Molecular Evolution, Genome-Wide Characterization and Codon Bias Analysis of the SBP-Box Family in *Cucumis sativus*

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Abstract

Background: SQUAMOSA promoter binding protein (SBP)-box genes encode a group of transcription factors which extensively play essential roles in plant development and stress responses. However, the SBP-box gene family has not been well characterized in cucumber (*Cucumis sativas*).

Results: In present study, 15 putative SBP-box genes were identified distributing on 4 chromosomes of cucumber. Evolutionary analysis showed that the green plant SBP family originated from a common ancestor. Phylogenic analysis divided CuSBPs into 6 groups similar to those of *Arabidopsis* and rice. Intron-exon and motif structure within each group shared common features according to evolutionary study. Expression pattern analysis of transcriptional data about flowering and resistance to powdery mildew demonstrated conserved SBP-box genes function in vegetative-to-reproductive transition and potential roles in other regulatory pathways. Moreover, codon bias analysis explained the mutation and selection pressure exerted on genes.

Conclusions: This study comprehensively characterized cucumber CuSBP gene families, which would provide a foundation to explore the functions of CuSBPs for improving yield, quality and stress tolerance of cucumber in the future.

1 Background

Transcription factors (TFs) are proteins binding to DNA sequence in specific manner and activate or inhibit transcription of target gene to specifically express in different tissues and environments. TFs have major roles in regulating plant defense and stress resistance[1]. SBP (SQUAMOSA promoter binding protein)-box genes are plant-specific TFs that regulate plant development [2, 3] and stress responses [4, 5] by targeting specific genes. Since the first identification in *Antirrhinum majus* [6], SBP-box genes have been identified and functionally characterized in various plant species, including angiosperms such as *Arabidopsis thaliana* [7], rice (*Oryza sativa*)[8], maize (*Zea mays*) [9], tomato (*Lycopersicon esculentum* Mill)[10] and silver birch (*Betula pendula*)[11], as well as early divergent land plants such as moss (*Physcomitrella patens*)[12]. Typically, SBP-box proteins tend to have a highly conserved SBP domain with 76 amino acids containing two zinc finger-like motifs and a nuclear location signal sequence [13].

SBP-box family has been reported to play an important role at various developmental stages of plants, including development of shoot [14], leaf [15], flower [16], the ripening of fruit [17], cellular homeostasis [18] and signal transduction [19]. A major function of the SBP-box family is to participate in the regulation of plant growth and development. In *Arabidopsis*, *SBP-LIKE* (*SPL*) genes, including *AtSPL3*, *AtSPL4* and *AtSPL5*, have been found to be involved in long floral induction [20], and promoting the floral meristem identity transition [3]. *AtSPL8* showed relationship with the pollen sac development [21]. Several *SPLs* were targeted by miR156 to regulate both the juvenile-to-adult vegetative transition and the vegetative-to-reproductive transition, including *AtSPL2*, *AtSPL9*, *AtSPL10*, *AtSPL11*, *AtSPL13* and *AtSPL15*[3]. In maize, it was demonstrated that SBP-box family might be involved in plant growth and development,

morphogenesis, adversity response, the development of flower organs and photosynthesis [22]. In addition, the SBP-box family is also involved in response to biotic and abiotic stresses. In *Arabidopsis thaliana*, *AtSPL 14* functioned to the presence of a programmed cell death (PCD)-inducing fungal toxin FB1 [23]. *AtSPL 6* could positively regulate N TIR-NB-LRR receptor-mediated plant innate immunity [24]. In birch (*Betula platyphylla* Suk), *BpSPL 9* improved the scavenging of ROS produced by salt and drought stress [25]. Moreover, a set of work of SBP-box gene families in other species indicated the diversity of the functions of SBP-box family. In rice, *OsSPL 14* encoded Ideal Plant Architecture 1 (IPA1), which is a target gene for miR156. A point mutation in *OsSPL 14* perturbing OsmiR156-directed regulation, increased the expression level of *OsSPL 14*, which would lead to reduced tiller number, increased lodging resistance, and enhanced grain yield [26]. In tomato, silencing the *LeSPL-CNR* gene could inhibit fruit ripening [17]. In pepper (*Capsicum annuum* L), *CaSBP* genes tended to be involved in pathogen resistance pathway against *phytophthora capsici*, which caused blight and fruit rot [27]. In sorghum (*Sorghum bicolor* L), *SbSBPs* tended to be involved in the ABA signaling pathway for abiotic stress response [28].

Cucumber is one of the most popular vegetables in the world. The whole genome sequencing of cucumber has been completed a decade ago [29] and refined in recent years (<http://cucurbitgenomics.org/>), which provided excellent resources to identify important gene families. Several important TF families, including *LOX* [30], *WRKY* [31] and *ERF* [32], have already been identified and analyzed in cucumber. However, research about SBP-box family has not been reported. In the present study, we conducted genome-wide identification and characterization and molecular evolution analysis of the SBP-box gene family in cucumber. Phylogeny, gene structure and the codon bias were systematically analyzed. Moreover, expression patterns comparison showed that the SBP-box genes potentially participated in flowering and contributed to powdery mildew resistance.

2 Results

2.1 Identification of SBP-box genes in cucumber and their chromosome location

A total of 15 *CuSBP* genes were identified in *cucumber*. They were named according to NCBI database (Table 1). The length of genomic sequences of these genes ranged from 2 kb to 12 kb, and the amino acid residues of the predicted proteins ranged from 141 to 1031. The results were quite similar to previous studies [27, 33], in which the lengths of genes and proteins showed significant variation. *CuSBPs* located on 4 out of the 7 chromosomes, including chr1, chr3, chr4 and chr6 of cucumber.

2.2 Conserved domain and phylogenetic analysis

The common feature of SBP-box gene members is that they all have SBP domain, which consists of two Zinc finger-like structures (C3H and C2HC), and a highly conserved nuclear localization signal peptide. In present research, conservative SBP domains were used to do the multiple alignment. The sequences

found in *Cucumis sativus* shared all of these three features (Table S1), which proved that these SBP domains were highly conserved in *Cucumis sativus* (Figure 1A). The sequence logo was drawn to show the distribution of amino acid with residue at each site (Figure 1B). Several highly conserved sequences were found at some positions within SBP domains, such as CQQC sequences and KRSCR sequences. Notably, the fourth His in Zn-1 of *CuSBP7a* was different from the others, which was replaced by Cys.

To further investigate the evolutionary relationship of SBP-box genes in *Arabidopsis*, rice and cucumber, a phylogenetic tree was constructed using 51 SBP sequences from three species (Figure 2). The sequences were clustered into 6 groups (Group I-Group VI). The number of group members varies greatly. The Group IV and VI had the largest number of members, both of which were 14. However, there were only three members in the Group I. The *CuSBP* family gene was distributed in all six groups and closely related to the *Arabidopsis AtSPL* family genes and had a relatively distant relationship with rice. Among them, *CuSBP7a*, *CuSBP8*, *CuSBP9*, *CuSBP14*, *CuSBP1a*, *CuSBP6*, *CuSBP13a* and *CuSBP13b* were distributed in the same sub-branches with *Arabidopsis* homologous genes.

2.3 Structural organization and conserved motif analysis of *CuSBP* genes

A phylogenetic tree for cucumber was constructed to further understand the structures of *CuSBP* (Figure S1). The exon-intron structures were generated based on their genome and coding sequences. The results revealed high variation in the number of introns, ranged from 2 to 10. Group I proteins contain 10 introns, Group II contains 2, Group III contains 2–3, Group IV contains 3, Group V contains 10, and Group VI contains 3 introns, which was in agreement with previous studies [27, 34].

Thirty conserved motifs were characterized in *CuSBPs* of cucumber by MEME (Figure S2). The number of motif types varied from 4 (*CuSBP1b*, *CuSBP1c*, *CuSBP3*) to 13 (*CuSBP1a*, *CuSBP14*). Generally, Motif1, 2 and 3 constitutes the SBP domain. Group VI had more diversity of motifs because it contained largest number of members in SBP gene family. *CuSBP1a* and *CuSBP14* both belonged to Group V, and shared exactly same motifs, in which motif 8 and 30 were specially presented. Motif 4, 17, 12, and 9 were shared by Group I and Group V. Motif 28 was the only repeated motifs, which repeated twice in *CuSBP3*.

2.4 Cis-element analysis of *CuSBP* genes

The analysis of cis component in promoter sequence is helpful to understand gene regulation patterns. In present study, cis-acting regulatory elements' function in promoter regions of *CuSBP* genes were classified into two types (Table S2). Type I involved in stress responses, such as ABRE (involved in the abscisic acid responsiveness), MBS (MYB binding site involved in drought-inducibility), ARE (essential for the anaerobic induction). Type II included elements involved in development process, like CAT-box (related to meristem expression), GCN4_motif (involved in endosperm expression), and P-box (gibberellin-

responsive element). The distribution of these elements varied a lot among *CuSBPs*, even if belonging to the same group.

2.5 Expression patterns of *CuSBP* genes in different periods of flowering

The transcriptomes dataset of different flowering periods in cucumber were downloaded from NCBI to investigate the expression patterns of *CuSBP* genes during flowering. Usually, flowers enter the stage of green bud at day 1, green yellow bud stage at day 3, yellow bud stage at day 4, and flowering stage at day 5. *CuSBP* genes with fragments per kilobase million (RPKM) value >5.0 were regarded as expressed genes (Figure 3). Most of gene members shared overlapping expression pattern during flowering period, only some of them had difference at specific stages. In general, we divided them into 3 types based on their expression. The first type expressed at a low level or had no expression, including *CuSBP1b*, *CuSBP6*, *CuSBP7b*, *CuSBP8*, *CuSBP13a* and *CuSBP13b*. *CuSBP1b* and *CuSBP8* both belonged to Group III. *CuSBP1b* had low expression at day1, while *CuSBP8* had no expression at day 5. The second type includes *CuSBP3*, *CuSBP7a*, *CuSBP9*, *CuSBP12* and *CuSBP16*. They had high expression level at the early flowering stage, and the expression level decreased along the timeline. The third type expressed at a high level and especially high at a specific development stage. *CuSBP1a* and *CuSBP14* were members in Group V. They were both up-regulated at day 3 and day 5 while at day 1 and day 4 they were down-regulated to same degree. Taking promoter analysis into consideration, type three genes' promoter regions all had light responsive elements (Table S2).

2.6 Expression profile of Cucumber SBP-box genes against powdery mildew

Powdery mildew is one of severe diseases in cucumber, which could decline the production dramatically. To study the role of SBP-box gene family against powdery mildew, we used the leaf transcriptional data of powdery mildew resistant segment substitution line SSL508-28 and the parent D8 with inoculation of 48h (PM) and no inoculation (CT) to explore the role of SBP gene families in powdery mildew resistance (Figure 4). *CuSBP* genes with FPKM value >5.0 are regarded as expressed genes.

Ten members of *CuSBPs* were clustered according to their expression level without *CuSBP3*, *CuSBP6*, *CuSBP8*, *CuSBP9* and *CuSBP13b*(Figure4). *CuSBP14* and *CuSBP1a* showed high expression level both in PM treatment and the control of the two germplasms. *CuSBP1c*, *CuSBP7a*, *CuSBP12* and *CuSBP13c* all showed down-regulated expression and *CuSBP7b*, *CuSBP16* and *CuSBP13a* showed opposite trends after PM treatment in two germplasms. Notably, *CuSBP13a* showed significantly higher expression in PM treatment of SSL508-28 than the control (about 20 folds). The expression of *CuSBP1b* were opposite in different germplasms.

2.7 Codon usage analysis of Cucumber SBP-box genes

Codon degeneracy and codon usage are interesting and challenging problems to be investigated, which are related to gene function or sequence properties. To study the codon usage in *CuSBPs*, we calculated the GC content on the 1st, 2nd and 3rd site of a synonymous codon (GC1, 2, 3s content), relative synonymous codon usage, codon adaptation index and effective number of codons. The calculated values are shown in Table S3. Firstly, the codon usage pattern was studied. The GC content is $46.32 \pm 3.49\%$ with GC1 $51.09 \pm 2.94\%$, GC2 $45.66 \pm 3.70\%$, GC3 $42.20 \pm 7.31\%$. The GC content is relatively lower at the 3rd site, while with relatively big variance. The neutrality plot (GC12 vs GC3s) was drawn (Figure 5a), in which the correlation of the points is not significant ($P > 0.05$) and the slope of the regression line is close to 0. Association between ENC and GC3s were learned with a standard curve shown in (Figure 5b) under the hypothesis that there is no selection.

3 Discussion

3.1 Evolution of cucumber SBP family genes

The SBP gene family plays an important role in various stages of plant development, such as stem growth[22], leaf development[35], flowering[14], fruit ripening[36] and signal transduction[21]. Phylogenetic analysis of multiple plants species focusing on SBP-box gene family has been reported, such as *Arabidopsis*(16 members), rice(19 members), *Betula luminifera*(18 members)[25], common bean(23 members)[37], grape(19 members)[38], *Ziziphus jujube*(16 members)[39], *Petunia*(21 members)[40]. In this study, we identified 15 SBP family genes, all of which contain a conserved SBP domain. To investigate the homology of SBP family genes in cucumber, *Arabidopsis* and rice, phylogenetic tree analysis showed that the SBP genes were clustered into 6 groups (Group I-Group VI) (Figure 2). Preston divided the SBP family genes of 9 species into eight clades[13]. In present study, dividing Group IV and Group VI from the first node respectively into Group IV -1, Group IV -2, Group VI -1, Group VI -2 generates 8 same clades as previous studies. However, there is no *CuSBP* in Group IV -2.

A recent study on SBP-box genes elaborated that land plants' SBP-box genes were generated through duplication events from one common ancestor. Based on the timeline of duplications, SBP-box genes are divided into group 1 and group 2-1, group 2-2, among which group 2-2 plays the major role of the expansion of SBP-box genes.[13] Group I in present study corresponds to group1, which is the first formed lineage. Group V in present study corresponds to group 2-1, which is the subgroup in the second group retaining similar evolutionary features to group 1 compared with group 2-2. We further constructed a phylogenetic tree of cucumber SBP genes and analyzed their conserved motifs and introns (Figure S1, S2). The number of introns in the *CuSBP* genes ranged from 2 to 10, motifs from 4 to 13. From the evolutionary point of view, the genes assigned under the same sub-group have similar numbers, types and distributions of motifs and introns, such as *CuSBP14* and *CuSBP1a* have identical conservative motifs in Group V, which further support the grouping of the phylogenetic tree. Some genes have different isoforms, which may be caused by paralogous genes of different chromosomes or by alternative splicing,

such as *CuSBP13a*, *CuSBP13b* and *CuSBP13c*, which are assigned to the same sub-group. However, *CuSBP1a*, *CuSBP1b* and *CuSBP1c* are assigned to different sub-groups.

3.2 Expression pattern and potential function analysis of *CuSBP* genes

SBP-box genes are plant-specific transcription factors, which play important roles in plant growth and development processes. They usually exhibit high expression level in panicles, flower buds, shoot apices in studied species[41-43]. At the same time, some SBP family genes are also involved in some biotic and abiotic stress responses.[4, 25] Currently, there are few functional data available describing SBP-box genes in cucumber.

To explore potential function of *CuSBPs* in cucumber, two sets of transcriptional data (flowering and PM) are used. Flowering serves as transition from vegetative phase to reproductive phase, which makes it crucial in reproductive plants. Therefore, it is important to study the expression pattern of *CuSBP* family genes in cucumber flowering process, discover its potential functions in flowering in order to provide potential resources for future cucumber breeding to improve cucumber yield. From the expression patterns of *CuSBP* family genes in different flower development stages, they could be divided into two groups, one group had lower expression levels during flowering (*CuSBP8/6/1b/13a/7b/13b*) The other group has a higher expression level (*CuSBP3/12/16/7a/9/1c/14/13c/1a*). It is speculated that genes with low expression during flowering may be involved in the vegetative growth process of plants. *CuSBP8*, *CuSBP1b* and *OsSBP10* are members of Group I, and their expression patterns are similar. *OsSBP10* was shown to be highly expressed in rice seedlings and young spikelets[44]. *CuSBP13* homologous gene *AtSPL13* expresses highly in hypocotyl, shoot apical meristem, leaf primordia and developing inflorescence. Disruption of *AtSPL13* regulation delays post-germinative transition from the cotyledon to vegetative-leaf stage.[35] In opposite, *CuSBP14* and *CuSBP1a* have high expression levels throughout the flowering process and are distributed in the Group II, which may play a key role in the flowering process. Its mutant *fbr6*, a homologous gene of *AtSBP14* in *Arabidopsis*, showed a transition to flowering later than wild type[23]. Group II contains *CuSBP1c*, *AtSPL3/4/5* and *OsSPL13*. In *Arabidopsis*, SPL3 3' UTR recognized by miR156/157 prevents early flowering[16]. SOC1 (Suppressor of overexpression of constans1) integrates photoperiod and GA signals to promote flowering via the SPL3/4/5[19]. *CuSBP1c* homologous gene *AmSBP1* also acts in the initiating flower development[45]. *OsSPL13* was found associated with spikelet size in cultivated rice by a GWAS analysis[46]. *CuSBP1c* shows decreased expression level as the flower buds develop, indicating similar roles with *AtSPL3/4/5*. In Group I, *CuSBP9* expresses less as the flowering time goes. *AtSPL2* have also been found to regulate ASYMMETRIC LEAVES 2(AS2) to control floral organ development[36]. *OsSPL14* controls shoot branching in the vegetative stage with the regulation of miRNA[26], pointing out that *CuSBP9* potential role in juvenile-to-adult vegetative transition.

Powdery mildew is one of the world most damaging diseases to cucumber, which is caused mainly by *Podosphaera fusca* [47]. It seriously affects photosynthesis and disturb metabolism, resulting in premature aging and declined production. Therefore, it is important to study the resistance functions of CuSBP family genes involved in the regulation of powdery mildew. In Group I, *CuSBP14* and *CuSBP1a* maintained high expression levels in both cultivars. After treatment with PM, the expression levels of the two genes in SSL508-28 were lower than those in D8. *CuSBP14*, a homologous gene of *CuSBP14* in *Arabidopsis*, is more sensitive to programmed cell death (PCD)-inducing fungal toxin FB1. This indicates that *CuSBP14* and *CuSBP1c* may be involved in the negative regulation of biotic stress. *CuSBP7a* has a lower expression level in SSL508-28 compared to D8, and *AtSBP7*, *OsSBP9* and *CuSBP7a* belong to the Group 1. *AtSPL7* and *OsSPL9* are considered functional genes in copper regulation pathways and Loss-of-function mutations in *SPL9* resulting in enhanced plant resistance to rice stripe virus[35, 48]. Both *CuSBP1b* and *CuSBP13a* were highly up-regulated after treatment with powdery mildew in SSL508-28. This indicates that these two genes may play a positive regulatory role in the regulation of powdery mildew resistance. *CuSBP12* was downregulated after treatment of two varieties of PM, but *CuSBP6* with the highest homology was not detected in this database. *AtSBP6* is a homologous gene of *CuSBP6/12*, which can actively regulate the defense genes and regulate Plant innate immune system. *NbSPL6* is essential for the N-mediated resistance to Tobacco mosaic virus.[24] This also indicates that homologous genes of different species may differ in functional evolution, but the specific function remains to be studied.

3.3 SBP-box genes had high codon usage bias in cucumber

Codon usage bias reflects the genetic information hidden in the RNA sequence and accessed us to the evolutionary process of genes in organism. In present work, we find high codon usage bias in cucumber SBP-box genes.

ENC is a parameter acknowledged to be used to measure the codon bias degree. Previous studies demonstrate that gene expression is negatively correlated with ENC value[49, 50], in other words, important highly expressed genes tend to have low ENC values. The ENC value of *CuSBPs* varies from 49 to 61 with the mean value 53.2, which indicates high codon bias. Lowest ENC values among them is 49, indicating severe selection exerted on them and they are well functionalized, which is identical to previous discussion. Group III members have top 3 ENC values, suggesting their roles may not be irreplaceable. In ENC-plot, notably, *CuSBPs* have low ENC value compared to the ENC expected value, rejecting the null hypothesis that there is no selection. The presented gene points are lying below the curve expect one and the genes were not narrowly distributed in the plot, which demonstrated that both mutation pressure and selection affected the codon usage pattern, reinforcing the theory of equilibrium between mutation and selection[51].

In the neutrality plot, the correlation of the points is not significant ($P>0.05$) and the slope of the regression line is close to 0. The weak correlation between two GC12 and GC3s suggests that there is

high mutation bias or low conservation of GC content levels, which means the mutation pressure rather than translational selection plays the major role.

RSCU is commonly used to analysis the synonymous codon usage (Table S4). Additionally, most abundantly used codons are A/T ended, as the result of compositional constrains (i.e., A and T)[52], which is part of the mutation pressure. For subsequent researches, if transgenic SBP-box genes are required to be expressed in cucumber, the insertion sequence can be modified by preferred codon patterns in this study.

4 Conclusion

In summary, the present provided a comprehensive understanding of SBP-box genes in cucumber. Genome-wide identification and characterization of SBP-box genes have been done in cucumber including phylogenic, gene structure and promoter analysis. Expression pattern analysis of flowering and powdery mildew resistance indicated that various potential roles of SBP-box gene in cucumber need to be explored. Moreover, codon usage analysis of *CuSBPs* could provide us the image of molecular evolution and further transformation work.

5 Materials And Methods

5.1 Identification of SBP-box genes in cucumber

The whole genome information of cucumber was downloaded from NCBI genome database (<https://www.ncbi.nlm.nih.gov>). The protein sequences of SBP-box genes from both *Arabidopsis thaliana* and *Oryza sativa* were downloaded from Uniprot as 'input files' (<https://www.uniprot.org/>). Then, two methods were used to genome-widely identify candidate SBP-box genes in *Cucumis sativus*: (1) the 'input files' were blasted against cucumber genome with E-value $< 1e^{-5}$ in order to obtain putative SBP-box family genes, (2) HMMER3 were used to get the HMM model and results [53]. These two methods led to the same result. After that, we conformed the sequences by Pfam (<http://pfam.xfam.org/>) and batch cd-search in NCBI.

5.2 Sequence alignment and phylogenetic analysis

Multiple amino acid sequence alignment was performed using DNAMAN software (Lynnon Biosoft, CA, USA). The sequence logo was obtained using the online platform Weblogo4 for conserved sequences. Phylogenetic trees were constructed using MEGA 7.0 (<https://www.megasoftware.net/index.php>) with the neighbor-joining method and 1000 bootstrap replicates for SBP-box family genes in *Arabidopsis thaliana*, *Oryza sativa* and *Cucumis sativas*.

5.3 Intron-exon structure analysis

Intron/Exon structures were determined by aligning coding sequences to their corresponding genomic sequences. A diagram of intron/exon structures was obtained using TBtools [54].

5.4 Motif analysis

Multiple EM for Motif Elicitation (MEME) software (<http://meme.nbcr.net/meme/>) was used to search for motifs in all 15 SBP-box genes. The number of motifs was set to 30.

5.5 Promoter analysis

Plantcare

(<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to analyze the cis-acting regulatory elements of *CuSBPs*.

5.6 Expression analysis of *CuSBPs*

Two sets of transcriptomes data of *Cucumis sativus* were obtained from NCBI database to study the expression profiles of *CuSBP* genes. Data (GSE76358) including samples of different flowering periods were downloaded from the database reported previously by Sun et al. [55]. The normal ovary blooms at 4-5 days after labeling (when the ovary is visible), with the majority blooms at day 5. Corolla of day1, day3, day4, and day5 after labeling were collected for RNA sequencing. Log₂ RPKM values were used to draw the heatmap. Another data set was genes expression pattern against powdery mildew with GEO accession: GSE81234. To investigate the candidate genes governing Pm5.1 and their effects on powdery resistance, the RNA-sequencing based transcriptomes of the powdery mildew resistant segment substitution line SSL508-28 and recurrent parent D8 were compared 48h after inoculation with the PM pathogen. Log₂ FPKM values were used to draw a heatmap.

5.7 *CuSBPs* codon usage analysis

To study the codon usage in *CuSBPs*, we calculated the GC content on the 1st, 2nd and 3rd site of a synonymous codon (GC1, 2, 3s content), relative synonymous codon usage (RSCU), codon adaptation index (CAI)[56], as well as effective number of codons (ENC) using online website Optimizer (<http://genomes.urv.es/OPTIMIZER/>) [57]. The correlation between exon length, CAI, GC, GC3, GC2, GC1, and ENC were calculated by R software.

To further analyze codon usage bias, plot of ENC vs GC3s (ENC plot) and plot of GC12 and GC3s (Neutrality Plot) were generated according to previous study. In ENC plot, the standard curve was calculated by:

$$ENC_{exp} = 2 + S + (29/(S^2 + (1 - S^2)))$$

Where, S is the frequency of G + C (i.e., GC3s). And in Neutrality Plot, GC12 is the frequency of GC at 1st and 2nd sites.

6 Declarations

6.1 Ethics approval and consent to participate

Not applicable.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and materials

The datasets analysed during the current study are available in the NCBI genome database (<https://www.ncbi.nlm.nih.gov>). The protein sequences of SBP-box genes from both *Arabidopsis thaliana* and *Oryza sativa* were downloaded from Uniprot as 'input files' (<https://www.uniprot.org/>). Two sets of transcriptomes data of *Cucumis sativus* were obtained from NCBI database to study the expression profiles of CuSBP genes. Data (GSE76358) including samples of different flowering periods were downloaded from the database reported previously by Sun et al. [55]

6.4 Competing interests

The authors declare that they have no competing interests.

6.5 Funding

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6.6 Authors' contributions

Conceived and designed the experiments: SX, YY, YZ, JW. Performed the experiments: YY, YZ, SL, JS. Analyzed the data: YY, YZ, GQ, FX, GW. Contributed reagents/materials/analysis tools: YY, YZ, ZC. Wrote the paper: YY, YZ, ZC, SX.

6.7 Acknowledgements

Not applicable

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Tables

Table 1 The SBP gene family in *Cucumis sativus*.

Gene Name	Gene ID	Chromosome	Start	End	Gene Length	Amino Acids Length
<i>CuSBP7b</i>	105434392	3	6445615	6448269	2655	339
<i>CuSBP1b</i>	101204516	6	27284737	27286763	2027	141
<i>CuSBP9</i>	101203301	3	30985523	30991713	6191	382
<i>CuSBP12</i>	101206747	1	3934091	3940111	6021	548
<i>CuSBP13b</i>	101222787	1	236064	239806	3743	379
<i>CuSBP8</i>	101204773	6	7313524	7317367	3844	297
<i>CuSBP16</i>	101217032	6	6509781	6513088	3308	328
<i>CuSBP14</i>	101219733	4	23212515	23218369	5855	1031
<i>CuSBP1c</i>	101215375	4	20657647	20658784	1138	202
<i>CuSBP13c</i>	101219589	3	22275396	22277307	1912	344
<i>CuSBP1a</i>	101203103	3	25952433	25964518	12086	1013
<i>CuSBP6</i>	101221286	1	2087516	2093881	6366	550
<i>CuSBP7a</i>	101205874	3	10311215	10321131	9917	793
<i>CuSBP13a</i>	101221267	1	6001467	6004185	2719	314
<i>CuSBP3</i>	101212580	1	7700070	7703680	3611	162

Figures

Figure 1

SBP domain alignment in CuSBPs. (A) Multiple alignment of SBP domain protein sequences of cucumber SBP-box proteins. The conserved zinc-finger structures (C3H, C2H) and NLS are marked. (B) Sequence logo of the SBP domain of CuSBPs.

Figure 2

Phylogenetic tree of cucumber, Arabidopsis and rice. The SBP domain protein sequences of cucumber, Arabidopsis and rice are used to construct to phylogenetic tree using Neighbor-Joining method. And the SBP-box genes are divided into 6 groups.

Figure 3

Expression analysis of CuSBPs in different flowering process. Ovaries are used to extract the RNA after which are visible at day1 (green bud stage), day3 (green yellow bud stage), day4 (yellow bud stage) and day5 (flower stage). The colored bar represents the expression values. Green represents low expression, and red represents high expression.

Figure 4

Expression analysis of CuSBPs in 'SSL508-28' and 'D8' with and without inoculation of powdery mildew. SS, 'SSL508-28'. D8, 'D8'. CT, control without inoculation. PM, inoculated treatment.

Figure 5

Codon usage analysis. (A) Neutrality Plot. GC12 and GC3s values of CuSBPs are used to draw a scatterplot with confidence interval. (B) ENC plot. ENC and GC3s values of CuSBPs are used to draw a scatterplot with a standard curve.

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