Comparative genome analysis of the SPL gene family reveals novel evolutionary features in maize

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Abstract

SPLs are plant-specific transcription factors that play important regulatory roles in plant growth and development. Systematic analysis of the SPL family has been performed in numerous plants, such as Arabidopsis, rice, and Populus. However, no comparative analysis has been performed across different species to examine evolutionary features. In this study, we present a comparative analysis of SPLs in different species. The results showed that 84 SPLs of the four species can be divided into six groups according to phylogeny. We found that most of the SPL-containing regions in maize showed extensive conservation with duplicated regions of rice and sorghum. A gene duplication analysis in maize indicated that ZmSPLs showed a significant excess of segmental duplication. The Ka/Ks analysis indicated that 9 out of 18 duplicated pairs in maize experienced positive selection, while SPL gene pairs of rice and sorghum mainly evolved under purifying selection, suggesting novel evolutionary features for ZmSPLs. The 31 ZmSPLs were further analyzed by describing their gene structure, phylogenetic relationships, chromosomal location, and expression. Among the ZmSPLs, 13 were predicated to be targeted by miR156s and involved in drought stress response. These results provide the foundation for future functional analyses of ZmSPLs.

Keywords: SPL, phylogenetic relationship, gene duplication, miR156 expression.

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Introduction

Transcription factors (TFs) are a large class of regulators controlling gene expression by activating or repressing target genes at the transcriptional level. Increasing evidence indicates that TFs have important roles in the regulating networks of plant growth and development processes (Riechmann et al., 2001). SPLs (SQUAMOSA promoter binding protein-like) comprise a family of plant-specific transcription factors that contain a highly conserved SBP domain consisting of about 76 amino acids (Chen et al., 2010). This domain has been implicated in DNA binding and nuclear localization, and also features two zinc-binding sites assembled as Cys-Cys-Cys-His and Cys-Cys-His-Cys, respectively (Klein et al., 1996; Yamasaki et al., 2004). Gene structure analysis indicated that the nuclear localization signal (NLS) region partially overlapped with the second Zn-finger located at the C-terminal of the SBP domain. SBP-domain encoding proteins were firstly isolated from Antirrhinum majus designated as AmSBP1 and AmSBP2. These two proteins can recognize a conserved motif in the promoter region of the floral meristem identity gene SQUAMOSA, which is a member of the MADS-box gene family based on its in vitro binding activity (Klein et al., 1996). Subsequent experiments indicated that the palindromic GTAC core motif of the cis-element is essential for efficient DNA binding by different SBP proteins (Birkenbihl et al., 2005; Cardon et al., 1997). To date, the SPL gene family has been identified in various plant genomes, such as Arabidopsis, rice, and Populus (Cardon et al., 1999; Xie et al., 2006; Guo et al., 2008; Li and Lu 2014).

In Arabidopsis, a total of 16 members have been identified as SPL proteins. Several biological experiments demonstrated that SPL proteins have important functions in plant development processes, especially flower development. For example, the AtSPL3 gene was shown to be involved in the floral transition, and it was the first SPL gene identified in Arabidopsis. As an ortholog of SQUAMOSA, AtSPL3 can interact with the promoter region of the floral meristem identity gene APETALA1 (API), and constitutive expression of this gene in Arabidopsis can result in an early flowering phenotype (Cardon et al., 1997). Loss-of-function mutation of the Arabidopsis SPL8 gene indicated...
that AtSPL8 can regulate pollen sac development (Unte et al., 2003). In maize, the tasselseath4 (tsh4) mutant of an SPL gene was shown to regulate bract development and the establishment of meristem boundaries (Chuck et al., 2010). In addition, SPL genes (SPLs) were also demonstrated to play crucial roles in fruit development (Manning et al., 2006), leaf development (Stone et al., 2005), plant hormone signaling (Zhang et al., 2007), male fertility (Xing, 2010), and shoot development (Wu and Poethig, 2006).

Besides transcription factors, miRNAs are another class of important regulators of gene expression, acting at the post-transcriptional level (Lee et al., 1993; Zhang et al., 2006). These small RNA molecules (20-24 nucleotides in length) can cause the degradation of mRNAs or repress translation by binding to the miRNAs of the target genes (Zhang et al., 2006). Most of the miRNAs in plants are evolutionarily conserved, encoded by gene families (Jones-Rhoades et al., 2006). Among them, miR156/157, a miRNA family that is highly conserved in plants (Axtell et al., 2008), is thought to be involved in important developmental processes. Previous studies demonstrated that half of the SPLs have been found to be targeted by miR156/157 family. For example, 10 of the 16 Arabidopsis SPLs, were targeted by the miR156 family (Rhoades et al., 2002; Schwab et al., 2005; Wu and Poethig, 2006; Wang et al., 2009; Yu et al., 2010). In rice, 11 of the 19 SPLs were found to be regulated by OsmiR156 (Xie et al., 2006).

Despite the progress in function studies of SPLs in many species, no comparative analysis has been reported across different species to study the evolution and functional relevance of this family. Although the maize SPL gene family has been reported by Hultquist and Dorweiler (Dorweiler, 2008), our understanding of this gene family in maize is still rather limited. Therefore, we firstly performed a comparative analysis of this family to dissect the evolutionary features in different species, and 31 ZmSPLs were further characterized, including gene structure, phylogenetic relationships, gene duplication, amongst others. Quantitative real-time PCR (RT-qPCR) analysis was performed to examine the expression pattern of miR156 targeted genes in different tissues and in response to drought stress. These results contribute to a basic understanding of the SPL gene family in different species, and provide a foundation to further elucidate the SPL gene function in maize.

**Material and Methods**

**Whole-genome identification and phylogenetic analysis of SPLs**

To identify maize SPL proteins, the Hidden Markov Model (HMM) profile of the SBP domain (PF03110.7) retrieved from Pfam database (http://pfam.xfam.org/) (Finn et al., 2006) was adopted as query against maize genome database (http://www.maizesequence.org/index.html), with an cutoff E-value of le^-5. Sequences of Arabidopsis and rice SPL proteins were also used to query against the maize genome to identify all possible maize SPL proteins (Cardon et al., 1999; Xie et al., 2006; Guo et al., 2008). The candidate sequences that met the standards were confirmed again by Pfam database and SMART (http://smart.embl-heidelberg.de/) (Lettunic, 2009). Finally, redundant sequences were removed manually after alignments using MUSCLE software (Edgar, 2004). To identify sorghum SPLs, the complete genome sequence of sorghum was obtained (ftp://ftpensemblgenomes.org/pub/plants/release-31/fasta/sorghum_bicolor/pep/), and the same method as described above was adopted. To understand the evolutionary relationships of the SPL family, full-length sequences of the SPL proteins were aligned using MUSCLE software. A phylogenetic tree was constructed using MEGA v4.0 (Tamura, 2007) by the neighbor-joining (NJ) method with 1,000 bootstrap replicates.

**Syntenic analysis, gene duplication, and evolution analysis**

Syntenic blocks among maize, rice, and sorghum were evaluated by MCScan software (Wang et al., 2012) and alignments with an E-value of le^-5 were considered significant matches. Then, the duplicated SPLs from these syntenic blocks were identified using a Perl script, and the relationships of the duplicated genes, including segmental and tandem duplications, were finally visualized using Circos (http://circos.ca) (Krzywinski et al., 2009; Wang et al., 2015). DnaSP v5.0 (Rozas et al., 2003) was used to estimate the number of nonsynonymous substitutions per non-synonymous site (Ka) and synonymous substitution per synonymous site (Ks) of the duplicated genes. The Ka/Ks ratios between duplicated genes were analyzed to deduce the selection model. To obtain further insight into selection pressure among duplicated gene pairs, a sliding window analysis of the Ka/Ks ratios was conducted with the following parameters: window size 150 bp and step size 9 bp. For duplication time analysis, the Ks value was translated into duplication time in million years based on a synonymous mutation rate of λ substitutions per synonymous site per year, as T = Ks/2A10^6 million years ago (Mya) (λ = 6.510^-9 for grasses) (Gaut et al., 1996; Quraishi et al., 2011).

**Sequence analysis and chromosomal locations of ZmSPL genes**

Information regarding the exon number, open reading frame (ORF) length, molecular weight (kDa), and isoelectric point (pI) of maize SPL proteins were determined by the Expasy program (http://www.expasy.org/tools/). Gene structure was predicted through alignments of the coding sequences (CDS) with corresponding genomic sequences using GSDS (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). Conserved motifs were investigated by MEME (Multiple Expectation Maximization for Motif Elicitation) (Bailey and Elkan, 1995) with the parameters used in our
previous study (Zhao et al., 2011). The chromosome location image was generated by MapInspect software (http://www.plantbreeding.wur.nl/uk/soft-ware_mapinspect.html) according to the starting positions of ZmSPLs on the 10 chromosomes.

Prediction of ZmSPL genes targeted by miR156

To predict ZmSPLs regulated by miR156, the sequence of maize miR156 was first obtained from miRBase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2010). Then, ZmSPLs targeted by miR156 were predicted by searching the coding regions and 3’ UTRs of all SPLs for complementary sequences to the maize miR156 sequence using psRNATarget server with default parameters (http://plantgrn.noble.org/psRNATarget/?function=3) (Dai and Zhao, 2011).

Expression pattern analysis using transcriptome data

Transcriptome data of the genome-wide gene expression atlas of the maize inbred line B73 was used to elucidate the expression pattern of ZmSPLs during different development stages (Sekhon et al., 2013). A heat map was generated based on the FPKM (fragments per kilobase of exon per million fragments mapped) values, which were initially transformed by taking log2 (FPKM + 1) and then loaded into R and the Bioconductor program (http://www.bioconductor.org/) (Ross and Robert, 2008).

Plant materials, stress treatments, RNA extraction, and RT-qPCR analysis

To examine the expression profile during different developmental stages, four representative tissues, including root, leaf, stem, and silk were collected from a life cycle of the maize inbred line B73. For stress treatment, maize seeds were surface-sterilized in 1 (v/v) Topsin-M (Rotam Crop Sciences Ltd.) for 10 min, washed in deionized water, and germinated on wet filter paper at 28 °C for 3 days. The germinated seeds were transplanted to enriched soil (turf to sorghum was the same as in a previous study. In addition, the number of sorghum SPLs was similar to that in rice and Arabidopsis. These genes were named ZmSPL1–ZmSPL31 and ShSPL1–ShSPL18 according to their order of distribution on the chromosomes (Tables S2, S3). It should be noted that the number of SPLs in the maize genome was greater than that in rice, Arabidopsis, and sorghum. This gives rise to the question, as to where did these additional genes originally come from in the maize genome. To elucidate the possible mechanism(s) of this phenomenon, we subsequently performed a comparative analysis of SPL gene family in these species.

Phylogenetic relationships of SPLs

To examine the evolutionary relationships of SPLs among different plant species, full-length sequences of the SPL proteins were aligned using MUSCLE, and then a combined phylogenetic tree of 84 SPL protein sequences

Results

SPL genes in different species

In previous studies, a total of 19, 16, and 31 SPLs were identified in rice, Arabidopsis, and maize, respectively (Cardon et al., 1999; Xie et al., 2006; Dorweiler, 2008; Guo et al., 2008). Due to maize genome database updates, we performed a BlastP search against the genome database to identify maize SPLs using the Hidden Markov Model (HMM) profile of the SPL domain, and the same strategy was used to identify sorghum SPLs. By this approach, a total of 31 and 18 non-redundant sequences in maize and sorghum were identified after searching against Pfam and SMART, respectively. The total number of SPLs in maize was the same as in a previous study. In addition, the number of sorghum SPLs was similar to that in rice and Arabidopsis. These genes were named ZmSPL1–ZmSPL31 and ShSPL1–ShSPL18 according to their order of distribution on the chromosomes (Tables S2, S3). It should be noted that the number of SPLs in the maize genome was greater than that in rice, Arabidopsis, and sorghum. This gives rise to the question, as to where did these additional genes originally come from in the maize genome. To elucidate the possible mechanism(s) of this phenomenon, we subsequently performed a comparative analysis of SPL gene family in these species.
from the four species, including 31 of maize, 19 of rice, 18 of sorghum, and 16 of *Arabidopsis*, was constructed using the NJ method with 1000 bootstrap replicates (Figure 1).

The 84 *SPL* genes were divided into six subfamilies (I-VI) according to phylogenetic relationship (bootstrap value > 50%). Although each of the subfamilies contained repre-

**Figure 1** - Phylogenetic relationships of maize, rice, sorghum, and *Arabidopsis* SPL proteins. The phylogenetic tree was constructed using MEGA4.0 with the NJ method. Bootstrap values above 50% are shown at each node.
sentative of rice, sorghum, and *Arabidopsis* SPLs, most maize SPLs showed closer relationships with sorghum SPLs than rice and *Arabidopsis*, suggesting a closer evolutionary relationship of the two species. For example, a total of 16 orthologous pairs were identified between maize and sorghum. We noted that the number of SPLs located in different subfamilies had a significant difference, ranging from 3 (III) to 20 (IV). Most of the members located in the same phylogenetic clade had well-supported bootstrap values, while some proteins showed unclear evolutionary relationships with lower bootstrap values, such as *AtSPL4*, *AtSPL5*, and *AtSPL6*. We also noted that the numbers of maize SPL proteins in most of the six groups were higher than other species, suggesting SPLs had especially expanded in the maize genome.

**Synteny analysis of SPLs among maize, sorghum, and rice**

To examine the origin and evolutionary history of SPLs among maize, sorghum, and rice, a comparative analysis was performed to identify SPL orthologous pairs. Because *Arabidopsis* belongs to the Dicotyledoneae group of plants, orthologous pairs were not detected with the three other species. Through the comparative analysis of the genomic regions hosting the SPLs using MCScan software, we observed strongly conserved synteny among the three species. A total of 104 orthologous gene pairs were found among maize, rice, and sorghum, including 38 pairs between maize and rice, 36 pairs between maize and sorghum, and 30 pairs between sorghum and rice (Figure 2, Table S4). The numbers of orthologous gene pairs among...

![Figure 2](image_url) - Synteny analysis of 68 SPLs from maize, sorghum, and rice. Maize, sorghum and rice chromosomes were labeled zm, sb, and os by different color boxes, respectively. The numbers along each chromosome box indicate sequence length of each chromosome in megabases. Black lines represent the syntetic relationships of orthologous gene pairs.
the three plants were similar, suggesting the conserved evolution of the SPL family. Some differences were also observed among the three species, for example, the ZmSPL16 and ZmSPL17 had two orthologous genes in rice (ZmSPL16/OsSPL4, OsSPL11; ZmSPL17/OsSPL3, OsSPL12), while only one was identified in sorghum (ZmSPL16/SbSPL8; ZmSPL17/SbSPL7), respectively, which might be related to gene loss in the evolution of sorghum. In addition, the syntactic information also provided important clues to study the putative function of the collinear gene. For example, ZmSPL4 encoding the lgl gene (Moreno et al., 1997) had one collinear gene in rice (OsSPL8) as well as in sorghum (SbSPL12). Especially, ZmSPL11 encoding the tga1 gene (Wang et al., 2005) had two orthologous genes in rice (OsSPL16 and OsSPL18) and sorghum (SbSPL3 and SbSPL13). These genes existing in different species might have originated from a common ancestor, which might share a similar regulatory role in plant growth and development.

Gene duplication of SPLs

The number of ZmSPLs (31) was almost twice that of Arabidopsis (16), and also much higher than that in rice (19) and sorghum (18) (Cardon et al., 1999; Xie et al., 2006). Gene duplication, including tandem and segmental duplications, are thought to have played important roles in the amplification of gene families in animals and plants (Moore and Purugganan, 2003). Thus, potential duplication events were analyzed to reveal the mechanism(s) behind the expansion of the maize SPL family. According to the syntactic regions and phylogenetic analysis, 18 ZmSPL gene pairs (24 genes) were located on the segmental duplication regions, accounting for 77.4% of the number of ZmSPLs (Figure 3, Table 1). In sorghum, six gene pairs (nine genes) were localized on the segmental duplication regions, accounting for 50% of the sorghum SPLs. In rice, 11 members forming seven gene pairs were detected, which accounted for 57.8% of the rice SPLs. In addition, no significant tandem duplication events were detected among the three plants. These findings indicated that segmental duplication was the major factor that contributed to the expansion of SPL gene family, especially for maize.

To further understand the duplication and divergence of SPLs, the Ka, Ks, and Ka/Ks ratio were calculated for each duplicated pair. The Ka and Ks results were used to examine the course of divergence after duplication, and the Ka/Ks ratio was applied to explore different selective constraints. Generally, a Ka/Ks ratio < 1 means purifying selection, a ratio = 1 indicates neutral selection, while a ratio > 1 stands for positive selection (Lynch and Conery, 2000). The results showed that the Ka/Ks ratio of the 18 duplicated ZmSPLs pairs ranged from 0.449 to 1.605. Among them, nine duplicated pairs had a Ka/Ks ratio <1. Moreover, the values of ZmSPL13/-5, ZmSPL15/-22 and ZmSPL22/-24 were less than 0.6, which suggests strong purifying selection during evolution. The other nine pairs showed a Ka/Ks ratio >1, indicating that these gene pairs evolved under positive selection (Table 1). In rice and sorghum, the Ka/Ks ratios of all gene pairs were < 1, except for OsSPL2/-18, suggesting that these gene pairs mainly evolved under purifying selection. To obtain further insight into the selection pressure of different sites/regions, we performed a sliding-window analysis of the Ka/Ks ratio for each duplicated gene pair. As shown in Figure 4, numerous sites/regions showed evidence of strong positive selection, especially for ZmSPL gene pairs. In contrast, the other sites/regions were conserved under purifying selection, such as OsSPL14/-17 and SbSPL2/-15.

According to the estimation for Ks, the dates for 31 segmental duplication pairs of maize, rice, and sorghum, were calculated based on a rate of 6.5 \times 10^{-9} substitutions per site per year (Gaut et al., 1996; Quraishi et al., 2011). The results indicated that the 18 maize duplication events were estimated to have occurred approximately between 4.81 to

![Figure 3](image-url) - Synteny analysis of maize (a), rice (b), and sorghum (c) SPLs. Maize, sorghum, and rice chromosomes were labeled zm, sb and os by different color boxes, respectively. The number along each chromosome box indicate sequence length of each chromosome in megabases. Black lines represent the syntetic relationships between SPLs.
50.08 Mya (Table 1), and the duplication events of rice and sorghum SPLs were estimated to have occurred between 34.46 to 47.00 Mya.

**Sequence analysis of maize SPLs**

Molecular weight (MW) and isoelectric point (pI) of the 31 ZmSPLs were determined using the Expasy server. The results showed that the ZmSPL proteins had a large variation in the length (bp) of the open reading frame (ranging from 300 to 3,339 bp) (Table S2). The 31 ZmSPLs were divided into six subfamilies based on the unrooted NJ tree (Figure S1a). Gene structure analysis indicated that the maize SPL family had highly diverse distributions of exon regions (Figure S1b). However, most SPLs within the same subfamilies of the phylogenetic tree had a similar gene structure. A total of 20 conserved motifs were identified in the maize SPL proteins (Table S5). Compared with the phylogenetic analysis, we found that genes located in the same subfamily had similar motif compositions (Figure S2). According to the starting positions of the maize SPL genes annotated by the maize B73 genome database, chromosome location analysis indicated that all of the 31 ZmSPLs were mapped to 9 of the 10 chromosomes with a clear non-random distribution (Figure S3) with approximately 45% of the SPLs on chromosome 4 (eight genes) and 5 (six genes).

**Identification of ZmSPLs targeted by miR156**

A series of SPLs have been confirmed to be targeted by miR156 in *Arabidopsis*, grape, and *Populus*. In general, the complementary sites of miR156 tend to be completely conserved and to locate in the coding regions or 3’ UTRs of

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**Table 1 - Ka/Ks analysis and estimated divergence time for the duplicated SPL paralogs**

<table>
<thead>
<tr>
<th>Duplicated pairs</th>
<th>Ka</th>
<th>Ks</th>
<th>Ka/Ks</th>
<th>Purifying selection</th>
<th>Date (Mya)</th>
<th>Duplicate type</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmSPL1-ZmSPL13</td>
<td>0.135</td>
<td>0.164</td>
<td>0.822</td>
<td>Yes</td>
<td>12.61</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL5-ZmSPL25</td>
<td>0.122</td>
<td>0.126</td>
<td>0.966</td>
<td>Yes</td>
<td>9.68</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL1-ZmSPL5</td>
<td>0.394</td>
<td>0.395</td>
<td>0.997</td>
<td>Yes</td>
<td>30.39</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL1-ZmSPL25</td>
<td>0.374</td>
<td>0.344</td>
<td>1.088</td>
<td>No</td>
<td>26.45</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL13-ZmSPL5</td>
<td>0.346</td>
<td>0.651</td>
<td>0.532</td>
<td>Yes</td>
<td>50.08</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL13-ZmSPL25</td>
<td>0.373</td>
<td>0.279</td>
<td>1.337</td>
<td>No</td>
<td>21.45</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL2-ZmSPL14</td>
<td>0.100</td>
<td>0.063</td>
<td>1.605</td>
<td>No</td>
<td>4.81</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL3-ZmSPL18</td>
<td>0.083</td>
<td>0.073</td>
<td>1.136</td>
<td>No</td>
<td>5.64</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL4-ZmSPL31</td>
<td>0.145</td>
<td>0.093</td>
<td>1.559</td>
<td>No</td>
<td>7.14</td>
<td>Segmental</td>
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<tr>
<td>ZmSPL6-ZmSPL11</td>
<td>0.480</td>
<td>0.565</td>
<td>0.849</td>
<td>Yes</td>
<td>43.49</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL8-ZmSPL27</td>
<td>0.114</td>
<td>0.122</td>
<td>0.934</td>
<td>Yes</td>
<td>9.35</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL9-ZmSPL29</td>
<td>0.124</td>
<td>0.100</td>
<td>1.242</td>
<td>No</td>
<td>7.65</td>
<td>Segmental</td>
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<tr>
<td>ZmSPL15-ZmSPL22</td>
<td>0.173</td>
<td>0.385</td>
<td>0.449</td>
<td>Yes</td>
<td>29.63</td>
<td>Segmental</td>
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<tr>
<td>ZmSPL22-ZmSPL24</td>
<td>0.365</td>
<td>0.611</td>
<td>0.598</td>
<td>Yes</td>
<td>46.98</td>
<td>Segmental</td>
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<tr>
<td>ZmSPL16-ZmSPL21</td>
<td>0.101</td>
<td>0.085</td>
<td>1.178</td>
<td>No</td>
<td>6.56</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL17-ZmSPL20</td>
<td>0.257</td>
<td>0.272</td>
<td>0.948</td>
<td>Yes</td>
<td>20.88</td>
<td>Segmental</td>
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<tr>
<td>ZmSPL17-ZmSPL19</td>
<td>0.553</td>
<td>0.526</td>
<td>1.051</td>
<td>No</td>
<td>40.46</td>
<td>Segmental</td>
</tr>
<tr>
<td>ZmSPL20-ZmSPL19</td>
<td>0.440</td>
<td>0.394</td>
<td>1.118</td>
<td>No</td>
<td>30.30</td>
<td>Segmental</td>
</tr>
<tr>
<td>ShSPL2-ShSPL5</td>
<td>0.228</td>
<td>0.467</td>
<td>0.488</td>
<td>Yes</td>
<td>35.923</td>
<td>Segmental</td>
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<tr>
<td>ShSPL3-ShSPL6</td>
<td>0.406</td>
<td>0.534</td>
<td>0.760</td>
<td>Yes</td>
<td>41.08</td>
<td>Segmental</td>
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<tr>
<td>ShSPL3-ShSPL13</td>
<td>0.206</td>
<td>0.2061</td>
<td>0.474</td>
<td>0.435</td>
<td>Yes</td>
<td>34.46</td>
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<tr>
<td>ShSPL6-ShSPL13</td>
<td>0.426</td>
<td>0.597</td>
<td>0.714</td>
<td>Yes</td>
<td>45.92</td>
<td>Segmental</td>
</tr>
<tr>
<td>ShSPL18-ShSPL7</td>
<td>0.265</td>
<td>0.611</td>
<td>0.433</td>
<td>Yes</td>
<td>47.00</td>
<td>Segmental</td>
</tr>
<tr>
<td>ShSPL17-ShSPL9</td>
<td>0.306</td>
<td>0.419</td>
<td>0.730</td>
<td>Yes</td>
<td>32.23</td>
<td>Segmental</td>
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<tr>
<td>OsSPL2-OsSPL16</td>
<td>0.380</td>
<td>0.519</td>
<td>0.732</td>
<td>Yes</td>
<td>39.92</td>
<td>Segmental</td>
</tr>
<tr>
<td>OsSPL2-OsSPL18</td>
<td>0.496</td>
<td>0.496</td>
<td>1.000</td>
<td>No</td>
<td>38.15</td>
<td>Segmental</td>
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<tr>
<td>OsSPL3-OsSPL12</td>
<td>0.487</td>
<td>0.524</td>
<td>0.929</td>
<td>Yes</td>
<td>40.31</td>
<td>Segmental</td>
</tr>
<tr>
<td>OsSPL4-OsSPL11</td>
<td>0.317</td>
<td>0.535</td>
<td>0.593</td>
<td>Yes</td>
<td>41.15</td>
<td>Segmental</td>
</tr>
<tr>
<td>OsSPL5-OsSPL10</td>
<td>0.338</td>
<td>0.412</td>
<td>0.820</td>
<td>Yes</td>
<td>31.69</td>
<td>Segmental</td>
</tr>
<tr>
<td>OsSPL14-OsSPL17</td>
<td>0.185</td>
<td>0.450</td>
<td>0.411</td>
<td>Yes</td>
<td>34.62</td>
<td>Segmental</td>
</tr>
<tr>
<td>OsSPL16-OsSPL18</td>
<td>0.292</td>
<td>0.461</td>
<td>0.633</td>
<td>Yes</td>
<td>35.46</td>
<td>Segmental</td>
</tr>
</tbody>
</table>
Figure 4 - Sliding window plots of segmental duplicated SPLs. Window size is 150 bp, and step size is 9 bp.
SPLs in different plants (Schwarz et al., 2008; Hou et al., 2013; Li and Lu, 2014). To identify the ZmSPLs targeted by miR156, we searched the coding regions and 3’ UTRs of all ZmSPLs for targets of maize miR156 using the psRNATarget online prediction tool with default parameters (Dai and Zhao, 2011). A total of 13 ZmSPLs were predicted to be potential targets of miR156 (Figure 5). We also found that the targeting sites of miR156 were located in coding regions for 11 ZmSPLs, and only two complementary sites were located in the 3’ UTRs (ZmSPL7 and ZmSPL26). Consistent with previous studies, the targeting sites of maize SPLs were highly conserved in the evolution by the alignments of miR156 with their complementary sequence of maize SPLs (Figure 6).

Expression patterns of ZmSPL genes in different developmental stages

The transcriptome data of the genome-wide gene expression atlas of maize was used to analyze the expression patterns of SPLs in different developmental stages (Sekhon et al., 2013) (Figure 7). The results showed that most ZmSPLs had ubiquitously expression in the 18 different tissues. The group IV members seem to play regulatory roles in maize at multiple development stages based on the constitutive expression at relatively high level in all of the 18 tissues. On the contrary, the group I genes were only expressed in one or a few tissues and at a very low expression level, for example, ZmSPL22 and ZmSPL31 are merely expressed in V3-Stem and SAM. Furthermore, ZmSPL15 was not expressed among the 18 tissues. By comparing the expression patterns of the duplicated gene pairs, we found that most of the duplicated gene pairs had similar expression patterns, but some with obvious divergence were also observed. For example, ZmSPL31 is only expressed in V3-Stem and SAM, while its paralog ZmSPL4 is expressed in V3-Stem and SAM, different stages of leaf and 10-DAP whole seed.

The expression patterns of the 13 ZmSPLs targeted by miR156 were further investigated by quantitative real-time PCR (RT-qPCR) in different tissues. Four representative tissues, including root, leaf, stem, and silk were used in this study. A total of 12 genes were detected in the four tissues (ZmSPL12 was not detected), and different expression levels were found. Most of the genes showed high expression in stem or leaves, especially ZmSPL5, ZmSPL7, ZmSPL9, ZmSPL10, and ZmSPL13. We also noted that segment duplicated genes had similar expression patterns of, for example ZmSPL5 and ZmSPL13, suggesting conserved evolution in maize (Figure 8).

Expression patterns of ZmSPL genes under drought stress

While most studies so far focused on divergent biological processes regulated by SPL genes, increasing evidence indicates that SPLs have also important roles in the response to abiotic stresses (Hou et al., 2013; Wang et al., 2009). To identify the possible members of ZmSPLs involved in drought stress, the expressions of the 13 miR156 targeted genes were further examined by RT-qPCR in maize leaves under slight, moderate, and severe stress (Figure 9). Consistent with the results of the expression at different developmental stages, the expression of ZmSPL12 was not detected, and all of the other 12 genes were responsive to drought stress, suggesting important functions in stress regulation. Among the 12 genes, the highest expression level was observed under severe stress treatment, espe-
cially for ZmSPL10, -13, -21, and -26. In addition, the
segment with duplicated genes showed similar expression
patterns, which might suggest their redundant function in
the regulation of maize drought response.

Discussion

SPLs encode a large gene family of plant-specific
transcription factors that play crucial roles in plant growth
and development (Klein et al., 1996; Cardon et al., 1997).
In the present study, we performed a comparative analysis
of the SPL family to examine the evolutionary history in
different species, thus providing a foundation for gene
function analysis. At least 16 SPLs were reported in
Arabidopsis, 19 in rice, and 28 in Populus (Cardon et al.,
1999; Xie et al., 2006; Li and Lu, 2014). In this study, a to-
total of 31 and 18 SPLs were identified in maize and sor-
ghum, respectively. The phylogenetic tree of the 84 SPL
proteins, including 31 of maize, 19 of rice, 18 of sorghum,
and 16 of Arabidopsis, were divided into six groups. It
should be noted that the number of maize SPL was much
higher than that in the mentioned species. With the purpose
of elucidating the expansion mechanism of the maize SPL
family, gene duplication events were investigated, which
are thought to have occurred during the process of evolu-
tion. Generally, gene duplications were major driving for-
tices in the evolution of genomes, and played vital roles in
the expansion of gene families in various species (Moore
and Purugganan, 2003; Mehan, 2004; Cannon et al.,
2004), such as NBS, HD-Zip, PHD, and others (Zhao et al.,
2011; Cheng et al., 2012; Wang et al., 2015).

According to the phylogenetic relationships and syn-
teny analysis, a total of 18 segmental duplicate gene pairs of
maize SPLs were identified, which accounted for 77.4% of
maize SPL family genes. However, only 50% and 57.8% of
the sorghum and rice SPLs, respectively, were detected to
be involved in segmental duplication. Among the 68
SPLs of the three species, no tandem duplication events were
detected. Thus, the segmental duplication was largely re-
sponsible for the expansion of SPL gene family. By comparing
the frequency of segmental duplication in the three species,
the segmental duplication of maize SPLs was seen to be
more prevalent than in the sorghum and rice genomes,
which provided a possible reason or explanation for why
the numbers of SPLs are significantly different among
maize, rice, and sorghum. In general, tandem duplication
often occurred in rapidly evolving gene families, while seg-
mental duplication was commonly reported in more slowly
evolving gene families, e.g. the HD-Zip gene family (Can-
non et al., 2004; Guo et al., 2008; Zhao et al., 2011). We
concluded that the prevalence of segmental duplication

Figure 7 - Expression profiles of ZmSPLs at different developmental stages. Blue and red indicate low and high levels of transcript abundance, respectively. Tissues from different developmental stages are shown at the bottom of the heat map.
demonstrated the slow evolutionary rate of the SPL gene family. In fact, a total of 38 orthologous gene pairs were identified between maize and rice, which was similar with the result between maize and sorghum (36), as well as between rice and sorghum (30). Therefore, these results suggested that the SPL gene family is a highly conserved and slowly evolving family in plants.

Whole-genome duplication (WGD) played crucial roles in plant diversification and evolution, and was often accompanied by polyploidization and gene loss (Otto and Whitton, 2000; Soltis et al., 2009). Previous studies showed that grass species have undergone several rounds of WGD. For example, maize experienced an ancient duplication prior to the divergence of grasses at approximately 50-70 Mya and a additional WGD at approximately 5 Mya, which separated maize from sorghum (Gaut, 2002; Salse et al., 2008; Schnable et al., 2009). The duplication time for the 18 ZmSPL segmental duplication pairs ranged from 4.81 to 50.08 Mya. Among them, seven pairs showed a duplication time of less than 10 Mya. However, all the segmental duplication events in the rice and sorghum genomes were shown to have occurred between 34.46 to 47.00 Mya. These results suggested that some segmental gene pairs of maize SPLs are due to a recent duplication. In addition, selection pressure analysis indicated that 50% of the maize duplicated pairs evolved under positive selection. Unlike in maize, SPL gene pairs of rice and sorghum mainly evolved under purifying selection, indicating novel evolutionary features of maize SPLs.

miR156 is one of the miRNA families that is highly conserved and functions in diverse processes associated with growth and development. It has been shown to mediate posttranscriptional regulation for a subset of SPLs through direct cleavage (Wu et al., 2009; Yu et al., 2010). For example, previous studies have identified 10, 11, and 18 potential SPLs as the targets of miR156 in rice, Populus, and tomato, respectively (Wu and Poethig, 2006; Xie et al., 2006; Addoquaye et al., 2008; Schwarz et al., 2008; Li and Lu, 2014). In this study, 13 of 31 ZmSPLs contained miR156 recognition sites. It is noteworthy that ZmSPL1 and ZmSPL17 are not regulated by miR156, while their duplicated genes ZmSPL13 and ZmSPL20 are targets of miR156. This finding suggested that some distinct regulatory mechanisms might exist in these duplicated genes. In most cases, the miR156-regulated SPLs are master regulators that play divergent and redundant roles in plant morphology and development (Schwab, 2012). For example, AtSPL3, AtSPL4, and AtSPL5 are mainly involved in the...
regulation of floral development (Cardon et al., 1997; Jung et al., 2011), while AtSPL2, AtSPL10, and AtSPL11 have been shown to be involved in lateral organ development in the reproductive phase (Shikata et al., 2009). However, whether the miR156-regulated ZmSPLs have similar regulatory roles remains to be further confirmed experimentally.

According to the microarray expression profile analysis, we found that some duplicated gene pairs have similar expression patterns, suggesting that the duplicated genes might have redundant functions in plant growth and development. Exceptions to this were also observed. The phylogenetic analysis showed that most of the maize SPL duplicated gene pairs located in the same branch had a high bootstrap value, and the duplicated gene pairs also exhibited similar exon/intron distribution and motif components. However, some duplicated gene pairs were shown to have significant divergence in expression patterns, such as ZmSPL31 and ZmSPL4. These results suggested that most of the duplicated gene pairs were still conserved in their evolution, but that functional diversification has also accompanied the evolutionary process, as a major feature of retained duplicated genes in long-term evolution (Blanc and Wolfe, 2004). The expression patterns of the 12 miR156-targeted genes were further investigated at different developmental stages by RT-qPCR. Among the 12 ZmSPLs, high expression was detected in leaf and stem. Especially, the results confirmed that some segment duplicated genes have similar expression patterns, suggesting their conserved evolution and redundant functions. The expression of the 12 ZmSPLs under drought stress was also examined. Since most of the studies about SPL family were related to developmental and biological processes, this result provided important information that the 12 miR156 targeted genes are involved in drought stress, which may have important implications in revealing the function and mechanism of SPL in the stress response.

With the advances of sequencing technologies, many new miRNAs have been identified, and an increasing number of studies on miRNAs are being reported. miR156-based regulation of SPL genes participates in various biological pathways and has been reported in many plants,
such as *Arabidopsis*, rice and others, but nearly no research is reported in maize. Based on our experimental results, we have identified several drought-response genes and cloned them, and this will be further studied by transgenic technology. In addition, we are verifying the actual regulatory relationship between miRNA156 and these cloned genes by 5’ RACE technology and degradation group sequencing technology, and we hope our research will reveal a new molecular mechanism in the maize abiotic stress response.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Author contributions**

XJP, QQW and QM conceived and designed the study; XJP, QQW, YZ, XYL and QM conducted the experiments; XJP, QQW, YZ and XYL analyzed the data; XJP, QQW and QM wrote the manuscript; all authors read and approved the final version.

**References**


Supplementary material

The following online material is available for this article:

Figure S1 - Phylogenetic relationships and gene structure of the ZmSPLs.

Figure S2 - Distribution of conserved motifs identified in the putative SPL proteins.

Figure S3 - Chromosomal locations of ZmSPLs on the 10 maize chromosomes.

Table S1 - List of gene-specific primers used in the present study.

Table S2 - Detailed information on the 31 SPLs in the maize genome.

Table S3 - Detailed information on the 18 sorghum SPLs.

Table S4 - Information about orthologous genes in maize, rice, and sorghum.

Table S5 - Detailed information on the 20 motifs identified in ZmSPLs.

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