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Identification and Characterization of SPL Transcription Factor Family Reveals Organization and Chilling-Responsive Patterns in Cabbage (*Brassica oleracea* var. *capitata* L.)

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Abstract: Squamosa promoter-binding protein-like (SPL) is a major family of plant-specific transcription factor, which is involved in multiple biological processes, such as plant growth and development, hormone response, light response and stress response. Therefore, it has been profoundly significant to systematically analyze the SPL Transcription Factors family in *Brassica oleracea*. In this study, a total of 33 *BoSPLs* were identified in the *B. oleracea* genome, and they were further divided into six subgroups based on the phylogenetic tree constructed from the SPL proteins of *B. oleracea*, *B. rapa* and *Arabidopsis thaliana*. The expression profile of *BoSPLs* in different organs/tissues showed that a large number of *BoSPLs* were expressed in the callus, root, stem, leaf, bud, flower and silique. In addition, the expression levels of two *BoSPLs* (*BoSPL9b* and *BoSPL10b*) were up-regulated in chilling tolerance cabbage 'CT-923' at 6 h after chilling stress when compared with normal treatment (mock), while two *BoSPLs* (*BoSPL9b* and *BoSPL15a*) in chilling sensitive cabbage 'CS-D9', five *BoSPLs* (*BoSPL1*, *-9a*, *-9b*, *-10b*, *-11b*) in 'CT-923' and two *BoSPLs* (*BoSPL9b* and *BoSPL16a*) in 'CS-D9' were up-regulated after 24 h chilling stress, indicated that these genes may play an important role in the chilling-tolerance of cabbage. We analyzed the characteristics of *BoSPLs* and provided the basis for further functional research.

Keywords: cabbage; bioinformatics; squamosa promoter-binding protein-like (SPL); expression patterns; chilling stress

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1. Introduction

Squamosa promoter-binding protein-like (SPL) is a plant-specific transcription factor family, which plays an important role in plant growth and development [1], plant architecture [2], primary and secondary metabolism [3], signaling [4] and biotic stresses [5]. *SPL* genes were first discovered in *Antirrhinum majus* [6], subsequently, several *SPL* families were identified in other species, such as *Arabidopsis thaliana* [7], rice [8,9], Chinese cabbage [10] and tomato [11]. The SPL protein contains a highly conserved DNA binding region, the SBP domain. The SBP domain is a typical zinc finger structure consisting of eight His or Cys residues, the C-terminus is Cys-Cys-His-Cys, the N-terminus is Cys-Cys-Cys-His or Cys-Cys-Cys-Cys [12]. These amino acid residues can bind to a single zinc ion, when zinc ion is absent, the SBP domain does not have the ability to bind to DNA. The *SPL* genes mainly regulate the expression of downstream genes by binding to

the complementary sequence of the promoter region of the downstream genes, thereby affecting the growth and development of the plants [13].

SPL genes were usually regulated by microRNA156 (miRNA156) in the regulatory network of plant growth and development [14], miRNA156 also targeted *SPL* genes to regulate the *Arabidopsis* response to environmental stress [15]. A total of ten *SPL* genes in *A. thaliana* [16], ten in tomato [11] and seventeen in soybean [17] were the targets of miRNA156, respectively. In rice, the OsmiRNA156-*OsSPL3/OsSPL12* module directly activates the nodes in *OsMADS50* to regulate the development of the rice crown root [18]. The miRNA156-*SPL4* module in switchgrass mainly regulates the germination of the aerial bud, *SPL4* inhibits the formation of the aerial bud and the basal bud, the genetic manipulation of *SPL4* could change the plant structure and increase yields [2]. The analysis of the spatiotemporal expression profile in wheat showed that *TaSPL16* expressed highly in young developing panicles, and its expression is almost undetectable in vegetative tissues; the ectopic expression of *TaSPL16* in *A. thaliana* leads to a delayed emergence of vegetative leaves, and promotes flowering early [19].

SPL genes also play an important role in temperature-sensitive flowering. The overexpression of *AtSPL1* and *AtSPL12* enhanced the tolerance to high temperature in *Arabidopsis* florescence [20]. In grapes, the expression of *VvSPL3* and *VvSPL5* were up-regulated, while *VvSPL4* and *VvSPL7* were significantly down-regulated under low temperature conditions (5 °C), indicating that *VvSPL3* and *VvSPL5* were involved in the low temperature stress [21]. The miRNA156 expression in *A. thaliana* increased under drought or salt stress, which decreased the expression of downstream target genes *AtSPL9* and *AtDFR*, which delayed the flowering of *A. thaliana* [5]. In contrast, in *Betula platyphylla*, the expression of *BpSPL9* in roots and leaves was induced, suggesting that it may be involved in drought and salt stress [22]. These findings indicate that *SPL* genes can respond to abiotic stresses in different plants, improve the disorder caused by stress in the metabolic balance system and, finally, improve the survival rate of plants.

Low temperature is a major environmental factor that limits plant growth, development and geographical distribution [23], and may significantly reduce crop yields, including vegetable crops. Cabbage (*Brassica oleracea* var. *capitata* L.) is a widely distributed cruciferous vegetable crop in the world. If cabbage encounters low temperatures before the heading stage, or seedlings meet their vernalization conditions, afterwards, they are easy to bolt in the long day light, resulting in the failure to form tight and leafy heads, which serves as a storage organ and edible product. If the temperature is lower than the tolerance of cabbage, the seedlings will freeze to death, which can cause serious economic losses. In *Arabidopsis*, the overexpression of miRNA156 causes delayed flowering at lower ambient temperatures, which is probably associated with the reduced levels of *SPL3* mRNA [24], while six *SPLs* were highly expressed in apices in response to vernalization in *Arabis alpine* [25]. Thus far, the analysis of the *SPL* family has focused on *A. thaliana*, rice and other plants, while the function and expression pattern of *SPL* genes in *B. oleracea* are little known. In this study, we systematically identified the *SPL* gene family at the whole genome level of *B. oleracea*. The results obtained from this study provide a theoretical foundation for further revealing the molecular characterization of the *SPL* family members of *B. oleracea*.

2. Materials and Methods

2.1. Identification of BoSPL Genes in B. oleracea

The whole genome sequences of *B. oleracea* [26], *B. rapa* and *A. thaliana* were downloaded from the *Brassica* Database (http://brassicadb.cn, accessed on 2 January 2021) [27] and the Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/, accessed on 2 January 2021), respectively. The SBP protein domain (PF03110) was used to search for the protein sequences of *B. oleracea, B. rapa* and *A. thaliana* using the hidden Markov model (Hmmer 3.0 software), with the E-value set to $\leq$1.0, and the candidate SPL proteins were obtained. These candidate SPL proteins sequences were submitted to

the Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/, accessed on 5 January 2021), Pfam (http://pfam.xfam.org/search, accessed on 5 January 2021) and SMART (http://smart.embl-heidelberg.de/, accessed on 5 January 2021) databases for conserved domain analysis, the candidate SPL proteins without an SBP domain will be discarded. The names of the SPL proteins of *B. oleracea* were according to the homology of the AtSPL1-16 protein sequences and the suffix was added (a, b, c..., etc.).

2.2. Characterization and Phylogenetic Analysis of BoSPL Proteins

The physical and chemical parameters of BoSPL proteins were predicted by the ProtParam tool (http://web.expasy.org/protparam/, accessed on 6 January 2021), including relative molecular weight, theoretical isoelectric point (pI), instability coefficient, aliphatic index and grand average of hydropathicity (GRAVY). By comparing the cDNA sequences of *BoSPLs* with their corresponding DNA genes, the exon-intron structure was determined using Display Server 2.0 (GSDS 2.0 http://gsds.cbi.pku.edu.cn/, accessed on 7 January 2021). In addition, the conservative motifs were analyzed using the MEME (http://meme-suite.org/tools/meme, accessed on 8 January 2021), the parameters were default values. The subcellular localization of SPL proteins was predicted using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 10 January 2021) and BaCelLo (http://gpcr2.biocomp.unibo.it/bacello/index.htm, accessed on 11 January 2021). The phylogenetic tree was constructed by MEGA7.0 [28] using the neighbor-joining (NJ) method, the remaining parameters were kept as the defaults, except that the bootstrap value was set to 1000.

2.3. Chromosomal Distribution

Mapchart software was used to map the chromosomal distribution of the *BoSPL* genes on the nine chromosomes. The BRAD database was used to identify the orthologous and paralogous of the *SPL* genes in *B. oleracea*, *B. rapa* and *A. thaliana* [29]. The relationships of orthologous and paralogous among the three species were plotted using the TBtools software [30].

2.4. Cis-Acting Element Analysis

The 2000 bp genomic DNA sequences upstream of the start codon (ATG) of each *BoSPL* genes were detected using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 13 January 2021) [31], which were regarded as putative promoter sequences. The specific *cis*-elements involved in hormone response, light response and stresses response were analyzed in this study.

2.5. Organs/Tissues Expression Analyses of BoSPLs

According to the obtained transcriptome data (NCBI GEO database: GSE42891) of *B. oleracea* in different organs/tissues (callus, root, stem, leaf, flower, bud and silique), the expression of *BoSPLs* in different organs/tissues of cabbage were evaluated using these transcriptome data. The FPKM (Fragments per kilobase of exon per million reads mapped) values were used to represent the expression abundance of *BoSPLs.* The expression heat map of *BoSPLs* was generated using TBtools based on $\log_2$ (FPKM + 1) values.

2.6. Chilling Stress, Sample Collection and RNA-Seq Analysis

The seeds of chilling-sensitive cabbage 'CS-D9' and chilling-tolerance cabbage 'CT-923' were germinated and sown in sterilized soil, and placed in the artificial climate chamber for growing. The temperature was set to 25 °C during the day and 18 °C at night, and there was light for 14 h each day. When the seedlings grew to six true leaves, they were moved to the vernalization chamber for chilling stress, and the seedlings as the mock were still under normal conditions. After 6 h and 24 h stress, the seedlings of chilling stress and normal treatment (Mock) were sampled at the same time (three biological replicates). The third fully expanded true leaves from the top of the plants were harvested, frozen in liquid

nitrogen and stored at −80 °C for RNA-Seq analysis. Sequencing of RNA-Seq libraries was performed on Illumina HiSeq™ 2500 platform.

3. Results

3.1. Identification and Phylogenetic Analysis of SPL Family Genes in B. oleracea

In this study, a total of 34 candidate *SPL* genes were identified in the *B. oleracea* genome through HMM profiles (Supplementary Tables S1 and S2). These 34 protein sequences were subjected to Batch CD-Search, Pfam and SMART analysis, and the sequence of BolC07g038540.2J was discarded without the SBP domain. These 33 BoSPL proteins were named BoSPL1 to BoSPL21 according to their homologs with *A. thaliana* (Table 1). The open reading frames (ORF) of *BoSPLs* ranged from 408 bp (*BoSPL3c*) to 4833 bp (*BoSPL10c*), and the corresponding proteins varied from 135 (BoSPL3c) to 1610 (BoSPL10c) amino acids, the predicted molecular weights ranged from 15.77 (BoSPL3c) to 178.85 (BoSPL10c) kDa. The isoelectric point (pI) varied from 5.80 (BoSPL1) to 9.67 (BoSPL19); there were 27 BoSPL proteins with pI values greater than 7.00, indicating that they are basic proteins; and the remaining proteins were acidic proteins with a pI value of less than 7.00. The instability index ranged from 46.11 (BoSPL19) to 111.81 (BoSPL3c). The Aliphatic Index and GRAVY of the BoSPL proteins ranged from 28.96 (BoSPL3c) to 78.89 (BoSPL7b), and −0.31 (BoSPL7b) to −1.52 (BoSPL3b and BoSPL3c), respectively. The results of the subcellular localization prediction showed that all the BoSPL proteins were in the nucleus, except for BoSPL19, which was in cytoplasm, and in secretory when Plant-mPLoc and BaCelLo were used, respectively (Table 1, Supplementary Table S3).

In order to investigate the evolutionary relationships of SPL proteins among *B. oleracea*, *B. rapa* and *A. thaliana*, an unrooted neighbor-joining phylogenetic tree was constructed using 79 full-length SPL protein sequences from *B. oleracea* (33), *B. rapa* (29) and *A. thaliana* (17) (Supplementary Table S4). The phylogenetic tree showed the group I, II, III, IV, V and VI contained seven, six, four, one, eight and seven BoSPL proteins, respectively (Figure 1).

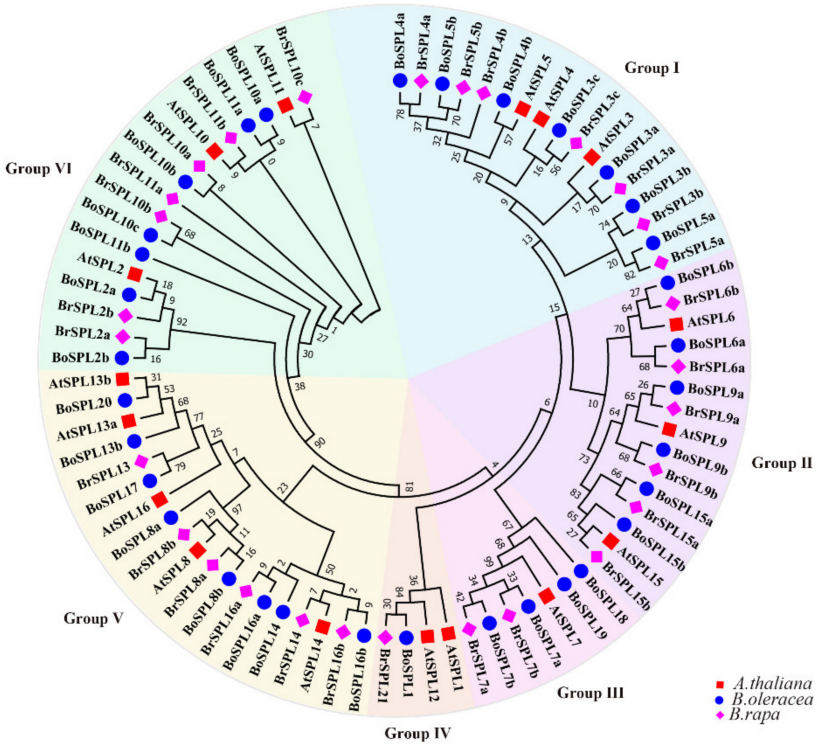

Figure 1. Phylogenetic tree of *B. oleracea*, *B. rapa* and *A. thaliana* SPL proteins. Phylogenetic analysis of 79 SPL proteins from *B. oleracea* (33), *B. rapa* (29) and *A. thaliana* (17) showing similar groups in the three species. Six groups were marked with different background colors.

Table 1. *SPL* family genes in *Brassica oleracea*.

Gene Name	PL (aa)	MW (kD)	pI	Instability Index	Aliphatic Index	GRAVY	Subcellular Localization Prediction
BoSPL1	888	98.89	5.80	52.76	78.52	−0.44	Nucleus
BoSPL2a	429	47.73	7.99	57.43	56.15	−0.70	Nucleus
BoSPL2b	361	40.55	8.83	51.95	59.97	−0.72	Nucleus
BoSPL3a	147	17.01	7.05	105.69	34.63	−1.38	Nucleus
BoSPL3b	141	16.48	6.25	108.70	31.21	−1.52	Nucleus
BoSPL3c	135	15.77	8.17	111.81	28.96	−1.52	Nucleus
BoSPL4a	179	20.42	9.59	51.65	49.55	−1.19	Nucleus
BoSPL4b	183	21.10	9.26	73.77	46.28	−1.24	Nucleus
BoSPL5a	179	20.71	9.45	67.67	50.17	−1.17	Nucleus
BoSPL5b	176	20.48	9.60	52.39	44.94	−1.22	Nucleus
BoSPL6a	318	36.03	8.33	60.86	57.04	−0.81	Nucleus
BoSPL6b	358	40.58	8.84	60.63	59.61	−0.70	Nucleus
BoSPL7a	781	87.38	6.71	50.64	78.35	−0.41	Nucleus
BoSPL7b	731	81.55	6.00	51.84	78.89	−0.31	Nucleus
BoSPL8a	328	36.54	8.85	56.37	48.17	−0.81	Nucleus
BoSPL8b	335	37.18	9.01	55.77	51.31	−0.77	Nucleus
BoSPL9a	370	40.39	8.62	55.79	45.84	−0.86	Nucleus
BoSPL9b	365	40.14	7.67	60.33	48.60	−0.84	Nucleus
BoSPL10a	366	40.71	8.89	56.22	50.82	−0.82	Nucleus
BoSPL10b	364	40.72	9.01	48.61	58.87	−0.71	Nucleus
BoSPL10c	1610	178.85	6.04	59.18	58.22	−0.88	Nucleus
BoSPL11a	394	44.44	8.51	51.25	61.85	−0.67	Nucleus
BoSPL11b	384	43.16	8.50	49.40	55.31	−0.81	Nucleus
BoSPL13b	348	38.28	7.64	61.89	52.41	−0.67	Nucleus
BoSPL14	1030	113.98	8.73	62.81	74.03	−0.50	Nucleus
BoSPL15a	324	36.13	9.14	55.22	57.50	−0.64	Nucleus
BoSPL15b	325	36.47	9.19	55.99	57.63	−0.72	Nucleus
BoSPL16a	1053	116.61	8.55	54.39	77.30	−0.38	Nucleus
BoSPL16b	989	109.02	8.80	55.53	75.01	−0.47	Nucleus
BoSPL17	341	37.48	8.29	74.14	54.57	−0.66	Nucleus
BoSPL18	743	84.89	6.47	51.73	78.51	−0.64	Nucleus
BoSPL19	181	20.15	9.67	46.11	68.40	−0.57	Cytoplasm
BoSPL20	359	39.02	8.45	74.21	59.50	−0.52	Nucleus

Note: PL: Protein length; MW: Molecular weight; aa: amino acid; pI: Isoelectric point; GRAVY: Aliphatic index and grand average of hydropathicity; SPL: Squamosa promoter-binding protein-like.

3.2. Chromosomal Distribution of BoSPL Genes

The chromosomal distributions of *BoSPLs* were analyzed using Mapchart software. Among them, a total of 33 *BoSPLs* were randomly distributed, anchored on nine chromosomes (C01-C09) of *B. oleracea* (Figure 2; Supplementary Table S4). The chromosomes C03 and C04 contain the largest numbers of *BoSPLs*, which account for six, while chromosome C01 and C08 only contain one *BoSPL*. Chromosome C02, C05, C06, C07 and C09 contained three, five, five, three and three *BoSPLs*, respectively. In addition, we easily found that *BoSPL10a* and *BoSPL10b,* and *BoSPL11a* and *BoSPL11b* may have occurred in a tandem duplication event. However, whether these genes were accompanied by functional similarities remains to be determined; further research should look for the functional differences of these tandem duplication genes using molecular biology methods.

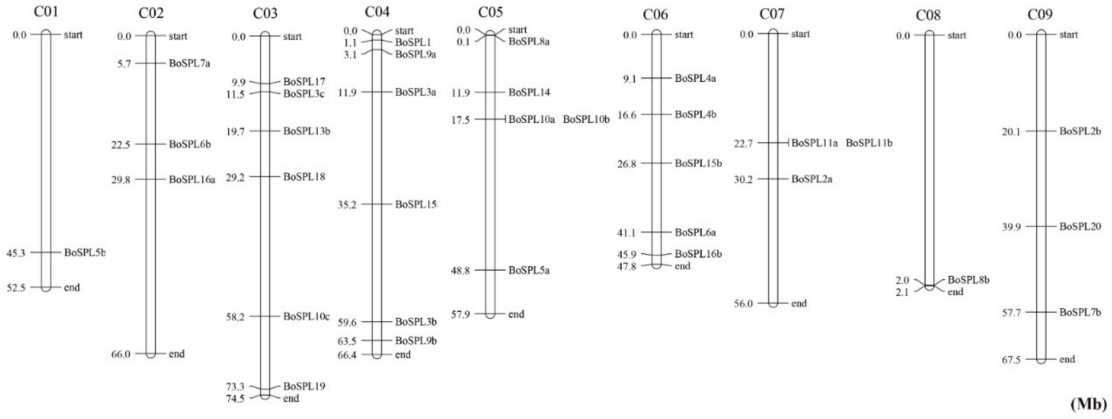

Figure 2. Distribution of *BoSPL* genes on nine *B. oleracea* chromosomes.

The orthologous and paralogous *SPL* genes relationships among *B. oleracea*, *B. rapa* and *A. thaliana* were analyzed using the BRAD database, the identified collinear and relationships of gene pairs in the *SPL*s are shown in Figure 3. A total of 27 orthologous *SPL* gene pairs between *B. oleracea* and *B. rapa*, 29 orthologous *SPL* gene pairs between *B. oleracea* and *A. thaliana* and 14 paralogous *SPL* gene pairs were identified in *B. oleracea*. Except for the loss of *BoSPL12*, all the *BoSPLs* were retained after the whole genome triplication event (WGT) and fractionation. Ten *BoSPL* genes (*BoSPL2*, *4*, *-5*, *6*, *-7*, *-8*, *-9*, *-11*, *-15*, *-16*,) retained double copies, *BoSPL3* and *BoSPL10* retained three copies.

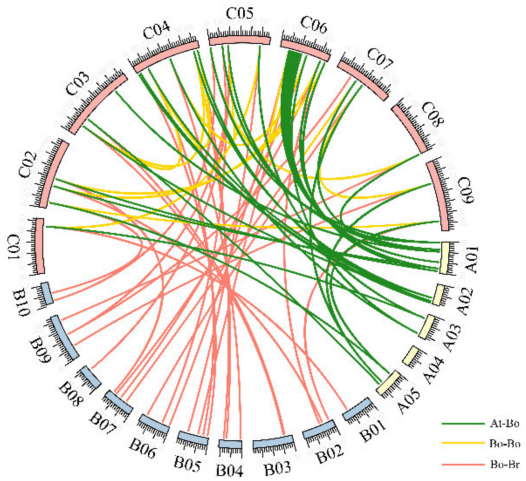

Figure 3. Syntenic relationship of *SPL* genes shown on chromosome maps among *B. oleracea*, *B. rapa* and *A. thaliana*.

3.3. Gene Structure and Conserved Protein Motifs of BoSPL Genes

The exon-intron structure is considered to play important roles in the evolution of multiple gene families. The exon-intron structure of 33 *BoSPLs* were mapped by comparing the CDS sequences of *BoSPLs* and the corresponding genomic sequences. As shown in Figure 4, the number of exons varied greatly, which ranged from two (*BoSPL-3a*, *-3b*, *-3c*, *4a*, *-4b*, *-5a*, *-5b*, *-6a*, *-6b*) to thirteen (*BoSPL18*). *BoSPLs* shared similar exon-intron structures in the same subgroup.

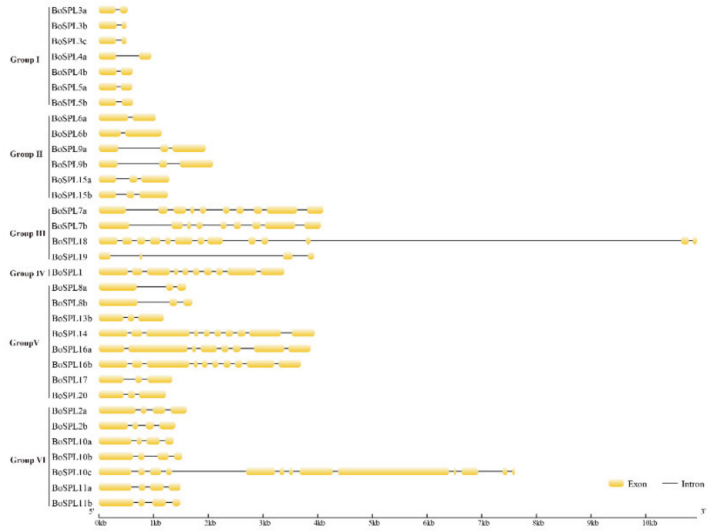

Figure 4. Gene structures of *BoSPL* genes. The structures of *BoSPL* genes were plotted using yellow boxes representing exons, black lines representing introns. The scale on the bottom is in the unit of kilobase (Kb).

In order to further understand the composition and diversity of the motifs in our predicted BoSPL proteins, the conserved motifs were searched using the MEME program. A total of fifteen conservative motifs were set and named as motif 1 to motif 15. The details of these conservative motifs are shown in Figure 5. Moreover, the logos of these motifs were obtained in MEME (Supplementary Figure S1). In our results, the BoSPL protein motifs were highly specific in different subgroups. All the BoSPL proteins contained motif one and motif two, except for BoSPL19, which was without motif one, and BoSPL7b and BoSPL18, which were without motif two. In contrast, the amino acid sequences of motif one and motif two were both predicted to be the conserved SBP domain (Supplementary Table S5), which indicates that all the BoSPL proteins contain a highly conserved domain.

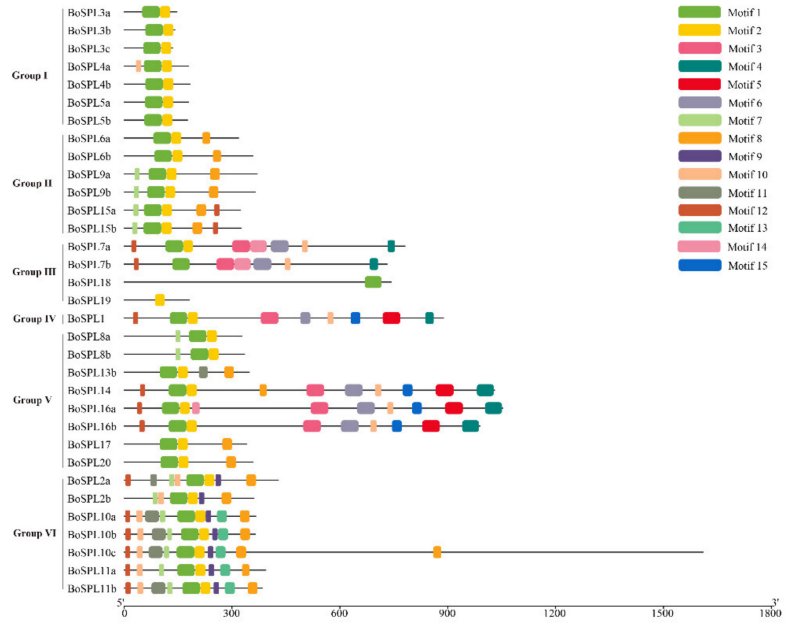

Figure 5. SPL proteins motifs. The motifs are shown as colored boxes. The scale on the bottom may be used to estimate the length of the motif (unit: amino acid).

3.4. Analysis of Putative Promoter Regions in BoSPL Genes

The gene expression patterns of stress response and tissue-specific expression are mainly regulated by *cis*-acting elements [32], and the *cis*-acting elements in the promoter are closed to various stress-responsive genes [33,34]. We found a long list of *cis*-acting elements in *BoSPLs*. There are three main types of *cis*-acting elements, including hormone response (ABRE, ERE, P-box, TCA-element, TGACG-motif and TGA-element), light response (GT1-motif, G-Box and MRE) and stress response (MBS, LTR, STRE and TC-rich repeats) (Supplementary Table S6). A total of 19 *BoSPLs* contain TCA-elements (salicylic acid response), 13 *BoSPLs* contain TGA-elements (auxin response) and 21 *BoSPLs* contain TGACG-motif (Methyl jasmonate salicylic acid response); these hormones are usually involved in signaling pathways for senescence and stress response, suggesting that *BoSPLs* are involved in the maturation of cabbage seeds. A total of 19 *BoSPLs* contain LTR (low temperature stress), 28 *BoSPLs* contain STRE (drought/salt stress response) and 17 *BoSPLs* contain TC-rich repeats (defense and stress responsiveness); these regulatory elements associated with environmental stress indicate that *BoSPLs* may respond to environmental stress.

3.5. Expression Profile of BoSPLs

To identify the tissue-specific expression profiles of *BoSPLs*, we analyzed the different expression levels of *BoSPLs* in seven organs/tissues (callus, root, stem, leaf, bud, flower and silique) by using the RNA-Seq dataset (GSE42891). These expression profiles of *BoSPLs* are presented using a heatmap (Figure 6; Supplementary Table S7). Only *BoSPL5b* showed organs/tissues-specific expression, which expressed only in the bud. Three *BoSPLs* (*BoSPL5a, BoSPL5b, BoSPL8a*) were not detected in the callus, five *BoSPLs* (*BoSPL4a, BoSPL4b, BoSPL5a, BoSPL5b, BoSPL13b*) were not detected in the root, *BoSPL5b* and *BoSPL13b* were not detected in the stem, the remaining *BoSPLs* were expressed in all the tissues. A total of twenty-one *BoSPLs* were expressed in the seven organs/tissues. The diversity of this expression patterns indicates that *BoSPLs* have a wide range of biological functions during the growth and development of cabbage.

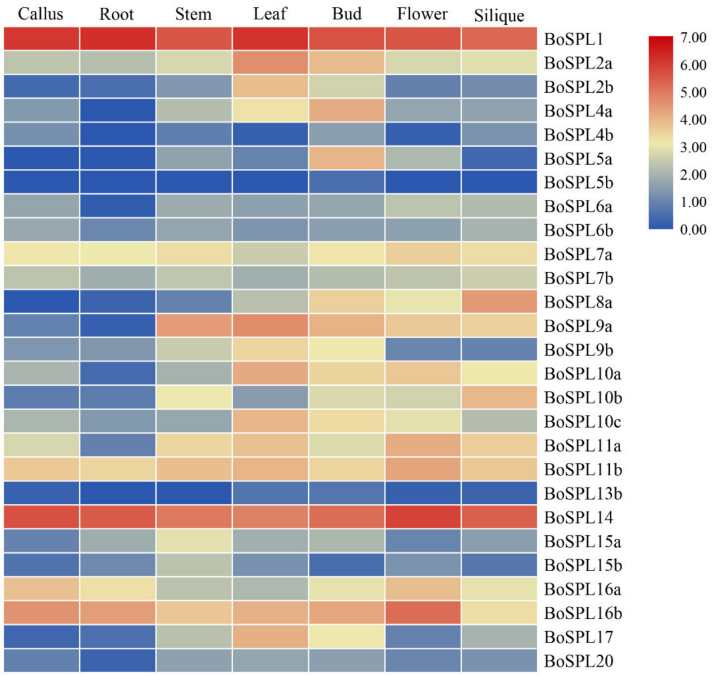

Figure 6. Expression profiles of *BoSPL* genes. Heat map representation of *BoSPL* genes in various organs/tissues, included callus, roots, stems, leaves, buds, flowers and siliques. Expression levels of the *BoSPL* genes are shown as the $\log_2$ (FPKM+1), transformed FPKM values obtained from the RNA-Seq data.

In addition, the expression patterns of *BoSPLs* in 'CS-D9' (chilling-sensitive cabbage) and 'CT-923' (chilling-tolerant cabbage) under 4 °C chilling stress were also analyzed (Figure 7; Supplementary Table S8; Supplementary Figure S2). A total of thirty-two *BoSPL* genes were detected in both 'CS-D9' and 'CT-923' under 6 h and 24 h chilling stress. In 'CS-D9', *BoSPL9b* and *BoSPL15a* were significantly up-regulated at 6 h, when compared with the mock-treated plants, and when chilling treated for 24 h, there were two *BoSPLs* (*BoSPL9b* and *BoSPL16a*) and three *BoSPLs* (*BoSPL3a, BoSPL4b* and *BoSPL10a*) significantly up- and down-regulated, respectively. In 'CT-923', compared with the mock-treated plants, two *BoSPLs* (*BoSPL9b* and *BoSPL10b*) and three *BoSPLs* (*BoSPL2b, BoSPL3a* and *BoSPL4b*) were significantly up- and down-regulated at 6 h, respectively, while five *BoSPLs* (*BoSPL1, -9a, -9b, -10b, -11b*) and two *BoSPLs* (*BoSPL3a* and *BoSPL4b*) were significantly up- and down-regulated at 24 h, respectively.

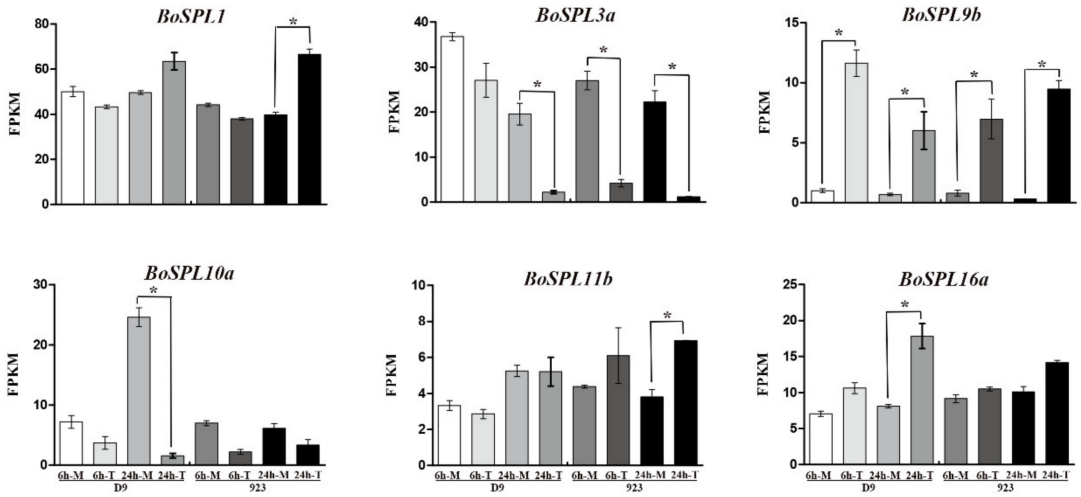

Figure 7. Expression profiles of six *BoSPL* genes under chilling stress. * indicates that the *p*-value was less than 0.05. Expression patterns of the thirty-two *BoSPL* genes under chilling stress are in Supplementary Figure S2.

4. Discussion

The *SPL* gene family is a plant-specific transcriptional regulator that has important regulatory functions in plant growth and development. There is no homology in humans, animals and bacteria [35]. With the completion of the genome sequencing of more and more plants, the *SPL* gene family has been identified and studied in many plants, including *A. thaliana*, *B. rapa* [10], *Betula platyphylla* Suk. [22], *Phaseolus vulgaris* [36] and *Castanea mollissima* [37]. Almost all the members of the family were found to be related to plant growth, morphological variation [38] and stress response [39–41]. *B. oleracea* var. *capitata* is an important cruciferous crop, and no systematic identification of the *SPL* gene family in *B. oleracea* was found. Therefore, it is significant to identify the *SPL* gene family in *B. oleracea* and its expression analysis under chilling stress.

In this study, a total of 33 *SPL* genes were identified in the *B. oleracea* genome, while there were 29 *BrSPLs* and 17 *AtSPLs* in *B. rapa* and *A. thaliana*, respectively. The difference in the number of *BoSPL*, *BrSPL* and *AtSPL* genes may be due to the *Brassica*-specific WGT and fractionation events [27]. Gene duplication leads to the functional differentiation and diversification of genes, which is thought to be the primary driver of evolution [42,43]. Studies have shown that the *SPL* gene family moves toward to an increasingly conservative direction after the encoding of the SPL domain has experienced replication events, forming and retaining multiple *SPLs* homologous branches [44,45], and multiple gene duplications occurred during evolutions [46]. Accompanied by genome triplication, we found ten *BoSPLs* had two separate orthologous genes, and two *BoSPLs* had three separate orthologous genes. In addition, we found evidence of *BoSPL* gene fractionation in *B. oleracea* after the split with *A. thaliana* from the recent common ancestor, with *BoSPL12* being lost.

These results indicated that the functional redundancy in *BoSPL* genes may lead to the loss of some gene copy. The *SPL* genes in *B. oleracea* are closely related to the similarity and conservation of *A. thaliana*. According to the structural similarity of the *SPL* gene family, it could be divided into six subgroups (Figure 1). The number of each subgroup in *B. oleracea*, *B. rapa* and *A. thaliana* was various; these showed that in different branches of subgroup, their retention of duplicates was different.

The functional diversities of *SPL* genes during plant growth and development may be related to the varieties of their protein motifs. The motifs of BoSPL proteins in *B. oleracea* were similar in the same subgroup, and the differences between the different subgroups were relatively large. BoSPL proteins contained motif one and motif two (Figure 3), except for BoSPL7b, BoSPL18 and BoSPL19, they only contain motif one or motif two. Some motifs only appear in specific subgroups, motif nine only in Group VI. The specific motifs may have unique functions, which indicates that many BoSPL proteins may experience functional differentiation, resulting in differences in different subgroups [47]. The exon/intron structure map of *B. oleracea* shows that the introns ranged from two to thirteen, while the number of introns in pepper was zero to eleven [48], and in moso bomboo was zero to ten [35]. While the number of introns in the same subgroup was similar, the structural variation could provide information for the further research. The *cis*-acting elements of the promoter region are closely related to the specific expression of genes and the stress response. We found a number of regulatory elements involved in hormone response, light response and stress response in the *BoSPLs* promoter regions of *B. oleracea*. The *cis*-acting elements of *BoSPLs* promoter regions differed significantly in the same subgroup, suggesting that the divergences in *BoSPLs*' function may be present in the promoter region and coding regions [47].

The *SPL* genes have essential roles in the regulation of plant growth and flowering, and exhibit species specificity. *OsSPL8* regulates the development of auricles unique to Gramineae [49], while it regulates male flower differentiation in Juglandaceae [45,50]. In this study, *BoSPL5b* showed organs/tissues-specific expression, which expressed only in the bud. It has been reported that *AtSPL14* has significant roles in plant architecture in *Arabidopsis* [51], while *BoSPL14* has high expression in the callus, root, stem, leaf, bud, flower and silique (Figure 6), which indicated that *BoSPL14* may also be involved in plant architecture. *AtSPL9* and *AtSPL15* have been reported to play redundant roles in reproductive transition and vegetative phase change [52]. In our study, *BoSPL9* and *BoSPL15* had two separate orthologous genes, *BoSPL9b, BoSPL15a* and *BoSPL15b* that had low expression in the flower and silique (Figure 6). Homology analysis is a relatively fast and effective way to understand the structure, function and evolution of unknown genes. We speculate that the homologous genes might have similar effects in *B. oleracea* through the function of *A. thaliana*, but the functions of these genes need further experimental verification.

Most of the *SPL* gene family had high expression levels at all stages of development [33]. In *A. thaliana*, *AtSPLs* were expressed in the roots, stems, leaves and floral organs (sepals, petals, carpels and stamens) [9]. Most of the *OsSPLs* were specifically expressed in young panicles in rice [8]. In *B. rapa*, more than half of the *BrSPLs* were expressed in the flowers more abundantly than in any other tissues [10], while in *B. oleracea*, the expression of only six *BoSPLs* (19.4%) was highest in the flower. It has been shown that *SPL* genes are involved in abiotic in several plants [53]. We found that *BoSPL9b* was up-regulated in both 'CS-D9' and CT-923 by chilling stress for 6 h and 24 h (Figure 7). In *A. thaliana*, sugar (such as glucose) was through inhibiting the expression abundance of miRNA156, to promote the transition of juvenile to adult stage; the expression of *SPLs* was then increased [48,54]. Whether the increase in *BoSPL9b* expression involved the chilling resistance of *B. oleracea* remains to be determined.

5. Conclusions

In this study, 33 *BoSPLs* were identified in the *B. oleracea* genome, according to the phylogenetic tree constructed with *B. rapa* and *A. thaliana*, they were further divided into

six subgroups. After the WGT and fractionation, ten *BoSPLs* retained double copies. The *BoSPLs* in one group have similar gene structure and protein motifs, which implies a potential similarity in the plant's biological functions. The expression patterns of seven organs/tissues showed that a large number of *BoSPLs* expressed in these organs/tissues. The RNA-Seq data analysis of chilling treatment indicated that the expression of *BoSPL9b* was up-regulated in 'CT-923' and 'CS-D9' at 6 h and 24 h chilling stress compared with the mock. The *cis*-acting elements analysis showed that *BoSPL9b* contained LTR in the upstream regions. Overall, this information will be important entry points for revealing potential candidate *BoSPLs* to participate in the response of cabbage to chilling stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11071445/s1, Table S1. The genomic sequences of the 33 *SPL* genes of *B. oleracea*; Table S2. The coding sequences of the 33 *SPL* genes of *B. oleracea*; Table S3. Amino acid sequences of 79 SPL proteins from *B. oleracea*, *B. rapa* and *A. thaliana*; Table S4. The characteristics of the 33 *SPLs* of *B. oleracea*; Table S5. Different motifs commonly observed in BoSPL proteins; Table S6. Known hormone-responsive, light-responsive and stress-responsive *cis*-acting elements in the promoter regions of *BoSPL* genes; Table S7. Expression of *BoSPL* genes in different organs/tissues of bud, callus, flower, leaf, root, silique and stem in *B. oleracea*; Table S8. Expression of *BoSPL* genes in leaves of chilling-sensitive ('CS-D9') and -tolerance ('CT-923') cabbage lines at chilling treat; Figure S1. Sequence logos of BoSPL proteins domains; Figure S2. Expression patterns of the thirty-two *BoSPL* genes under chilling stress.

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