Genome-Wide Identification and Characterization of SPL Family Genes in Chenopodium quinoa

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Abstract: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes encode a large family of plant-specific transcription factors that play important roles in plant growth, development, and stress responses. However, there is little information available on SPL genes in Chenopodiaceae. Here, 23 SPL genes were identified and characterized in the highly nutritious crop Chenopodium quinoa. Chromosome localization analysis indicated that the 23 CqSPL genes were unevenly distributed on 12 of 18 chromosomes. Two zinc finger-like structures and a nuclear location signal were present in the SBP domains of all CqSPLs, with the exception of CqSPL21/22. Phylogenetic analysis revealed that these genes were classified into eight groups (group I–VIII). The exon–intron structure and motif composition of the genes in each group were similar. Of the 23 CqSPLs, 13 were potential targets of miR156/7. In addition, 5 putative miR156-encoding loci and 13 putative miR157-encoding loci were predicted in the quinoa genome, and they were unevenly distributed on chromosome 1–4. The expression of several Cqu-MIR156/7 loci was confirmed by reverse transcription polymerase chain reaction in seedlings. Many putative cis-elements associated with light, stress, and phytohormone responses were identified in the promoter regions of CqSPLs, suggesting that CqSPL genes are likely involved in the regulation of key developmental processes and stress responses. Expression analysis revealed highly diverse expression patterns of CqSPLs among tissues. Many CqSPLs were highly expressed in leaves, flowers, and seeds, and their expression levels were low in the roots, suggesting that CqSPLs play distinct roles in the development and growth of quinoa. The expression of 13 of 23 CqSPL genes responded to salt treatment (11 up-regulated and 2 down-regulated). A total of 22 of 23 CqSPL genes responded to drought stress (21 up-regulated and 1 down-regulated). Moreover, the expression of 14 CqSPL genes was significantly altered following cadmium treatment (3 up-regulated and 11 down-regulated). CqSPL genes are thus involved in quinoa responses to salt/drought and cadmium stresses. These findings provide new insights that will aid future studies of the biological functions of CqSPLs in C. quinoa.

Keywords: Chenopodium quinoa; SPL gene family; Cqu-MIR156/7; promoter analysis; expression pattern; stress response

1. Introduction

The expression of genes plays a critical role in the growth and development of plants, and transcription factors (TFs) are essential for regulating gene expression. TFs are classified into several families according to their sequences, structures, domains, motifs, and other molecular features. SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) proteins are a family of plant-specific TFs that contain a conserved SBP domain of 76 amino acids. Two zinc finger sites are present in the N-terminal and C-terminal of the SBP domain, and the C-terminal zinc finger site overlaps with a nuclear localization signal (NLS) [1,2]. The first two SBP proteins, AmSBP1 and AmSBP2, were identified in snapdragon (Antirrhinum majus) based on their binding activity to the promoter region of the flower meristem identity genes.
gene SQUAMOSA [3]. Another two SBP-box genes, SPL3 and liguleless1 (LG1), were later described from Arabidopsis and maize, respectively. SPL3 is involved in floral transition [4], and LG1 is required for ligule and auricle formation during leaf development [5]. Additional SBP-box genes were later identified in Arabidopsis and rice [6,7]. A total of 4168 sequences encoding SBP proteins from green algae to land plants have been deposited in the database of plant TFs (http://planttfdb.gao-lab.org/, accessed on 2 March 2022). The SPL gene family has been analyzed at the genome-wide level in more than 60 species (Table S1), including 15 SPL genes in grape, 28 in poplar, 31 in maize, 15 in potato, 24 in buckwheat, and 56 in wheat [8–14]. More SPL genes will likely be discovered as more plant genome resources become available.

SPL was the first SPL gene to be functionally analyzed in Arabidopsis using a mutant. Three alleles of spl8 mutants were obtained, and all evidence indicated that SPL8 plays a role in microsporogenesis [15]. Overexpression analysis indicated that SPL8 is involved in gibberellin signaling [16]. SPL9 and SPL15 have redundant functions in regulating shoot maturation and plastochron length [17,18]. Similar to the proteins AmSBP1 and AmSBP2 in snapdragon, SPL3 and SPL9 promote flowering by directly binding to the cis-elements of the flowering genes LFY, FUL, API, and SCo1 [19,20]. In addition to the functions mentioned above, SPL genes play key roles in phase transitions [21,22], lateral root development [23], trichome formation [24–26], fertility [27,28], embryogenesis [29], and seedling growth [30]. In rice, SPL genes control grain size, shape, yield, and quality as well as plant architecture [31–34]. Rice yield can be enhanced by fine-tuning the expression of SPL genes [35]. In maize, SPL genes regulate bract development, primordium initiation, and domestication [36–38]. SPL genes regulate inflorescence morphogenesis, ovule development, and fruit ripening in tomato [39–42] and nodulation in soybean [43]. Recently, TaSPL3/17 have been shown to be involved in tilling/branching in wheat [44].

SPL genes control copper (Cu) homeostasis and the response to Cu deficiency [45,46], cadmium (Cd) tolerance [47,48], and iron (Fe) homeostasis [49]; they are also involved in responses to other abiotic and biotic stresses, such as cold, heat, salt, drought, and wounding, as well as hormone signaling [50–57]. Many SPL genes are targeted by miR156/7; for example, 10 out of 16 SPL genes in Arabidopsis and 11 out of 19 from rice contain miR156/7 complementary sequences. This miR156/7-SPL module is a regulatory hub that links plant developmental stage transitions with other biological processes [58].

Quinoa (Chenopodium quinoa Willd.) is an annual grain-like crop that was originally grown in South Africa [59]. It began to be cultivated and domesticated over 7000 years ago [60]. The planting area of quinoa has significantly increased over the past decades, and it is now grown in North and South America, Europe, and Asia. Quinoa has received increased attention because of its highly nutritious seeds, which are rich in essential amino acids, minerals, and vitamins; it is also gluten-free and possesses a superior ratio of protein, lipids, and carbohydrates compared with other grains and grain-like crops [61]. In addition, quinoa shows high tolerance to environmental stresses, such as drought, salinity, and low temperature [62]. This, coupled with its low nutrient requirements, enables it to grow on arid soil and marginal land. Quinoa was declared by the United Nations Food and Agricultural Organization as a major crop for ensuring world food security and sustainability under global climate change, and 2013 was designated as the year of quinoa [63]. The first chromosome-scale reference genome of quinoa was published in early 2017 [64], and this genomic resource has provided key insights into the mechanisms underlying agronomically important traits of quinoa. The genes involved in some key biological processes have been studied [65,66]. Some TF families, such as NAC, GRAS, ARF, bZIP, and WRKY, have been studied at the genome level in quinoa [59,67–70]. However, SPL genes, which comprise a conserved gene family that might play a role in quinoa growth, development, and stress responses, have not yet been studied at the genome scale. Here, we identified SPL genes in quinoa on a genome-wide scale, determined the chromosomal locations of these genes, and analyzed the structure, phylogenetic relationships, conserved motifs, cis-acting elements, and expression profiles of these genes in various tissues under normal
growth conditions. miR156/7 family genes were also identified, and their expression patterns in quinoa seedlings were examined. Putative targeted SPL genes were predicted. The expression responses of these quinoa SPL genes to salt, drought, and Cd stress were investigated to reveal their potential functions in stress tolerance. The results of this study provide new insights that enhance our understanding of the evolution and function of CqSPL genes. Our findings also contribute novel information that could be used to screen specific CqSPL genes involved in various developmental processes and the response to different types of abiotic stress.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions, and Stress Treatments

Quinoa plants were grown in a growth chamber under controlled conditions: 60% relative humidity, 418.2 ppm CO₂, a 16 h/8 h light/dark cycle, and temperature of 23 ± 1 °C. The light was produced from three band fluorescent lamps (T5/28W), and the light intensity was 120 µmol/m²/s. The plant samples were taken from two-week-old seedlings and seeds produced after flowering.

To determine the expression of CqSPL genes under stress conditions, quinoa seeds were germinated on moist filter paper in a growth chamber. Three-day-old uniform seedlings were transplanted into the holes of a 96-hole plate in a 2-L container filled with 1/2 Hoagland nutrient solution. On the seventh day after transplanting, the seedlings were subjected to different treatments: (1) control—seedlings were kept in nutrient solution throughout the experiment; (2) drought stress—seedlings were transferred to nutrient solution containing 15% PEG6000; (3) salt stress—seedlings were transferred to nutrient solution with 200 mM NaCl; and (4) Cd stress—seedlings were transferred to nutrient solution with 100 µM CdCl₂. Samples were collected 2 h after each treatment and stored at −80 °C until analysis.

2.2. Identification of SPL and MIR156/7 Family Members in the Quinoa Genome

SPL8 from Arabidopsis thaliana (AtSPL8) was used as a query to conduct BLAST searches against the Chemopodium DB (http://www.cbrc.kuak.edu.sa/chenopodiumdb/, accessed on 25 February 2022) and Phytozome 13 (http://www.phytozome-next.jgi.doe.gov/, accessed on 25 February 2022) to identify SPL genes in the quinoa genome. Twenty-three sequences homologous to AtSPL8 were recovered in the BLAST results, and each sequence was further confirmed using SMART (http://smart.embl-heidelberg.de/, accessed on 26 February 2022) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/, accessed on 26 February 2022) from the National Center for Biotechnology Information (NCBI) based on the presence of the SBP domain. Other SPLs from A. thaliana were also used as queries to conduct BLAST searches, but no additional sequences were recovered. The molecular weight (MW) and isoelectric point (pI) were predicted using a EXPASy Proteomics Server (https://web.expasy.org/compute_pi/, accessed on 5 March 2022). Subcellular localization prediction was performed using Plant-mSubP (http://www.bioinfo.usu.edu/Plant-mSubP/, accessed on 5 March 2022).

To search for MIR156/7 genes, precursor sequences of MIR156/7 from A. thaliana were used to conduct BLAST searches against the Chemopodium DB, and the secondary structures of the obtained sequences were predicted using MFold (http://www.mfold.org/, accessed on 20 March 2022). Cqu-MIR156 family members in PmiREN (http://www.pmiREN.com/, accessed on 25 March 2022) were individually compared with sequences obtained from our BLAST searches. All web softwares mentioned above were used with default parameters.

2.3. Location of Genes on Chromosomes

The physical map of the chromosomes of quinoa was scaled following Jarvis et al. [64]. The distribution of CqSPL and Cqu-MIR156/7 genes in the quinoa genome was determined using the ChenopodiumDB V 1.0 (V1 pseudomolecule) annotation database (https://www.
2.4. Prediction of CqSPL Genes Targeted by miR156/7

Based on information from *Arabidopsis*, most sequences targeted by miR156/7 are present in the coding region of the target genes, and this conserved region encodes six conserved amino acids, ALSLLS [27]. A search of the conserved ALSLLS sequence in 23 CqSPLs revealed that 12 CqSPLs contained this conserved amino acid sequence, suggesting that these 12 CqSPLs are putative targets of Cqu-miR156/7. The full-length genomic sequences of 23 CqSPL genes were analyzed using the psRNATarget online tool (http://plantgrn.noble.org/psRNATarget/, accessed on 25 April 2022) with default parameters. We detected another gene with this same target element in the 3′ UTR. Multiple sequence alignment of the miR156/7-targeted CqSPL genes and reverse-complement sequences of Cqu-miR156/7 was performed using the ClustalW method in MEGA11. This same method was used to compare the miR156/7 sequences from both *A. thaliana* and *C. quinoa*.

2.5. Sequence Alignment and Phylogenetic Analysis

*AISPL* sequences were obtained from the TAIR home page (http://www.arabidopsis.org/, accessed on 10 February 2022). CqSPL sequences were downloaded from Phytozome (http://www.phytozome-next.jgi.doe.gov/, accessed on 10 February 2022). The phylogenetic tree was generated using ClustalW alignment and the neighbor-joining method in MEGA 11 with 1000 bootstrap replicates and provided settings (Jones–Taylor–Thornton model, uniform rates, pairwise deletion, and number of threads 7). The orthologship of the genes in the phylogenetic tree was confirmed as reciprocal best hits using BLAST in NCBI.

2.6. Gene Structure and Conserved Motifs

To clarify the structures of CqSPL genes, genomic sequences and coding sequences (CDSs) were downloaded from Phytozome and the Chenopodium DB. Graphs of exon–intron structures were obtained using GSDS 2.0 (gsds.gao-lab.org/, accessed on 13 April 2022) by inputting genomic sequences and related CDSs. The MEME online tool (http://meme-suite.org/tools/meme/, accessed on 20 April 2022) was used to analyze the full-length CqSPL proteins to predict the conserved motifs using the following parameters: motif width, 10 to 50; maximum number of conserved motifs, 20; and site distribution, zero or one occurrence per sequence. The SBP-domain sequence logo was prepared using the WebLogo online tool (weblogo.berkley.edu/logo.cgi/, accessed on 25 April 2022).

2.7. Identification and Analysis of Promoters

The 3.0-kb promoter sequences of the 23 CqSPL genes were retrieved from the Chenopodium DB. MEME (http://meme-suite.org/tools/meme/, accessed on 15 April 2022) was used to predict the conserved motifs; the maximum number of motifs was set to 30 with a motif width of 6 to 50. Zero or one occurrence per sequence was chosen as the site distribution. The promoters were also analyzed using PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html/, accessed on 20 April 2022) and the New PLACE database (https://www.dna.affrc.go.jp/PLACE/, accessed on 20 April 2022) to identify cis-regulatory sequences [71].

2.8. RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from harvested samples using an RNeasy Plant Mini Kit (Qiagen), following the manufacturer’s instructions. The RNA quality and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and 2.0 µg of total RNA was used for first-strand cDNA synthesis with a reverse-transcription kit (Takara Bio Group, Shiga, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a BIO-RAD CFX Connect™
Real-Time System with ChamQTM Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The primer sequences used are listed in Table S2. The total volume of the qRT-PCR reaction was 20 µL, and it contained 10 µL of 2× SYBR qPCR Master Mix, 0.8 µL of primers (10 µM), 2 µL of cDNA templates, and 6.4 µL of ddH2O. The reaction was conducted under the following thermal cycling conditions: 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, and 60 °C for 31 s. CqACT2 was used as an internal control. The experiments were conducted in three biological replicates. The relative expression levels of genes were calculated using the 2^−ΔΔCT method.

2.9. Data Analysis

Statistical analyses were carried out by one-way ANOVA, followed by Tukey’s HSD test with SPSS v16.0. All data are shown as mean ± SE, and statistical significance was considered at p < 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).

3. Results

3.1. Identification of SPL Family Genes in the Quinoa Genome

In the quinoa genome, 23 SPL genes were retrieved using BLASTP. Two orthologous genes of AtSPL8 were obtained and designated as CqSPL8A and CqSPL8B. Other genes were named CqSPL1 to CqSPL22 based on their order of appearance in the BLAST results. CqSPL8A was located on chromosome 1, and CqSPL8B was located on chromosome 10. Other CqSPLs were unevenly distributed on chromosome 3, 6–12, and 14–16 (Figure 1). The predicted CDSs of the CqSPL genes ranged between 609 and 3570 bp, and the deduced length of the proteins ranged from 202 to 1189 amino acids (Table 1, Supplementary File S1). A short version of the two CqSPL sequences, which have 849-bp and 861-bp CDSs, respectively (Table 1). The predicted MW of these CqSPLs ranged from 21.12 to 132.14 kD, and the pI that ranged from 5.74 to 9.81. CqSPL3, CqSPL4, and CqSPL5 were predicted to be localized to both the nucleus and cytoplasm; the rest of the CqSPLs were exclusively localized to the nucleus (Table 1).

![Figure 1. Chromosomal locations of CqSPL and Cqu-MIR156/7 genes. The scale bar represents 10 megabases (Mb).](image-url)
Table 1. Molecular features of SPL genes identified from the genome of C. quinoa.

| Gene Name | Gene ID     | CDS (bp) | Amino Acids | MW (kD) | pI   | Subcellular Localization |
|-----------|-------------|----------|-------------|---------|------|-------------------------|
| CqSPL1    | AUR62028919 | 1389     | 462         | 50.27   | 8.90 | Nucl                    |
| CqSPL2    | AUR62005629 | 1389     | 462         | 50.31   | 8.67 | Nucl                    |
| CqSPL3    | AUR6202563  | 2937     | 978         | 108.45  | 6.44 | Nucl/Cyto               |
| CqSPL4    | AUR62029983 | 3570     | 1189        | 132.14  | 7.01 | Nucl/Cyto               |
| CqSPL5    | AUR62029984 | 2943     | 980         | 108.87  | 6.30 | Cyto/Nucl               |
| CqSPL6    | AUR62019452 | 609      | 202         | 21.12   | 9.81 | Nucl                    |
| CqSPL7    | AUR62024322 | 1050     | 349         | 37.73   | 7.64 | Nucl                    |
| CqSPL8A   | AUR62004146 | 849      | 282         | 31.95   | 9.41 | Nucl                    |
| CqSPL8B   | AUR62013707 | 861      | 286         | 32.59   | 9.41 | Nucl                    |
| CqSPL9    | AUR62012061 | 1122     | 373         | 39.56   | 8.45 | Nucl                    |
| CqSPL10   | AUR62011728 | 2955     | 984         | 110.16  | 5.76 | Nucl                    |
| CqSPL11   | AUR62029416 | 1044     | 347         | 38.02   | 8.76 | Nucl                    |
| CqSPL12   | AUR62039662 | 1638     | 545         | 60.40   | 6.26 | Nucl                    |
| CqSPL13   | AUR62032118 | 1689     | 562         | 62.08   | 6.87 | Nucl                    |
| CqSPL14   | AUR62003425 | 1050     | 349         | 38.42   | 8.74 | Nucl                    |
| CqSPL15   | AUR62042534 | 2769     | 922         | 101.71  | 6.75 | Nucl                    |
| CqSPL16   | AUR62035190 | 3321     | 1106        | 121.76  | 7.06 | Nucl                    |
| CqSPL17   | AUR62005645 | 1080     | 359         | 39.00   | 9.36 | Nucl                    |
| CqSPL18   | AUR62028905 | 954      | 317         | 34.78   | 9.05 | Nucl                    |
| CqSPL19   | AUR62003075 | 1500     | 499         | 55.39   | 5.89 | Nucl                    |
| CqSPL20   | AUR62007890 | 1272     | 423         | 46.75   | 6.57 | Nucl                    |
| CqSPL21   | AUR62042853 | 2151     | 716         | 80.13   | 6.32 | Nucl                    |
| CqSPL22   | AUR62042654 | 2124     | 707         | 79.25   | 5.74 | Nucl                    |

In addition, consistently with findings in other plant species, we found that 13 of 23 CqSPL genes contained miR156/7-targeted elements, and all were located in the coding regions, with the exception of CqSPL6, which had the mir156/7-targeted sequence in its 3′UTR (Figure 2A).

Accordingly, 18 MIR156/7 genes in the quinoa genome were revealed (Supplementary File S2), including 16 that had previously been added to the PmiREN database (https://pmiren.com/, accessed on 2 April 2022). We renamed these MIR156/7 genes based on the mature sequences of miR156/7; miR157 subfamily sequences contain a U at the tenth position from the 3′ end, and miR156 subfamily sequences contain a U/A at the seventh position from the 3′ end (Figure 3). Five MIR156 genes were named Cqu-miR156a to Cqu-miR156e. The other 13 were MIR157 subfamily genes and were named Cqu-miR157a to Cqu-miR157m (Table 2). Eleven Cqu-MIR156/7 genes were located on chromosome 4, and the Cqu-MIR157a-d cluster and Cqu-MIR157e-h cluster are duplicates of each other. Cqu-MIR156a and Cqu-MIR157l were located near both ends of chromosome 4, and Cqu-MIR156b was located below the Cqu-MIR157e-h cluster. Cqu-MIR156e and Cqu-MIR156c were located on chromosome 2 and 3. Cqu-MIR156d, Cqu-MIR157i-k, and Cqu-MIR157m were located on chromosome 1 (Figure 1). Cqu-MIR156e and Cqu-MIR157m were newly discovered in this study. The precursor sequences of these two miRNAs can both form a hairpin structure with the mature sequence of miR156/7 on the stem (Figure 2B).

We examined the expression of several Cqu-MIR156/7 loci in quinoa seedlings. All the Cqu-MIR156/7 genes examined were expressed in the seedlings (Figure S1).

3.2. Phylogeny of CqSPL Genes

To explore the evolutionary relationship between the SPL genes from both quinoa and A. thaliana, a phylogenetic tree of 23 CqSPLs and 17 AtSPLs was constructed based on the conserved SBP-domain sequences (Figure 4). The tree can be divided into eight groups, with at least one gene from both species in each group. Group I contained four genes (CqSPL12, CqSPL13, CqSPL19, and CqSPL20) from C. quinoa and only one gene (AtSPL6) from A. thaliana. Group II had four genes: two (CqSPL7 and CqSPL9) from C.
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quinoa and two (AtSPL9 and AtSPL15) from A. thaliana. Three genes (AtSPL11, AtSPL10, and AtSPL2) from A. thaliana and two genes (CqSPL1 and CqSPL2) from C. quinoa were present in Group III. Two SPL13 duplicates (AtSPL13A and AtSPL13B) from A. thaliana and four CqSPL genes (CqSPL17, CqSPL18, CqSPL11, and CqSPL14) from C. quinoa were present in Group IV. Group V comprised SPL8 orthologs (CqSPL8A, CqSPL8B, and AtSPL8). There were ten genes in group VI: six (CqSPL3, CqSPL4, CqSPL5, CqSPL10, CqSPL15, and CqSPL16) from C. quinoa and four (AtSPL1, AtSPL2, AtSPL14, and AtSPL16) from A. thaliana. Group VII contained four genes: three genes (AtSPL3, AtSPL4, and AtSPL5) from A. thaliana and one gene (CqSPL6) from C. quinoa. Group VIII comprised three genes: two (CqSPL21 and CqSPL22) from C. quinoa and one (AtSPL7) from A. thaliana. The genes in group I–IV and group VII were targets of miR156/7 in both species (Figure 2A), and the genes in the other groups were not targets of miR156/7. Ten genes from group VII encoded large proteins consisting of more than 800 amino acids. AtSPL7 in group VIII encoded a protein of 818 amino acids, but the orthologs CqSPL21 and CqSPL22 in this group encoded proteins of approximately 710 amino acids. The length of the other SPLs from both species was less than 600 amino acids. Phylogenetic analysis revealed that every group of CqSPL genes had at least one ortholog in A. thaliana.

A

![Hairpin structures predicted for Cqu-miR156e and Cqu-miR157m by MFold](http://www.mfold.org/, accessed on 5 April 2022). The mature sequences of miR156e and mi157m on the stems are indicated by red lines.

Figure 2. Sequence alignment of miR156/7 complementary sequences within CqSPL genes and predicted stem-loop hairpin structures. (A) Sequence alignment of miR156/7-targeted elements located in the coding regions of CqSPLs, with the exception of CqSPL6, which has the miR156/7-targeted sequence in its 3′ UTR. Reverse complementary sequences of the mature miR156/7 are shown below the alignment for comparison. (B) Hairpin structures predicted for Cqu-miR156e and Cqu-miR157m by MFold (http://www.mfold.org/, accessed on 5 April 2022). The mature sequences of miR156e and mi157m on the stems are indicated by red lines.
Figure 3. Sequence alignment of mature Cqu-miR156/7 and Ath-miR156/7. A U/A is present at the seventh position from the 3′ end of miR156 subfamily members, and a U is present at the tenth position from the 3′ end of miR157 subfamily members. The asterisk indicates the conserved nucleotide.

Table 2. List of identified miR156/7 loci in Chenopodium quinoa.

| Name           | Chr. | Precursor | Length (bp) | Position Database | Original Name  |
|----------------|------|-----------|-------------|-------------------|----------------|
| Cqu-MIR156a    | 4    | 166       | 1458734-1458899 | PmiREN*          |                |
| Cqu-MIR156b    | 4    | 85        | 13677430-13677514 | PmiREN         |                |
| Cqu-MIR156c    | 3    | 197       | 75723156-75723352 | PmiREN      |                |
| Cqu-MIR156d    | 1    | 86        | 107403506-107403591 | PmiREN     |                |
| Cqu-MIR156e    | 2    | 87        | 22325967-22326053 | PmiREN     |                |
| Cqu-MIR156f    | 4    | 87        | 6735955-6736041  | PmiREN     |                |
| Cqu-MIR156g    | 4    | 82        | 6736193-6736274  | PmiREN     |                |
| Cqu-MIR156h    | 4    | 87        | 6743900-6743986  | PmiREN     |                |
| Cqu-MIR156i    | 4    | 84        | 6744076-6744159  | PmiREN     |                |
| Cqu-MIR156j    | 4    | 87        | 6786472-6786558  | PmiREN     |                |
| Cqu-MIR156k    | 4    | 82        | 6794596-6794680  | PmiREN     |                |
| Cqu-MIR156l    | 4    | 83        | 115037617-115037699 | PmiREN |                |
| Cqu-MIR156m    | 4    | 87        | 6794506-6794596  | PmiREN     |                |

3.3. Structure of CqSPL Genes and Domain/Motif Analysis of Their Proteins

To compare the 23 CqSPL genes directly, their exon–intron structures were first predicted. In general, the number of introns within the coding regions of CqSPL genes ranged from 2 to 14. Eight genes (CqSPL6, CqSPL8A, CqSPL8B, CqSPL11, CqSPL12, CqSPL13, CqSPL14, and CqSPL20) had two introns, and two genes (CqSPL4 and CqSPL15) had fourteen introns. The largest intron was intron 2 (8584 bp), which was present in CqSPL19, and the smallest intron was intron 5 (75 bp), which was present in CqSPL16. The widest exon was present in CqSPL12 (3854 bp), and the shortest exon was present in CqSPL15 (exon 9, 24 bp). SBP-box sequences in most CqSPLs spanned two exons, but they were present in only one exon in a subset of large CqSPLs (CqSPL3, CqSPL4, and CqSPL5 in group VI). One SBP-box sequence in CqSPL6 spanned three exons (Figure 5). Several pairs of CqSPL genes were identified, including CqSPL1/2, CqSPL8A/B, CqSPL11/14, and CqSPL12/13. Each pair of CqSPL genes had the same number of introns with the same intron phase and were in the same group (Figure 4).
Table 2. List of identified miR156/7 loci in Chenopodium quinoa.

| Name          | Chr. | Precursor Length (bp) | Position            | Database    | Original Name            |
|---------------|------|-----------------------|---------------------|-------------|--------------------------|
| Cqu-MIR156a   | 4    | 166                   | 1458734-1458899     | PmiREN *    | Cqu-MIR156a              |
| Cqu-MIR156b   | 4    | 85                    | 1367430-13677514    | PmiREN      | Cqu-MIR156b              |
| Cqu-MIR156c   | 3    | 197                   | 75723156-75723352   | PmiREN      | Cqu-MIR156c              |
| Cqu-MIR156d   | 1    | 86                    | 107403506-107403591 | PmiREN      | Cqu-MIR156d              |
| Cqu-MIR156e   | 2    | 87                    | 22325967-22326053   | This study  |                          |
| Cqu-MIR157a   | 4    | 87                    | 6735955-6736041     | PmiREN      | Cqu-MIR156e              |
| Cqu-MIR157b   | 4    | 82                    | 6736193-6736274     | PmiREN      | Cqu-MIR156f              |
| Cqu-MIR157c   | 4    | 87                    | 6743900-6743986     | PmiREN      | Cqu-MIR156g              |
| Cqu-MIR157d   | 4    | 84                    | 6744076-6744159     | PmiREN      | Cqu-MIR156h              |
| Cqu-MIR157e   | 4    | 87                    | 6786472-6786558     | PmiREN      | Cqu-MIR156i              |
| Cqu-MIR157f   | 4    | 82                    | 6786710-6786791     | PmiREN      | Cqu-MIR156j              |
| Cqu-MIR157g   | 4    | 87                    | 6794420-6794506     | PmiREN      | Cqu-MIR156k              |
| Cqu-MIR157h   | 4    | 85                    | 6794596-6794680     | PmiREN      | Cqu-MIR156l              |
| Cqu-MIR157i   | 1    | 83                    | 115037617-115037699 | PmiREN      | Cqu-MIR156m              |
| Cqu-MIR157j   | 1    | 87                    | 115046902-115046988 | PmiREN      | Cqu-MIR156n              |
| Cqu-MIR157k   | 1    | 144                   | 46037964-46038107   | PmiREN      | Cqu-MIR156o              |
| Cqu-MIR157l   | 4    | 132                   | 52526028-52526159   | PmiREN      | Cqu-MIR156p              |
| Cqu-MIR157m   | 1    | 85                    | 115031692-115031776 | This study  |                          |

* Plant MicroRNA Encyclopedia (https://pmiren.com).

Figure 4. Phylogenetic analysis of SPLs from C. quinoa and A. thaliana. The phylogenetic tree was constructed based on the SBP-box domain sequences with MEGA 11 using the neighbor-joining method and 1000 bootstrap replicates. The defined groups were labeled with different colors and filled circles.
The domain conserved among all CqSPLs was the SBP domain, which comprised 76 amino acids; in CqSPL21 and CqSPL22, several parts of the SBP domain were missing (Figure 6A). As in other species, the SBP domain of CqSPLs contained two zinc finger structures: one (Zn1, CCCH) in the N-terminal and another (Zn2, CCHC) in the C-terminal. The conserved bipartite NLS overlapped with the second zinc finger structure (Zn2) (Figure 6A,B). The CqSPL21 had 17 fewer amino acids around the Zn2 motif, and 32 amino acids were absent around both the Zn1 and Zn2 motifs in CqSPL22 (Figure 6A).

Additional motifs were identified in CqSPLs using MEME. Twenty motifs were identified from all CqSPLs, and the SBP-box domain was present in the connected motifs 3, 1, and 4. Of the 20 motifs, 18 were identified in the 3 large proteins CqSPL3, 4, and 5. CqSPL10 and CqSPL16 both had 13 motifs, and CqSPL15 had 11 motifs. No additional motifs were detected in CqSPL6 aside from motifs 3, 4, and 1 (SBP domain). There was only one motif (motif 8) that overlapped with the N-terminal part of the SPL8-specific motif (RIGLNLGRTYF from AtSPL8) upstream of the SBP domain in CqSPL8A and CqSPL8B. The motif composition was similar among CqSPLs from the same group; for example, CqSPL1 and CqSPL2 in Group III (Figure 4) each had an upstream motif (motif 15) and downstream motif (motif 11 containing the miR156/7 target site) with the SBP domain, as well as the connected motifs 3, 1, and 4 containing the SBP-box domain. CqSPL21 and 22 from group VIII had 3 motifs (motifs 2, 9, and 10) downstream of their partial SBP domains (motifs 3 and 4, Figure 7).

### 3.4. Promoter Analysis of CqSPL Genes

Cis-acting elements are required for the proper expression of genes. We studied the motifs and cis-elements in the 3.0-kb promoters of 23 CqSPL genes (Supplementary File S3). We first generated a phylogenetic tree of these promoters (Figure 8A). Promoters from gene pairs or genes from the same group in Figure 4 were generally clustered (Figure 8A). Multiple motifs were determined in both strands of the CqSPL promoters using MEME. The number of the motifs ranged from 7 (CqSPL20) to 23 (CqSPL14). Of the 23 CqSPL promoters,
17 had 10 or more motifs. The spatial distribution of these motifs was similar within promoter groups but differed among promoter groups. For example, the CqSPL3/4/5 group contained 12 or 16 motifs in their promoters, and most of these motifs were located within 1 kb upstream (−1 to 0 kb) of the start codon, and motif 22 and 23 in the CqSPL11/14 group occurred further upstream (−3 to −1 kb) of the promoters (Figure 8A,B).

Figure 5. Gene structure of SPL family members in C. quinoa. The exons and introns are indicated by blue boxes and black horizontal lines, respectively. The pink color indicates the SBP box, and the numbers refer to the intron phase.

Figure 6. SBP-box domain alignment of CqSPLs. (A) Multiple alignment of SBP-box domains of CqSPL proteins. All CqSPL proteins contain an SBP-box domain of 76 amino acids, with the exception of CqSPL21 and CqSPL22, which have some missing parts of the SBP-box domain. The two conserved zinc finger motifs (Zn1 and Zn2) and the nuclear localization signal (NLS) are indicated. The asterisk indicates the conserved nucleotide. (B) The sequence logo of SBP-box domains (with the exception of that from CqSPL21 and CqSPL22) was prepared using the WebLogo online tool. The overall height of the stack reflects the extent of sequence conservation at that position, and the height of the letters within each stack indicates the relative frequency of each amino acid at that position.

To obtain detailed information on the cis-elements or motifs in the CqSPL promoters, PlantCARE software was used to analyze promoter sequences. A total of 94 types of cis-elements were identified, and they were unevenly distributed in the 3.0-kb upstream region of the CqSPL genes. The copy number of these cis-elements ranged from 171 in CqSPL22 to 272 in CqSPL8B. The basic cis-elements TATA-box and CAAT-box were the most common in these motifs; for example, 115 and 81 copies of TATA-box and CAAT-box were present in CqSPL8A, and 42 TATA-boxes and 54 CAAT-boxes were present in CqSPL16. Other cis-elements were classified into several categories: light responsiveness, development, hormone responsiveness, stress responsiveness, elicitor induction, and unknown function (Table S3). Cis-elements involved in stress responsiveness, including abiotic and biotic stress, were detected in all 23 CqSPL promoters and were further validated in New PLACE database. MYB and MYC elements are drought-responsive and dehydration-responsive elements, respectively, and both were present in the 23 CqSPL promoters (Table S4). The copy numbers of these cis-acting elements in each promoter are shown in Figure 9. All these promoters contained more than 20 copies of these elements, with the exception of CqSPL8A, which had only 12 copies. A maximum of 43 copies of these elements were detected in CqSPL7, and 40 were present in both CqSPL9 and CqSPL10 (Figure 9 and Table S5). These data suggest that CqSPL genes play a role in developmental processes and stress responses.
Figure 7. Distribution of conserved motifs in SPL family members of *C. quinoa*. Motifs indicated by different colored boxes were identified using the MEME online tool (meme-suite.org/, accessed on 2 May 2022). A total of 20 motifs were identified, and the conserved SBP-box domain was embedded in the connected motifs 3, 1, and 4.
Multiple motifs were determined in both strands of the CqSPL promoters using MEME. The number of the motifs ranged from 7 (CqSPL20) to 23 (CqSPL14). Of the 23 CqSPL promoters, 17 had 10 or more motifs. The spatial distribution of these motifs was similar within promoter groups but differed among promoter groups. For example, the CqSPL3/4/5 group contained 12 or 16 motifs in their promoters, and most of these motifs were located within 1 kb upstream (−1 to 0 kb) of the start codon, and motif 22 and 23 in the CqSPL11/14 group occurred further upstream (−3 to −1 kb) of the promoters (Figure 8A,B).

![Figure 8. Phylogenetic analysis and motif distribution of CqSPL promoters. (A) A phylogenetic tree generated based on 3-kb promoters of 23 CqSPL genes with MEGA 11 using the neighbor-joining method and 1000 bootstrap replicates. (B) The motifs in the promoters were identified using the MEME online tool (meme-suite.org/, accessed on 4 May 2022). The 30 motifs identified are indicated by different colored boxes. The consensus sequence of each motif is shown at the bottom of the figure.](image)

3.5. Tissue Expression Patterns of CqSPL Genes

To have a general idea where the CqSPL genes function, the tissue expression patterns of the CqSPL genes were determined by performing qRT-PCR on samples taken from several tissues, including root, seedling, stem, leaf, flower, and seed tissue. The expression level of most CqSPL genes was lower in the roots and higher in flowers and leaves. The expression of some CqSPL genes was higher in flowers compared with other tissues, such as CqSPL6, CqSPL8A, CqSPL8B, CqSPL11, and CqSPL14. CqSPL21 was most highly expressed in the leaves, CqSPL4 and CqSPL17 were most highly expressed in seeds, and CqSPL18 was most highly expressed in seedlings. Variation in the expression levels of the other genes among tissues was low. Genes with similar motif compositions in their promoters
had similar expression patterns (Figures 8 and 10). For example, CqSPL1/2, CqSPL8A/8B, CqSPL11/14, and CqSPL15/16 were all from the same group and had similar motif compositions and expression patterns (Figures 4 and 8). However, this was not the case for the CqSPL3/4/5 group, as CqSPL4 was highly expressed in flowers and seeds, CqSPL3 was highly expressed in leaves, and CqSPL5 was highly expressed in flowers (Figure 10). These variable expression patterns indicate that the CqSPLs involved in development and other physiological processes are functionally diverse.

Figure 9. Cis-element analysis of CqSPL promoters involved in stress responses. Cis-elements associated with similar functions are shown in the same color.

3.6. Expression of CqSPL Genes under Drought, Salt, and Cd Stress

To evaluate the functions of the CqSPL genes in response to various types of stress, qRT-PCR was performed after quinoa seedlings were exposed to salt (NaCl), drought (PEG), and Cd treatment. Comparison of the expression level between the control and stress-treated seedlings revealed that the expression of 13, 22, and 14 CqSPL genes significantly responded to salt, drought, and Cd treatment, respectively. In the salt treatment, the expression of 11 genes was up-regulated, and the expression of 2 genes was down-regulated. Of the 11 up-regulated genes, the expression of CqSPL8B, CqSPL11, and CqSPL18 was up-regulated more than 2.5, 2.2, and 8.2-fold, respectively, and the expression of the two down-regulated genes CqSPL6 and CqSPL19 was decreased more than 9.7 and 2.4-fold, respectively (Figure 11A). In the drought stress treatment, the expression of 21 CqSPL genes was up-regulated, and the expression of CqSPL3, CqSPL4, CqSPL5, CqSPL12, and CqSPL18 was increased more than 15.1, 14.0, 30.6, 13.2, and 27.0-fold, respectively. The expression of all other up-regulated genes, with the exception of CqSPL1 and CqSPL2, was increased by at least more than 2-fold. The expression of CqSPL6 was down-regulated more than 2.4-fold in the drought treatment (Figure 11B). The expression of only three CqSPL genes (CqSPL4, CqSPL17, and CqSPL18) was up-regulated in the Cd treatment, and the expression of these three genes was up-regulated 1.6, 1.9, and 10.1-fold, respectively. The expression of 11 CqSPL genes was down-regulated; the expression of CqSPL6 and CqSPL22 was decreased more than 4.5 and 2.6-fold, respectively, and the expression of the other 9 down-regulated CqSPL genes was decreased by less than 2-fold (Figure 11C). Generally, the expression of CqSPL4, CqSPL18, and CqSPL6 responded to all three types of stress and exhibited similar expression patterns; the expression of CqSPL4 and CqSPL18 was up-regulated, and the expression of CqSPL6 was down-regulated. These findings indicated that many CqSPL genes are involved in the response to two types of stress.
CqSPL3 was highly expressed in leaves, and CpSPL5 was highly expressed in flowers (Figure 10). These variable expression patterns indicate that the CqSPLs involved in development and other physiological processes are functionally diverse.

Figure 10. Expression patterns of CqSPL genes under normal growth conditions. The expression levels of 23 CqSPL genes were determined in different tissues at different developmental stages, and the fold change in the expression level is shown for each CqSPL. The expression level in root tissue was arbitrarily set to 1. CqACTIN2 was used as an internal control. Different letters indicate significant differences in expression.

3.6. Expression of CqSPL Genes under Drought, Salt, and Cd Stress

To evaluate the functions of the CqSPL genes in response to various types of stress, qRT-PCR was performed after quinoa seedlings were exposed to salt (NaCl), drought (PEG), and Cd treatment. Comparison of the expression level between the control and...
Figure 11. Expression profiles of CqSPL genes under salt, drought, and Cd treatments as determined by qRT-PCR. (A) 2-h treatment with 200 mM NaCl. (B) 2-h treatment with 15% PEG6000. (C) 2-h treatment with 100 µM CdCl₂ (Cd). The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method. The expression level of the control (CK) was arbitrarily set to 1. Error bars indicate standard deviations of the mean value from three biological replicates and three technical replicates. Asterisks indicate significant differences in expression between control and stressed samples (* $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$).
4. Discussion

Since the first two SBP-box genes were identified in snapdragon [3], the number of SPL genes identified has significantly increased in many plant species. The SBP-box family is now a large plant gene family. SPL genes have only been detected in green plants to date, including green algae and land plants. Phylogenetic analysis has shown that the SPL genes of green algae and land plants form a monophyletic group and that each lineage of SPL genes has undergone duplication events followed by divergence; this suggests that SPL genes might have originated from a common ancestor of green plants and that the origin of SPL genes might predate the divergence between green algae and the ancestors of land plants [72,73]. Analysis of CqSPL proteins using SMART (http://smart.embl-heidelberg.de/, accessed on 4 March 2022) and grouping of proteins with similar domain architecture recovered a sequence with an SBP domain comprising 84 amino acid residues in the bacterium TMED181, and this sequence was similar to a sequence in green algae Micromonas commode (Supplementary File S4). This potentially suggests that SPL genes might also occur in bacteria. However, additional work is needed to confirm this finding, given that this sequence might be derived from the contamination of bacterial samples. Thus, the origin of the SPL genes still remains ambiguous.

In this study, a total of 23 CqSPL genes were identified in the quinoa genome, and these genes were clustered into 8 groups with 17 members from Arabidopsis (Figure 4). Similar groupings of SPL genes have been reported in petunia, tomato, tartary buckwheat, sugarcane, and Jatropha curcas; however, the group order of these genes might vary among species [13,74–77]. These data suggest that CqSPL genes might have originated from their close relatives. Quinoa is an allotetraploid plant (2n = 4x = 36) that might be derived from a cross between two diploids such as Chenopodium pallidicaule (2n = 2x = 18, A sub-genome) and Chenopodium suecicum (2n = 2x = 18, B sub-genome). We identified 10 and 12 SPL genes in C. pallidicaule and C. suecicum from the Phytozome database (https://phytozome-next.jgi.doe.gov/, accessed on 25 May 2022), respectively (Supplemental File S5), and this might provide insight into the distribution and divergence of CqSPL genes in the quinoa genome.

Notably, two studies examining the evolution of SPL genes have suggested that SPL genes from land plants can generally be divided into two distinct groups. Group I members generally occur in single copies or a low number of copies in each species; group II contains several members in each species and can be further classified into several subgroups [72,73]. One marked difference between group I and II members is that the N-terminal zinc finger of the SBP domain is C4 (CysCysCysCys) in group I but C3H (CysCysCysHis) in group II (the C-terminal zinc finger is C2HC in both group I and II). AtSPL7 from Arabidopsis belongs to group I. It plays an important role in Cu homeostasis [45] and is functionally related to CRR1 in the single-celled green alga Chlamydomonas reinhardtii [78], but CRR1 possesses a C3H zinc-finger in the N-terminal of the SBP domain, similar to other SPL members in green algae (Figure S2) and group II members in land plants. In our phylogenetic analysis, AtSPL7, CqSPL21, and CqSPL22 were classified into group VIII (Figure 4). Although CqSPL21 and CqSPL22 only contained a partial SBP domain, CqSPL21 still had a full N-terminal zinc finger C4 (Figure 6). A deletion of the same region within the SBP domain of CqSPL22 has also been observed in C. pallidicaule (A sub-genome contributor), but this was not the case for CqSPL21, as its ortholog in C. suecicum (B sub-genome contributor) has the full-length SBP domain. No deletion has been observed in other members of this group, including orthologs from Beta vulgaris and Spinacia oleracea (Figure S2, Supplementary File S6), two species from the same family of quinoa (Chenopodiaceae). These findings indicate that the deletion of 32 amino acids in the SBP domain of CqSPL22 predated the speciation of quinoa, and the deletion of 17 amino acids in the SBP domain of CqSPL21 occurred following the speciation of quinoa. Whether these deletions affect the DNA-binding activity of CqSPL21 and CqSPL22, and whether these genes play a role in Cu homeostasis, requires further investigation. The rest of the groups (I to VII) in our study corresponded to the several subgroups (Ia to IIi) of group II in Guo et al. [72]. Group V (IIb) contained AtSPL8, CqSPL8A, and CqSPL8B (or the orthologs from other species). Recently, SPL8 in alfalfa has
been shown to play a role in biomass yield and the response to salt and drought stress [79]. SPL8 has also been shown to promote flowering in switchgrass [80]. There has been much interest in studying the function of SPL8, but no study has yet addressed whether SPL8 has a conserved function in early anther development and sporogenesis [15]. Members of group I–IV (IIc, IIe, and IIff) and group VII (IIld) targeted by miR156/157 (or miR529) have been identified in land plants [72]. miR156/529 sequences have been identified in the red alga Eucheuma denticulatum [81], and this finding has raised questions regarding the origin of these miRNAs. These miR156/7-targeted SPL genes also show more diverse tissue-specific expression patterns (Figure 10) [82], which is consistent with the finding that this miR156/7-SPL module plays a key role in many developmental processes [58,82]. These miR156/7-targeted SPL genes encode smaller proteins than the SPL genes in group VI (IIa), which are not targeted by miR156/7. AtSPL14, AtSPL16, and AtSPL11/12 from Arabidopsis in group VI are ubiquitously expressed in most tissues [82]. AtSPL14 and AtSPL11/12 have been shown to play a role in toxin resistance and thermotolerance, respectively [83,84]. However, the function of AtSPL16 has not yet been identified. Gene structure analysis has revealed that there are ten or more exons in the members of this group, with the exception of ppSBP2 and ppSBP10 from moss [72,82,85]. The six members in this group from quinoa (CqSPL3/4/5, CqSPL10, and CqSPL15/16) were highly expressed in most tissues examined, with the exception of root tissue (Figure 10). The structure of CqSPL3/5/10/16 was similar to that of AtSPL1/12/14, and CqSPL4/15 have more exons in their C-terminal region compared with other CqSPL genes (Figure 5). Another distinctive feature of CqSPL3/4/5 was that the SBP domain-encoding sequence was only present in one exon, rather than two as in most other SPL genes. This pattern has also been observed in moss ppSBP2 and ppSBP10, AAA-12591 (C. pallidaule), BBB-13790 and BBB-14488 (C. suecicum), Spov_chr3.03860 (S. oleracea), and EL10Ac3q06264 (B. vulgaris), which all belong to the same group. Whether this stems from an intron loss in these SPL genes or an intron gain in other SPL genes remains unclear.

Finally, the promoter of each CqSPL gene was analyzed to gain further insight into their functions. Many cis-elements involved in stress responses were identified in these SPL genes (Figure 9), suggesting that they play a key role in stress responses. Quinoa is a salt and drought-tolerant plant, but the mechanisms underlying its salt and drought tolerance remain unclear. miR156 and SPL genes have recently been reported to be involved in salt and drought responses [51,54–56,86–90]. miR156 and SPL genes have also been shown to play a role in responses to heavy metal stress, such as Cd and Fe stress [48,49]. Our qRT-PCR data revealed that the expression of many CqSPL genes responded to salt, drought, and Cd treatment (Figure 11), which suggests that these CqSPL genes are involved in these stress responses. Additional studies are needed to clarify the regulatory roles of these CqSPL genes in the response to salt, drought, and Cd stress in quinoa.

5. Conclusions

We identified and characterized 23 SPL genes in the quinoa genome, and these SPL genes could be divided into 8 groups with similar exon–intron structures and motif compositions. Of 23 CqSPL genes, 13 were putative targets of miR156/7. A total of 18 MiR156/7 loci were predicted, and the expression of several of these genes was further confirmed in seedlings and seeds. Many cis-acting elements involved in light, hormone, and stress were identified in the promoter regions of these CqSPL genes. The diverse expression patterns of CqSPL genes among tissues and in response to salt, drought, and Cd stress suggest that these CqSPL genes play an important role in the growth, development, and stress responses of quinoa. The findings of this study provide new insights into the molecular mechanisms by which CqSPL genes regulate developmental and physiological processes, as well as information that will aid future studies of SPL genes in the Chenopodiaceae family.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13081455/s1, Figure S1: Expression of several CqSr-MiR156/7 loci in quinoa seedlings; Figure S2: Multiple sequence alignment of the SBP-domains within the AtSPL7 orthologs from different species (Supplementary File S1); Table S1: List of species where
the genome-wide identification of SPL genes was performed; Table S2: List of primers used in this study; Table S3: Categories of cis-elements presented in the promoters of CqSPL genes; Table S4: The number of cis-regulatory motifs in 23 CqSPL promoters; Table S5: The number of stress-related cis-regulatory elements in 23 CqSPL promoters; Supplementary File S1: CqSPL CDS and deduced protein; Supplementary File S2: Cqu-mir156/7 precursor sequences; Supplementary File S3: 3.0 kb promoter sequences of CqSPL genes; Supplementary File S4: Similar SPL sequences found in Bacterium TMED181 and Micromonas commode; Supplementary File S5: SPLs genes identified from C. pallidicaule (A sub-genome) and C. suecicum (B sub-genome); Supplementary File S6: 39 sequences of AtSPL7 orthologs from different species.

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