPL* genes in *A. thaliana* have two or more counterparts in *M. acuminata* DH Pahang. For example, Group IV contained seven *M. acuminata* DH Pahang *SPL* genes, but there was only one *A. thaliana* member; Group II contained twelve *MaSPL* genes, but there were only two *A. thaliana* *SPL* genes. The results may indicate that the *SPL* genes of different plant species have undergone differential expansion.

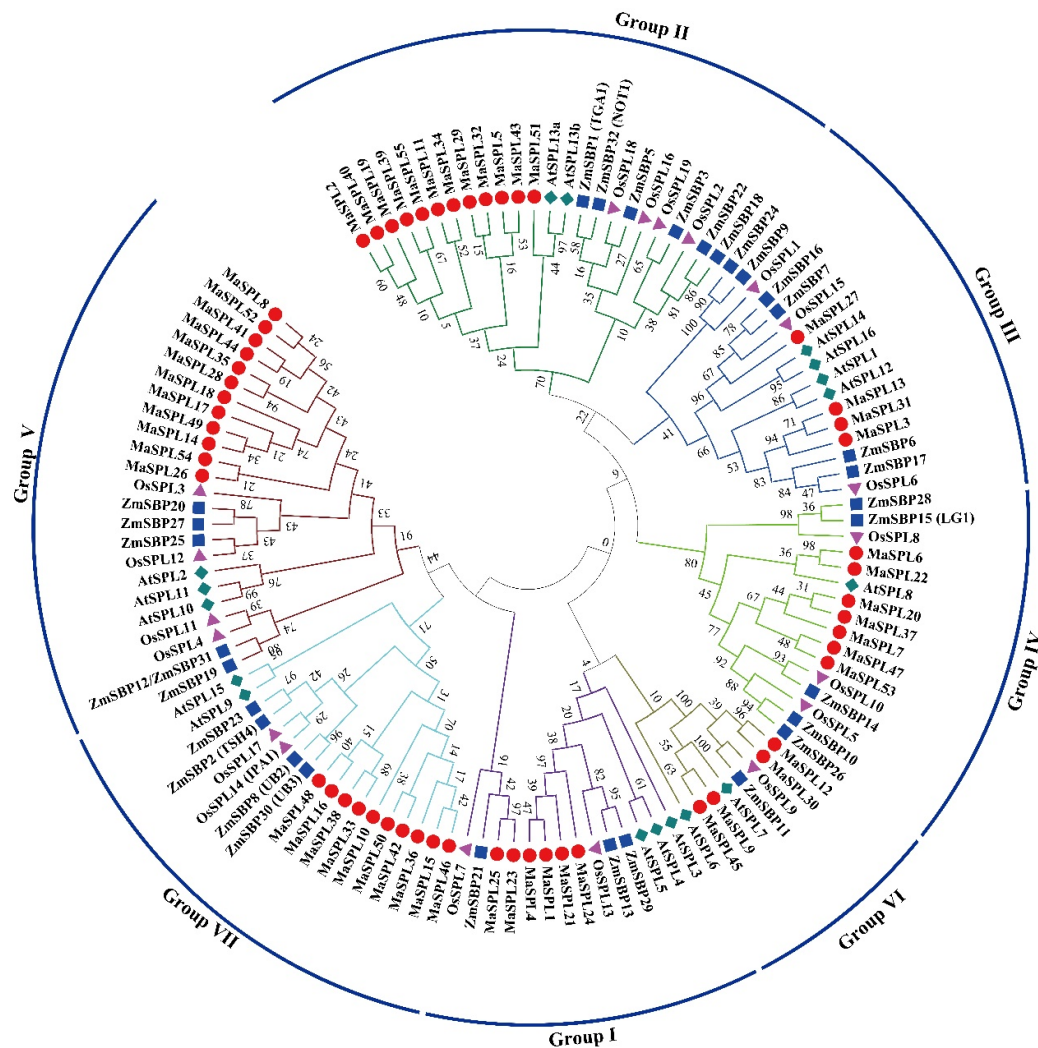


Figure 1. Phylogenetic tree of *M. acuminata* DH Pahang, *A. thaliana*, *O. sativa*, and *Z. mays* SPL proteins. The phylogenetic tree was constructed using MEGA 7.0 software with the Maximum Likelihood (ML) method, aligning the SPL proteins from the *Musa acuminata* ssp. *malaccensis* var. Pahang (*M. acuminata* DH Pahang) (55), *A. thaliana* (17), *O. sativa* (19), and *Z. mays* (30), followed by performing 1000 bootstrap replicates. They were divided into seven groups according to the evolutionary tree and were represented by different colors. The various icons represent the SPL genes in diverse species, with dark red circles representing *M. acuminata*, green squares representing *A. thaliana*, light fuchsia triangles representing *Oryza sativa*, and dark blue squares representing *Zea mays*. At, *A. thaliana*; Ma, *M. acuminata*; Os, *O. sativa*; Zm, *Z. mays*.

3.3. The Chromosomal Distribution, Duplication Events, and Syntenic Analysis of the Maspl Genes

Furthermore, we constructed a chromosome distribution map for the SPL gene family in *M. acuminata* DH Pahang. A total of 55 *MaSPL* genes identified in *M. acuminata* DH Pahang are distributed across eleven chromosomes (Figure S1). Ten *MaSPL* genes were present on chromosome 4, nine on chromosome 9, eight on chromosome 3, seven on chromosome 5, four on chromosomes 2, 6, and 7, three on chromosomes 8 and 10, two on chromosome 1, and only one on chromosome 11. Moreover, the evolutionary relationships among the *MaSPL* genes in *M. acuminata* DH Pahang were investigated through a duplication analysis. Among the collection of forty-five *MaSPL* genes, forty-four pairs of duplication genes were identified (Figure S1; Table S2).

To better understand the molecular functions and evolutionary origin of *MaSPL* genes, a comparative syntenic analysis between *M. acuminata* DH Pahang, *A. thaliana*, *O. sativa*, and *Z. mays* was established (Figure 2; Table S3). The synteny block analysis revealed that 46 orthologous *SPL* gene pairs were found between *M. acuminata* DH Pahang and *Z. mays*, 42 orthologous *SPL* gene pairs between *M. acuminata* DH Pahang and *O. sativa*, whereas two orthologous *SPL* gene pairs were identified between *M. acuminata* DH Pahang and *A. thaliana* (Figure 2; Table S3). In addition, 23 *MaSPL* genes (e.g., *MaSPL1*, *MaSPL3*, and *MaSPL4*) were not found in any duplication blocks, indicating that these *SPL* genes might have independently duplicated during evolution. It was also found that some *MaSPL* genes, such as *MaSPL10*, *MaSP29*, and *MaSPL46*, exhibit four collinear gene pairs with *Z. mays*. The results suggest the importance of these genes during the evolution of *MaSPL* genes in *M. acuminata* DH Pahang. Notably, *MaSPL10* and *MaSPL43* show collinear relationships with the *SPL* genes from *A. thaliana*, *O. sativa*, and *Z. mays* (Table S3).

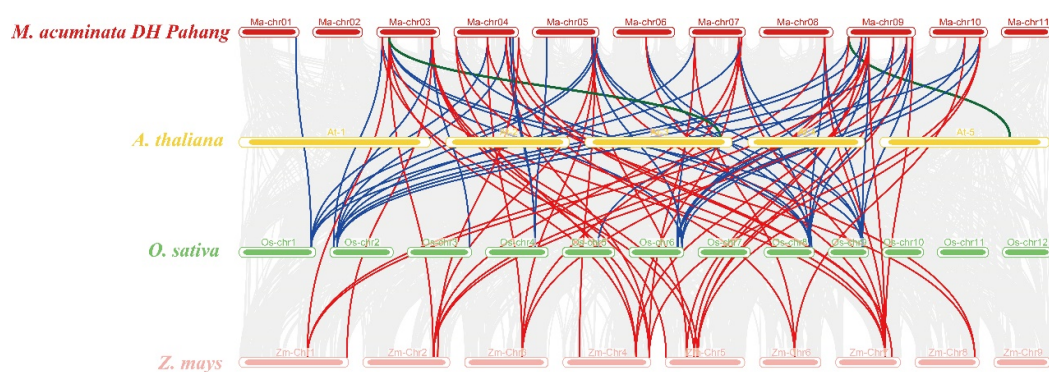


Figure 2. Synteny analysis of *SPL* genes between *M. acuminata* DH Pahang and three representative plant species (*A. thaliana*, *O. sativa*, and *Z. mays*). Green lines delineate the syntenic *SPL* gene pairs between *M. acuminata* DH Pahang and *A. thaliana*. Blue lines delineate the syntenic *SPL* gene pairs between *M. acuminata* DH Pahang and *O. sativa*. Red lines delineate the syntenic *SPL* gene pairs between *M. acuminata* DH Pahang and *Z. mays*.

3.4. Phylogenetic Analysis, Conserved Motifs, and Gene Structure Analysis of *MaSPLs* in *M. acuminata* DH Pahang

To better understand the diversification of *MaSPL* genes in *M. acuminata* DH Pahang, the phylogenetic tree, gene structure, and motif diversity were analyzed (Figure 3). The phylogenetic tree showed that Groups I, II, III, IV, V, VI, and VII contained six, twelve, four, seven, twelve, four, and ten *MaSPL* proteins, respectively (Figure 3A). Further studies conclusively showed that all 55 *MaSPL* proteins have a complete SBP conserved domain (Figure S2). In addition, to gain further insight into the structural features of *MaSPLs*, a total of 10 conserved motifs encoding amino acid sequences of *MaSPLs* were identified in this study using the MEME (Multiple EM for Motif Elicitation) program (Figure 3B). The results demonstrated that all members of the *MaSPL* family contain conserved motifs, including motif 1, motif 2, and motif 3. *MaSPLs* in Group V exhibited the highest number of conserved motifs, except for *MaSPL28*, which lacked motifs 8 and 10. The remaining 11 members (e.g., *MaSPL8*, *MaSPL14*, and *MaSPL17*) were found to contain eight conserved motifs. Furthermore, the eight motifs exhibited a consistent distribution and were arranged in the following order: motif 8, motif 6, motif 3, motif 1, motif 2, motif 9, motif 10, and motif 5. Additionally, the *MaSPL* family members in Group I and Group IV exhibited a distinct pattern, with only three conserved motifs (motif 1, motif 2, and motif 3). In contrast, the *MaSPLs* in the remaining four groups exhibited a more diverse range of motif distributions, with four to seven motifs. The results indicate that the discrepancies in motif

position and number between members of the same phylogenetic branch were minimal; however, the differences between members of different branches were more pronounced.

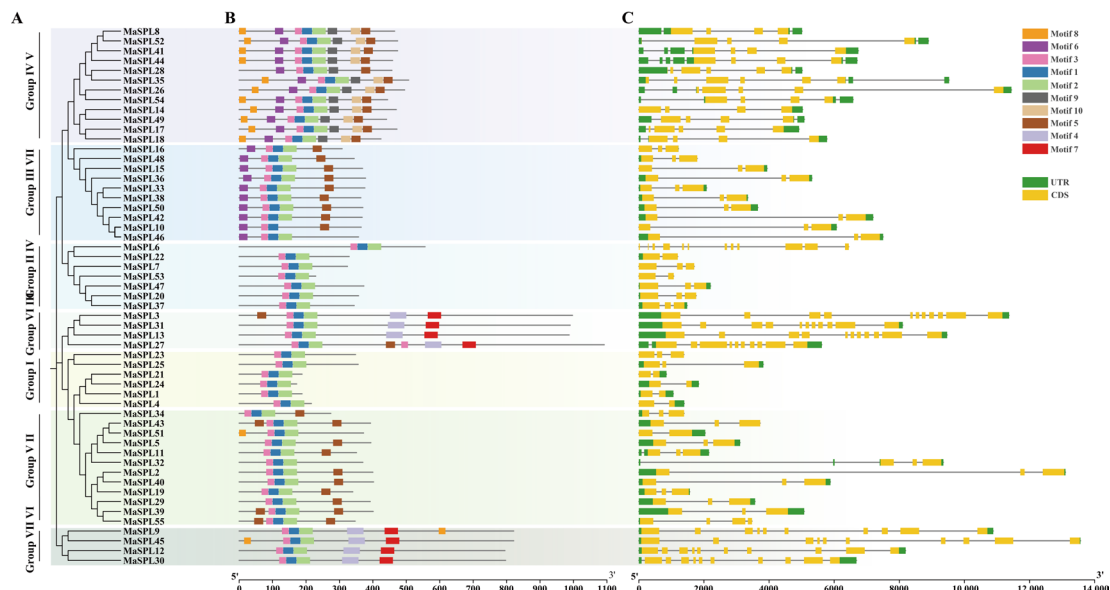


Figure 3. Phylogenetic tree, compositions of the conserved protein motifs, and gene structures of *MaSPL* genes in *M. acuminata* DH Pahang. (A) A phylogenetic tree was constructed based on 55 *MaSPL* proteins divided into seven groups; (B) Conserved motifs of the *MaSPL* proteins; the conservative motif of the *MaSPL* gene is predicted by the MEME method, the numbers (1–10) in the colored rectangles indicate motif 1–10, and the length of the rectangle indicates the size of the motif; (C) Exons-introns and untranslated regions (UTRs) of *MaSPL* genes. Yellow boxes indicate exons; black horizontal lines indicate introns; Green boxes represent UTRs. The length of the protein can be estimated using the scale at the bottom.

The *MaSPL* gene family also exhibited considerable variability within the gene structure, as illustrated in Figure 3C. Each *MaSPL* gene contains between two and twelve exons, with a maximum of twelve exons (*MaSPL6*) and a minimum of two exons (*MaSPL1/4/21/22/24/51/53*). Except for *MaSPL6* in Group IV and *MaSPL55* in Group II, the remaining *MaSPL* genes in Groups I, II, IV, and VII exhibited two to three exons, while those in Group V displayed four to six exons. The *MaSPL* genes in Groups III and VI exhibited ten to eleven exons. The preceding results indicate that the structure of *MaSPLs* in *M. acuminata* DH Pahang has undergone evolutionary change, with differences in the distribution position and number of exons observed among different members.

3.5. Analysis of Cis-Acting Elements in the Promoter Regions of *MaSPL* Genes in *M. acuminata* DH Pahang

The different *cis*-acting elements in the promoter of a gene indicate possible factors that affect the regulation of gene expression. The promoters of the 55 *MaSPL* genes (2 kb upstream of the start codon) in *Musa acuminata* DH Pahang were analyzed for putative *cis*-acting elements by using PlantCARE. There are four main types of *cis*-acting elements, including growth and development (e.g., CAT-box and L-box), hormone response (e.g., ABRE, P-box, TGACG motif, and TGA-box), light response (e.g., GT1 motif, G-box, and AT1 motif), and stress response (e.g., MBS, ARE, and TC-rich repeats) (Figure 4). For example, *MaSPL22* contained the largest number of *cis*-acting elements (forty-nine), and *MaSPL17* contained the smallest number of *cis*-acting elements (twelve). Notably, all *MaSPL* genes contained at least one *cis*-acting element for light, biotic/abiotic stress, growth and development, and phytohormone response (Figure 4). These results indicate the significant

contributions of *MaSPL* genes to various biological processes as well as their involvement in response to light, abiotic/biotic stress, and hormones in *Musa acuminata* DH Pahang.

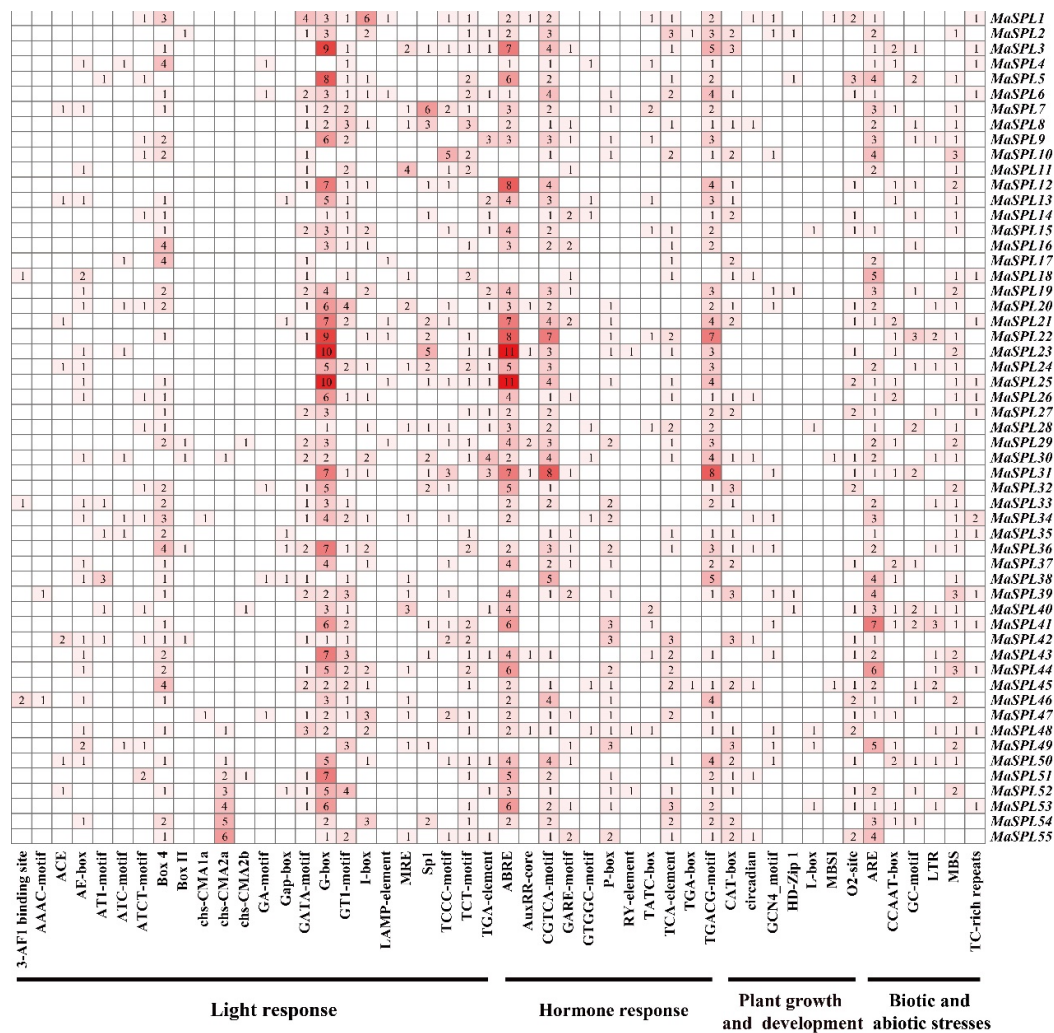


Figure 4. Cis-acting element in the promoter region of *MaSPLs* in *M. acuminata* DH Pahang. Numbers indicate the number of cis-regulatory elements in the promoter region of the *MaSPL* gene. Based on functional annotation, cis-acting elements of *MaSPL* genes can be classified into four categories: phytohormones, light response, plant growth and development, and biotic and abiotic stress. The intensity of the red color indicates the number of cis-acting elements.

3.6. Expression Profile of *MaSPL* Genes in Different Tissues

To further identify the tissue-specific expression profiles of *MaSPL* genes, we used the transcriptome data (NCBI BioProject accessions: PRJNA394594) [55] and analyzed the differential expression of *MaSPLs* in banana roots, leaves, and fruits at different stages of development (0, 20, and 80 days after flowering, DAF) and ripening (8 and 14 days postharvest, DPH) (Figure 5). In general, several similarities in the *SPL* expression patterns could be identified. For example, *MaSPL35* was identified as being predominantly expressed in the roots. *MaSPL26* and *MaSPL30* exhibited a predominant expression in the roots and leaves. Five *MaSPLs* (*MaSPL3*, *MaSPL9*, *MaSPL13*, *MaSPL27*, and *MaSPL31*) exhibited high expression in roots, leaves, and fruits at different stages of development and ripening. It is noteworthy that twelve *MaSPLs* (*MaSPL5*, *MaSPL7*, *MaSPL24*, *MaSPL28*, *MaSPL33*, *MaSPL36*, *MaSPL39*, *MaSPL41*, *MaSPL47*, *MaSPL49*, *MaSPL52*, and *MaSPL55*) were identified as being predominantly expressed in banana during the fruit development stage. *MaSPL14* and *MaSPL19* exhibited a progressive expression throughout the

fruit development and post-harvest stages. In particular, *MaSPL1* exhibited predominant expression during fruit development and early post-harvest stages.

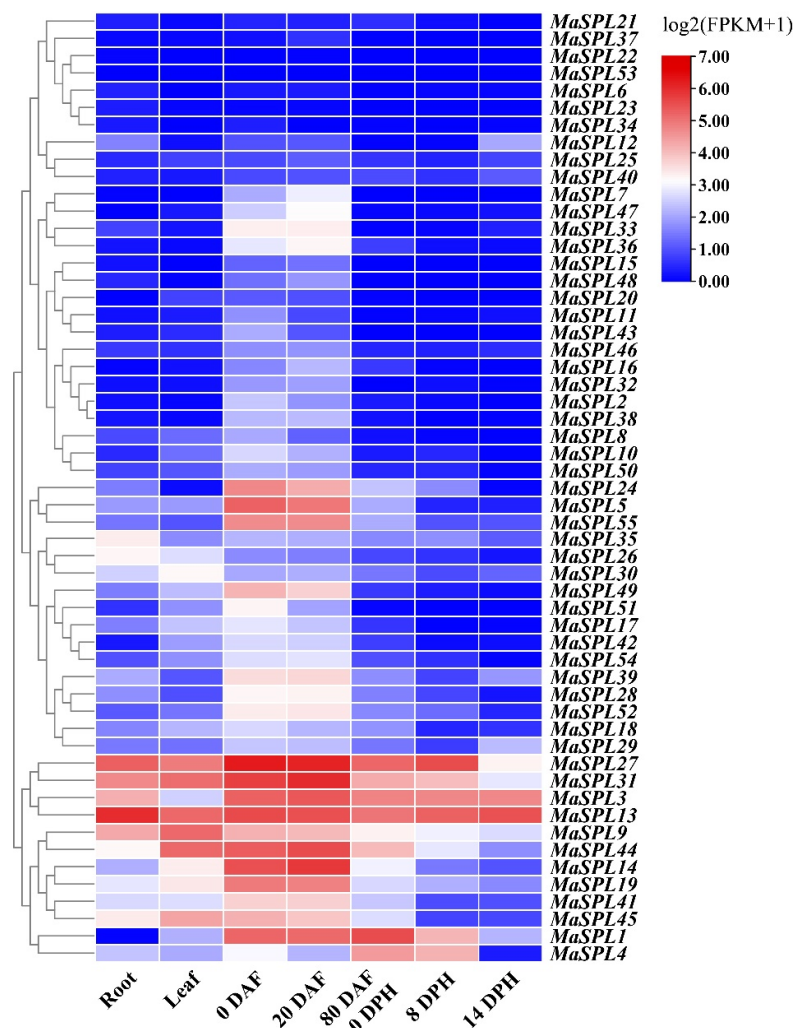


Figure 5. Expression profiles of *MaSPL* genes in different tissues. Heat map showing the expression profiles of 55 *MaSPL* genes in various tissues, including roots, leaves, and fruits at different stages of development (0, 20, and 80 DAF) and ripening (8 and 14 DPH). Expression levels of the *MaSPL* genes are shown as the log2 (FPKM+1), transformed FPKM values obtained from the RNA-Seq data. High expression levels are shown in red, and low expression levels are shown in blue. Zero DAF, 0 days after flowering; 20 DAF, 20 days after flowering; 80 DAF, 80 days after flowering; 0 DPH, 0 days postharvest; 80 days after flowering, corresponding to the day of harvest (0 days after harvest); 8 DPH, 8 days postharvest; 14 DPH, 14 days postharvest; FPKM, fragments per kb of transcript per million fragments mapped.

3.7. Transient Overexpression of *MaSPL1* in Banana Fruits Accelerates the Fruit Ripening

To investigate the function of *MaSPL1* in fruit ripening, *MaSPL1* was transiently overexpressed in banana fruits (Figure 6A,B). The expression level of *MaSPL1* in the transgenics was examined by RT-qPCR (Figure 6A). In contrast to the empty vector (control), the transient overexpression of *MaSPL1* enhanced fruit ripening in banana, resulting in a faster yellowing (ripening) phenotype (Figure 6B). In *MaSPL1*-overexpressing fruits, the decline in the hue angle was advanced by three days during maturation (Figure 6C); meanwhile, the firmness level of fruits reduced throughout the ripening process (Figure 6D). These findings indicate that *MaSPL1* plays a positive role in regulating fruit ripening in bananas.

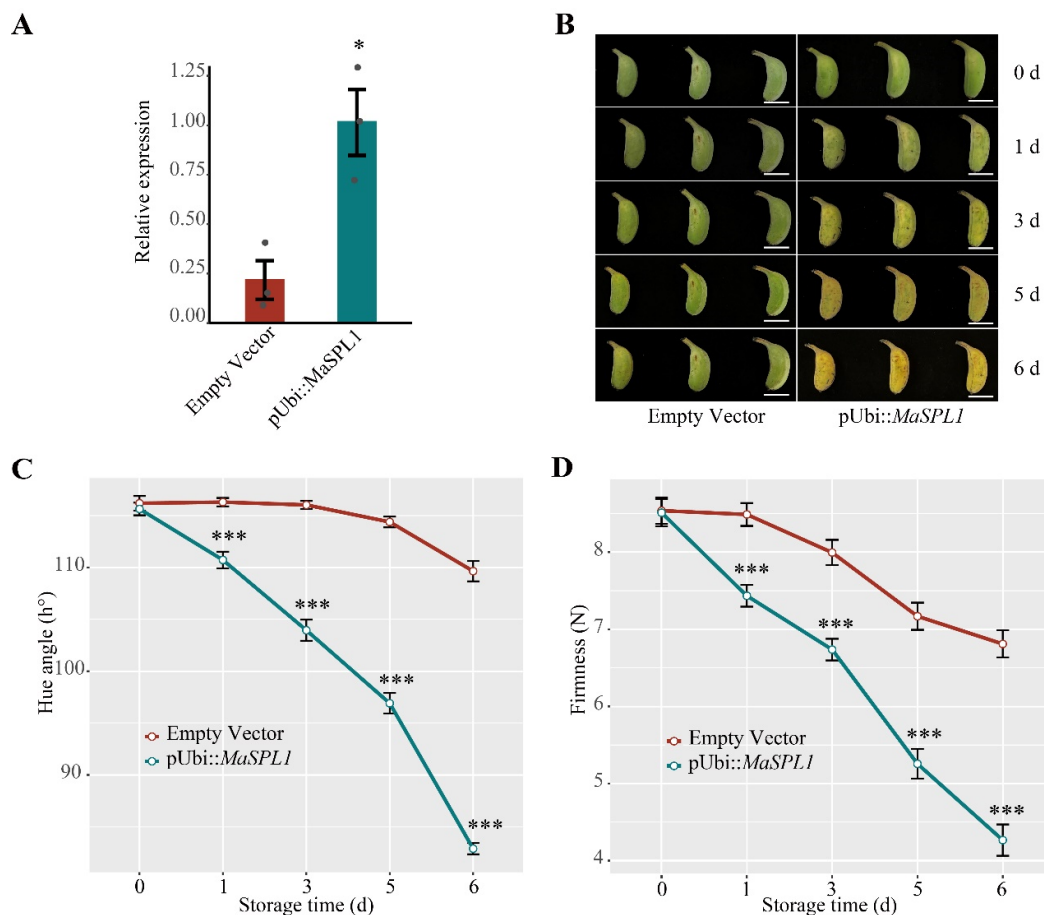


Figure 6. Transient upregulation of *M. acuminata* SPL (*SQUAMOSA promoter binding protein-like*) 1 (*MaSPL1*) in banana fruits accelerates fruit ripening. **(A)** RT-qPCR analysis showed the expression of *MaSPL1* in *MaSPL1* overexpression and control banana fruit; **(B)** the appearance of banana fruits that transiently overexpressed *MaSPL1* and the empty vector throughout ripening. Scale bar, 5 cm; **(C)** hue angle and **(D)** fruit firmness in *MaSPL1* overexpression and control banana fruit. The data are presented as the mean $\pm$ S.E. of three or four biological replicates, and the statistical significance was estimated by the two-sided Student's *t*-test (* $p < 0.05$, *** $p < 0.001$).

4. Discussion

Over the past decades, the members of *SPL* (*SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE*) transcription factors have been functionally characterized as crucial regulators for various plant developmental and stress-related processes [57,58]. Given the well-established roles in model plants like *Arabidopsis* and important crops, the *SPL* gene family in bananas has not been extensively explored. In this study, a total of 55 *MaSPL* genes were identified from the *M. acuminata* DH Pahang and classified into seven groups with the orthologs in *A. thaliana*, *O. sativa*, and *Z. mays* (Figure 1). By means of the phylogenetic analysis (Figure 3A), the conserved motif (Figure 3B) and the gene structure analyses (Figure 3C), it was revealed that *MaSPL* genes within the same group exhibited a similar motif arrangement and gene structure, which suggested conserved evolutionary relationship among these genes and also supported the phylogenetic analysis (Figure 1).

Here, based on the expression patterns in different tissues, the *MaSPL* family genes were mainly classified into three groups, including the constitutional high-expression group, the tissue-specific expression group, and the constitutional low-expression group within the given tissues (Figure 5). These expression patterns may reflect the fine regulation and selection of *SPL* gene family members in different tissues, particularly during the fruit development

and post-harvest stages in bananas. In addition, the potential function of *MaSPL* genes in bananas can be more accurately predicted by analyzing expression patterns and phylogenetic relationships with *SPL* genes from other plant species (e.g., rice, maize, and *Arabidopsis*). For example, most *MaSPL* genes cluster independently into a specific branch on the phylogenetic tree, yet exhibit diverse gene expression patterns (Figures 1 and 5). It is notable that *TGA1* [31] and *OsSPL16* [27–29] play a pivotal role in glume development and regulate the size, shape, and quality of the seed. In banana, a total of 12 genes homologous to *TGA1* and *OsSPL16* were identified, yet significant differences in expression patterns were observed (Figure 5). Only 4 of the 12 genes, *MaSPL5*, *MaSPL19*, *MaSPL39*, and *MaSPL55*, were found to be predominantly expressed during the developmental stages of banana fruit (Figure 5). Notably, previous studies have already confirmed that *MaSPL39* (also known as *MaSPL16*) can directly bind with the “GTAC” element of the *MaNAC029* promoter, activate the transcription level, and positively regulate fruit ripening in bananas [30]. Hence, these findings suggest the potential for significant differences in the functional evolution of Group II *SPL* genes and further corroborate that the *MaSPL* gene family has abundant biological functions. Further investigation, particularly through the development and characterization of transgenic banana lines overexpressing or silencing *MaSPL* genes [59], is necessary to validate their diverse biological functions, including the regulation of fruit morphology, postharvest characteristics, and other agronomic traits.

As a typical climacteric fruit, banana undergoes postharvest ripening through a genetically programmed, highly coordinated, and irreversible process regulated by plant hormones, environmental signals, and both transcriptional and post-translational mechanisms [60–62]. At the transcriptional level, several transcription factors, such as *MabHLH28* [40], *MaNAC169* [44], and *MaSPL16* [30], have been shown to regulate key ripening-related genes associated with cell wall degradation and starch conversion. These transcription factors function by binding to the promoters of target genes, modulating their expression in response to developmental and hormonal cues. Importantly, in this study, *MaSPL1* exhibited predominant expression during fruit development and the early postharvest stages (Figure 5). Transient overexpression of *MaSPL1* in banana fruit provided compelling evidence of its pivotal role in postharvest ripening (Figure 6). While this study elucidated the biological function of *MaSPL1* in banana fruit ripening, its specific regulatory mechanisms remain to be clarified. Key questions for future investigation include the following: (1) Does *MaSPL1* regulate ethylene biosynthesis genes to enhance ethylene production and release? (2) Does *MaSPL1* modulate the ethylene signaling pathway to amplify ethylene responses and accelerate fruit ripening? (3) Does *MaSPL1* regulate the expression of cell wall-degrading enzyme genes to promote fruit softening? (4) Does *MaSPL1* co-regulate banana fruit ripening by forming complexes with other regulatory factors or modulating their transcript levels? We should focus our research on these key issues to further elucidate the molecular regulatory mechanisms of *MaSPL1* in banana fruit ripening. Further research, particularly using *MaSPL1* transgenic lines developed through banana transgene transformation [59], is needed to confirm the critical role of *MaSPL1* in fruit ripening and other agronomic traits, and to elucidate its precise molecular regulatory mechanisms.

5. Conclusions

In this study, we identified 55 putative SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) transcription factors in *M. acuminata* ssp. *malaccensis* var. Pahang, classifying them into seven groups based on phylogenetic analysis. Further research revealed that the expression patterns of *MaSPLs* across different tissues and developmental stages exhibit distinct spatiotemporal characteristics, suggesting a potential role in banana

growth and development. Notably, we found that *MaSPL1* is predominantly expressed during fruit development and early postharvest stages, positively regulating fruit ripening in bananas. In summary, this study provides comprehensive information for further investigation of the specific roles of *SPL* genes in developmental processes, particularly during fruit development and post-harvest stages, in bananas. Future work involving transgenic banana lines overexpressing or silencing *MaSPL* genes [59] will be essential to elucidate their biological functions, including roles in fruit morphology, ripening, and other agronomic traits.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae11060576/s1>, Figure S1: The chromosomal distribution, and duplication events of the *MaSPL* genes in *M. acuminata* DH Pahang; Figure S2: Schematic diagram of the conserved domain of *MaSPL* proteins in *M. acuminata* DH Pahang; Table S1: Identification of *SPL* Family Genes in *Musa acuminata* DH Pahang, *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays*; Table S2: Duplication events for *MaSPL* gene pairs in *Musa acuminata* DH Pahang; Table S3: Synteny analysis of *SPL* genes between *Musa acuminata* DH Pahang and other plants (*Arabidopsis thaliana*, *Oryza sativa* and *Zea mays*); Table S4: Summary of primers used in this study.

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