Genome-wide identification and expression analysis of *SBP-box* gene family reveals their involvement in hormone response and abiotic stresses in *Chrysanthemum nankingense*

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Abstract

SQUAMOSA promoter-binding protein (SBP)-box family proteins are a plant-specific class of transcription factors in plants, which widely regulate the development of floral and leaf morphology in growth and environmental signal response. In this study, we identified and isolated 21 non-redundant *SBP-box* genes in *Chrysanthemum nankingense* with bioinformatics analysis. Sequence alignments of the CnSBP proteins discovered a highly conserved SBP domain including two zinc finger-like structures and a nuclear localization signal region. According to the amino acid sequence alignments, 67 *SBP-box* genes were divided into eight groups, and the motif analysis and gene structures also sustained this classification. The gene evolution analysis indicated the *CnSBP* genes experienced a duplication event about 10 million years ago (MYA), and the *CnSBP* and *AtSPL* genes occurred a divergence at 24 MYA. Transcriptome data provided valuable information for tissue-specific expression profiles of the *CnSBPs*, which highly expressed in floral tissues and differentially expressed in different organs. Quantitative Real-time PCR data showed variable expression patterns of the *CnSBPs* under exogenous hormone and abiotic stress treatments, such as abscisic acid, salicylic acid, gibberellin A3, methyl jasmonate and ethylene spraying as well as salt and drought stresses, discovering that the candidate *CnSBP* genes potentially involved in plant regulation pathways. Our study represents the first genome-wide systematic analysis of the *SBP-box* gene family in *C*.

nankingense. In general, this research provides a reference on the role of *SBP-box* gene family in regulating hormone signal and abiotic stress pathway, and lays a foundation for subsequent studies of *CnSBP* genes functions in plant growth and development and other biological processes.

Introduction

Plants may encounter a variety of environmental stresses during their growth and development; such as extreme temperatures, water-deficiencies, drought and salinity stress, may adversely affect the growth and productivity of plants. Plants have evolved a variety of mechanisms to overcome abiotic stresses, which involve the expression patterns modulation of stress response gene for adaptive development and growth (Skirycz & Inze 2010). Transcription factors (TFs) are important groups of regulatory genes, which appropriately regulate growth, differentiation and metabolism during respond to endogenous hormones and environmental factors and play critical roles in flowering plants (Liu et al. 2021b; Song et al. 2022). Plant hormones are the center of regulating plant growth and development, because they not only integrate the internal development programs, but also transmit exoteric environmental inputs (Glazebrook 2005). Signaling pathways depend on the phytohormones like abscisic acid (ABA), jasmonic acid (JA), gibberellin (GA), ethylene (ETH), and salicylic acid (SA) to mediate the early responses of plants under environmental stresses (Hou et al. 2013; Colebrook et al. 2014).

SQUAMOSA promoter-binding protein (*SBP*)-box genes encode plant-specific TFs that possess approximately 76 amino acid and a highly conserved DNA-binding domain comprising two typical zinc-finger structures, C3H and C2HC, and a nuclear localization structure, NLS (Yamasaki et al. 2004; Birkenbihl et al. 2005; Guo et al. 2008). *SBP-box* genes, *AmSBP1* and *AmSBP2*, were initially discovered in snapdragon (*Antirrhinum majus*), which were identified due to their interactions with the promoter sequence region of the floral meristem identity gene *SQUAMOSA* (a kind of *MADS-box*) (Klein et al. 1996). It is an important phase to transit from vegetative to reproductive stage of life during time of flowering in higher plants. The *MADS-box* family genes are relevant to the origin and evolution of reproductive structures such as flowers and ovules, so it is of great significance to research the functions of *SBP-box* gene family (Ning et al. 2019). Since then, *SBP-box* genes had been isolated and characterized in many plants ranging from the single-celled alga (*Chlamydomonas reinhardtii*) (Kropat et al. 2005) to model plant, *Arabidopsis thaliana* (Cardon et al. 1999) as well as from world-wide important crops like rice (*Oryza sativa*) (Xie et al. 2006), Chinese cabbage (*Brassica rapa*) (Cheng et al. 2016) and wheat (*Triticum aestivum*) (Li et al. 2020) to fruits like sweet orange (*Citrus sinensis*) (Song et al. 2021), apple (*Malus×domestica* Borkh.) (Li et al. 2013) and sugarcane (*Saccharum spontaneum*) (Feng et al. 2021).

SBP-box genes control many aspects of development and physiology and related to innovations in flowering plants, including the vegetative phase change (Xu et al. 2016), flowering time (Xu et al. 2016), leaf initiation (Preston et al. 2016), shoot and inflorescence branching (Shao et al. 2019; Cui et al. 2020), fruit development and ripening (Ferreira e Silva et

al. 2014), floral organ development and fertility (Liu et al. 2017b), pollen sac development (Unte et al. 2003), trichome development and root development (Lan et al. 2019; Shao et al. 2019). In *Arabidopsis*, *AtSPL3/4/5* were previously reported to redundantly promote the floral meristem identity transition through direct activation of *LEAFY* (*LFY*), *FRUITFUL* (*FUL*), and *APETALA1* (*AP1*), and they may act synergistically with the *FLOWERING LOCUS T* (*FT*)-FD module toinduce flowering under long-day (LD) condition (Yamaguchi et al. 2009). *AtSPL9* and *AtSPL15* were showed to control the transition from juvenile to adult stage and combined the floral promoters *SOC1* and *AGL42* to play a positive role in floral development (Dorca-Fornell et al. 2011). In monocotyledons, rice, several *OsSPLs* were directly involved in yield-related traits, for instance, *OsSPL8* was reported to control the development of panicle branch angle and *OsSPL16* participated in the regulation of size, shape and quality of grains (Wang et al. 2012; Yang et al. 2019). Complicating regulatory network was reported that *OsmiR156k-OsSPL18-DEP1* module controlled the weight and number of grains (Yuan et al. 2019). Significantly, some specific *SBP-box* genes were proved to play essential roles in tolerating various stresses and response hormone signal transduction pathway (Wang et al. 2009). *AtSPL7* and *AtSPL14* separately played a significant regulator for copper homeostasis and cell death-inducing fungal toxin fumonisin B1 (FB1) (Stone et al. 2005; Yamasaki et al. 2009). Over-expression *AtSPL1* and *AtSPL12* enhanced thermos-tolerance during reproductive growth in inflorescence (Chao et al. 2017), and OsSPL10 negatively regulated salt tolerance but positively participated in trichome formation (Lan et al. 2019). Besides, the *VpSBP* genes in grape overexpressed in *Arabidopsis* improved the tolerance of salt and drought stress by regulating salt hypersensitivity (SOS) and reactive oxygen species (ROS) signaling cascades (Hou et al. 2018), and *CiSPL* genes in pecan (*Carya illinoinensis*) displayed apparent spatiotemporal expression patterns under salt and drought treatments (Wang et al. 2021). Futhermore, *VvSBP* and *MdSBP* genes in grape and apple revealed potentially involvement in regulation mechanism against abiotic stresses, possibly dependent on hormonal signaling pathway (Hou et al. 2013; Li et al. 2013).

Some *SBP-box* genes were proved to target by microRNAs (miRNAs, 20-24 nucleotides) and formed a RNA-induced silencing complex to regulate functions in plants. In *Arabidopsis* and rice, 11 of 17 and 11 of 19 *SBP-box* genes possessed the *miR156*-target-site, which were located in either coding region (CDS) or 3' untranslated region (3'UTR) (Xie et al. 2006; Xing et al. 2010). The *miR156-SBP/SPL* regulation modules involved in lots of plant developmental processes and stresses had come to light, recently. *MiR156*-targeted *CmSPL6/9/16* and *SlSPL13* genes were separately reported to play important roles in floral bud and inflorescence morphogenesis development in chinese chestnut and tomato (Chen et al. 2019; Cui et al. 2020). *MiR156/529/535-SPL* gene modules regulated the cereal panicle development and higher cytokinins accumulation in female inflorescence in oil palm (Tregear et al. 2022). Besides, the *miR156*-targeted *AtSPL* genes improved the tolerance of heat stress recurring and *TcSPLs* in tamarisk developed a critical post-transcription regulation at 1 h time point under salt stress (Stief et al. 2014; Wang et al. 2019).

Chrysanthemum, *C. nankingense* (2n=2x=18), a diploid native species of China, which holds well adaptability to harsh environments and tolerance to cold, drought and soil infertile as well as ornamental and medicinal value (Yang et al. 2006; Ren et al. 2014). The morphological and physiological diversity of chrysanthemum create great demand prospects in the market. However, complex flowering regulation mechanism and diversification of growth environment relatively restrict the development of the native chrysanthemum resources. In order to improve the ornamental value and benefit of *C. nankingense*, it is necessary to discuss the relationship between environment and plant growth, and further exploration on flowering factors and flower bud differentiation. It is worth noting that *SBP-box* gene family plays a pivotal regulatory in integrating growth and environmental signals. The success of the *C. nankingense* whole genome sequencing is doubtlessly a milestone in the direction of herbaceous plants molecular research, and makes it possible to excavate gene families from genome-wide to provide molecular basis in genetic evolution mechanism and plant growth regulation (Song et al. 2018). In this study, we performed a genome-wide identification of the *SBP-box* gene family in *C. nankingense*, and the characterization, phylogeny, gene structures, *miR156*-targeted genes and tissue-specific expression analysis were investigated by bioinformatics and experiments. We also endeavored to analyse the expression levels of 21 *CnSBP* genes under exogenous phytohormone and abiotic stresses treatments. This research provided a fundamental theoretical basis of candidate hormone- and stress-responsiveness *CnSBP* genes and further elucidated the potential function in response to biotic and abiotic stresses dependent on hormone signal pathway.

Materials & Methods

Plant materials and treatments

The seeds of *C. nankingense* were preserved with 4°C in College of Landscape Architecture, Northeast Forestry University (Harbin, Heilongjiang). Lay the soaked seeds flat on a petri dish with wet filter paper at low density, and seeds germinated in two days. The seedlings were cultivated in a growth chamber at a temperature of 25 ± 2°C with a light/dark cycle of 16/8h for vegetative growth and 60%–70% relative humidity (Wang et al. 2022). At the one-month-old seedlings in age, the fourth to sixth fully expanded leaves beneath the apex were sprayed with 100 μM salicylic acid (SA), 50 μM methyl jansmonate (MeJA), 100 μM gibberellin A3 (GA_3), 100 mM abscisic acid (ABA) and 0.5 g/L ethylene (ETH) hormone. The roots of seedings were soaked in 200 mmol L^{-1} NaCl and 20% polyethylene glycol (PEG) 6000 to simulate salty and drought environment. Leaves were sampled followed by 0, 3, 6, 12, 24 and 48 h and immediately stored at -80 °C in preparation for subsequent experiment (Li et al. 2013; Liu et al. 2021a; Wang et al. 2021). The leaf samples of each treatment repeated three times and sprayed with sterile water as the control.

Identification and analysis of *SBP-box* genes in *C. nankingense*

The related genome data of *C. nankingense* was downloaded from chrysanthemum genome database (http://www.amwayabrc.com/zh-cn/index.html), and the blast installation package was

constructed with localization environment for efficient sequence alignments. Protein sequences, coding sequences and genome data of *Arabidopsis*, rice and *Artemisia annua* were obtained from website (https://www.A.thaliana.org/index.jsp), (http://O.sativa.plantbiology.msu.edu/cgi-bin/gbrowse/O.sativa/) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, localized NCBI and Pfam (http://www.sanger.ac.uk) were used to search with a hidden Markov model (HMM) profile of the SBP domain (Accession No. PF03110) with a cut-off E-value of 1×10^{-5} (Finn et al. 2014; El-Gebali et al. 2019). NCBI-CDD (http://www.ncbi.nlm.nih.gov/structure/cdd/) and SMART (https://smart.embl-heidelberg.de/) were used to identify whether a complete SBP domain or not, and removed incomplete domain or redundant sequences. The selected SBP proteins were renamed CnSBP1-CnSBP21 according to the ascending order of genomic protein IDs. The physicochemical properties of the CnSBP proteins, including relative molecular mass, isoelectric point, average hydrophilic coefficient and others were analysed by ExPASy (https://web.expasy.org/protparam/) and subcellular localization was predicted by WoLF PSORT (https://www.genscript.com/psort.html) . The secondary and tertiary structures of proteins were predicted by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl) and SWISS-MODEL (https://swissmodel.expasy.org).

Sequence alignments, phylogenetic and gene structure analysis

Multiple alignments were carried out by DNAMAN 7.0 and Clustal X software. Phylogenetic trees were constructed by MEGA 7 software with parameters of neighbor-joining (NJ) method, 1000 times bootstrap replications and p-distance substitutions model with 50% cut-off partial deletion (Kumar et al. 2016). The conserved motifs of CnSBP proteins were showed through MEME website (http://meme.nbcr.net/meme/intro.html) with 6 minimum and 50 maximum width amino acids, 8 motifs found (Bailey et al. 2006). The conserved sequence logos were obtained through Weblogo (http://weblogo.berkeley.edu) website. The exon-intron structure of *CnSBPs* was obtained by TBtools software according to the genome generic feature format (gff) files (Chen et al. 2020).

Calculation of Ka/Ks values

Due to the degeneracy of codons, the difference of paralogous and orthologous gene sequences during species evolution resulted in an amino acids change in the encoded protein, which was known as a non-synonymous substitution (Ka), conversely, the existence of synonymous codon held same amino acids was called a synonymous substitution (Ks). Software DnaSP5 was used to calculate the Ka and Ks rates aiming to analyze gene duplication events (Librado & Rozas 2009). The Ka/Ks rate of orthologous and paralogous *SBP-box* gene pairs between *C. nankingense* and *Arabidopsis* was used to determine the selection pressure, and the Ks value can reflect the divergence time during large-scale duplication events. Divergence time (T) was calculated with the formula $T = Ks/2\lambda$ MYA for each gene pair to estimate the date of duplication events , where the approximate clock-like synonymous substitution rate (λ) was 1.5×10^{-8} substitutions synonymous/site/year in dicots (Blanc & Wolfe 2004; Won et al. 2017).

Promoter *cis*-elements, protein interaction and *miR156*-targeted sites prediction

We extracted 2000 bp sequences regarded as promoter region of the *CnSBP* genes from TBtools software. The *cis*-regulator elements were predicted by PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) website and visualized by TBtools (Lescot et al. 2002). STRING (https://string-db.org) website was used to predicte the interaction of the homologous proteins of CnSBPs, AtSPLs in *Arabidopsis* with other proteins. We obtained *ath-miR156* mature sequences in *Arabidopsis* from miRBase (https://www.mirbase.org/) through the alignments of *C. indicum* miRNA high-throughput sequencing to search against the *miR156*-targeted sites of the *CnSBP* genes by website psRNATarget (http://plantgrn.noble.org/v1_psRNATarget) (Dai et al. 2018).

Expression profiles of *CnSBP* genes

For increasing insight into potential functions of *CnSBPs*, we analyzed the tissue-specific expression patterns of 21 *CnSBP* genes. RNA-seq data of 6 various plant tissues and organs (leaves (L), stems (S), roots (R), buds (B), ligulate flowers (LF) and tubular flowers (TF)) were downloaded from *C. nankingense* genome database (http://www.amwayabrc.com/zhcn/blast/blast.html). The tissue-specific expression data was extracted by transcripts per kilobase of exon model per million (TPM) mapped reads using TBtools software. The expression levels of 21 *CnSBPs* were showed by TBtools in the form of heatmaps with parameters of normalized scale method and log scale.

Quantitative real-time PCR analysis

Total RNA was extracted from the frozen samples using Plant RNA Extract Kit R6827 (Omega Bio-Tek, Guangzhou). Single-strand cDNA was synthesized from 0.5 μg total RNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Japan). Quantitative Real-time PCR was conducted with the UltraSYBR Mixture (Low ROX) (CWBIO, Beijing). The sequences of the specific primers were listed in Table S1. All groups of qRT-PCR experiments were performed with three biological duplication, and gene *CmEF1α* (GenBank Accession No. KF305681) was determined for reference gene (Zhu et al. 2020). The relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Results

Identification and characteristics of *SBP-box* family genes in *C. nankingense*

We used the AtSPL and OsSPL protein sequences to identify the *CnSBP* gene family members in the localized BLAST program with E-values less than $1*e^{-5}$. We obtained 28 *CnSBP* genes preliminarily across analysis in HMMER software with a profile Hidden Markov Model (pHMM) of the SBP domain (PF03110). However, 7 of them (CHR00008556, CHR00054349, CHR00065414, CHR00077268, CHR00077269, CHR00078717, CHR00084913) were excluded from further analysis in SMART and NCBI-CDD database for their incomplete or redundant

SBP domains. Eventually, 21 *CnSBP* genes were determined in *C. nankingense* genome, and we renamed *CnSBP1* to *CnSBP21* based on ascending order of genomic gene IDs. The number of *CnSBP* genes was consistent with some flowering plants, such as petunia (*Petunia hybrida*) (21), *Prunus persica* (17) and *Prunus mume* (17), but far less than the vegetable crops wheat (48), oilseed rape (58) and Euphorbiaceae (77) . It could be a consequence of the divergence of flowering responsive function of *SBP-box* genes.

The amino acid length (aa), relative molecular weight (MW), isoelectric point (PI) and average hydrophilic coefficient (GRAVY) of the 21 CnSBP proteins were summarized in Table 1. The amino acid length was ranged from 142 to 954 aa and the molecular weight were in a range of 116447.45-106321.94 Kd. The 21 CnSBP proteins were mostly basic amino acids and unstable proteins, in that the isoelectric point was above 7.0 and the instability coefficient was over 40. It indicated that all the CnSBP proteins were provided with hydrophobicity due to the negative value of GRAVY except CnSBP14 which was hydrophilic protein. Subcellular localization results showed 19 CnSBP proteins were predictably located in the nucleus but both of CnSBP3 and CnSBP14 were mainly existed in endoplasmic reticulum, meaning additional functions in CnSBP3 and CnSBP14. All of 21 CnSBP proteins possessed the main protein secondary structures including α -helix, β-helix, random coli and extended strand but the proportion of each structure was distinct (Table S2). The results were consistent with the analysis of *SBP-box* gene family in *Arabidopsis*. It was concluded from the analysis of the tertiary structures of 21 CnSBP that all the proteins have similar structures except for subtle diversities which led to various functions (Table S2).

Sequence alignments and phylogenetic analyses

Multiple sequence alignments of 21 CnSBP protein sequences were carried out by DNAMAN 5.0 and Clustal X software in order to determine the structure of each gene and domain composition in details (Fig. 1). The 21 CnSBP proteins of conserved domain sequences were showed in Table S3. As shown in Fig. 1, 21 CnSBP proteins all have an intact SBP conservative domain (SBP-DBD) which was generally composed of 72-80 amino acid residues. The sequences found in *C. nankingense* contained three features, the two zinc finger-like structures (Zn1 and Zn2) and a nuclear localization signal region (NLS). CysCysCysHis (C3H) was Zn1 structure for all members except CnSBP14 with another Zn1-like structure CysCysCysCys (C4) which was consistent with AtSPL7 in *Arabidopsis*. While CysCysHisCys (C2HC), the Zn2 structure existed in 18 CnSBP proteins, with the exception of CnSBP2, CnSBP3 and CnSBP12 which lack part of the C2HC structure. Similar to *Arabidopsis*, the C-terminus SBP structural domain of the chrysanthemum CnSBP proteins highly conserved nuclear localization signal region (NSL) consisting of a large number of basic amino acid residues. The NLS region possessed a partial sequence coincidence with Zn2 structure and specifically identified GTAC motif and may play an important role in regulating the accurate binding of SBP proteins to target DNA sequence and locating in nucleus after extranuclear translation and processing (Fig. 1) (Birkenbihl et al. 2005; Riese et al. 2007).

To gain further insight into the evolutionary relationships between CnSBP and various species of SBP family members, an unrooted NJ phylogenetic tree was constructed with bootstrap analysis (1000 replicates) based on the multiple sequence alignments of 69 *SBP-box* genes from four species, including monocotyledons (*O. sativa*) and dicotyledons (*Arabidopsis, A. annua,* and *C. nankingense*) (Fig. 2). According to the results, the 69 *SBP-box* genes were clustered into eight groups (GI - GVIII). The 21 *CnSBPs* were distributed in all eight groups and the largest group (GVIII) contained 7 *CnSBPs* which accounted for 33.3 % of the total *CnSBPs*, whereas GII, GIII, GIV and GVI contained only one *CnSBP* member. The phylogenetic tree showed that there were 4 groups of paralogous genes in *C.nankingense*, *CnSBP2*/*CnSBP3*, *CnSBP1*/*CnSBP16*, *CnSBP12*/*13*/*19* and *CnSBP9*/*CnSBP17*, meanwhile, 10 groups of orthologous genes were found in *Arabidopsis* and *A. annua,* suggesting that the *SBP-box* family diverged earlier than the species divergence of *C. nankingense* and *Arabidopsis*. It was worth noting that most *CnSBPs* were highly homologous with *AaSBPs* due to similar evolutionary relationships in *Asteraceae* species. Apart from GII, each of the remaining groups contained *CnSBP* and *AtSPL* gene family members, s. Based on these findings, it was speculated that the *CnSBP* genes have undergone multiple gene replication events of the same ancestral gene and distinct patterns of differentiation appear have occurred among many family members after the separation of each lineage.

Motif composition and gene structures analysis of *CnSBPs*

The typical evolutionary blots and gene biological functions of TF families were linked with the intron/exon structure and the type and quantity of intron, therefore, we analyzed the structural characteristics between 21 *CnSBP-box* genes and 17 *AtSPL* genes (using the genome IDs in *Arabidopsis*) and constructed the intron-exon structures (Fig. 3A). The results revealed that CnSBP8 contained additional gene and motif structures with low complexity sequence repeats regarded as the ankyrin repeat domain (ANK) analysed by NCBI CD-Search. The genes with ANK-domain generally have diverse and complex biological functions, in that it functionality mediates the protein-protein interactions.

The intron–exon structures indicated that different *CnSBP* genes were diverse, while the same subgroup genes usually possessed similar intron–exon structures, for instance, *CnSBP12*/13/18/19, which owned three exons, were all in GVIII (Fig. 2, Fig. 3A). Statistical analyses showed that most *CnSBP* genes contained 2-4 exons, but *CnSBP3*, *CnSBP8* and *CnSBP14* contained 6, 11 and 13, respectively (Fig. 3A). Motif, which is shared by most members of gene family, is likely to be an indispensable part to implement important functions or structure compositions. It is particularly critical to use the identification and analysis of these features of gene sharing motifs to discover new members of gene families. From Fig. 3B, eight motifs were required to predict within AtSPL and CnSBP proteins by MEME website and the sequence logos were showed in Fig. S1. It showed that most CnSBP proteins possessed three to six motifs and motif 1, 2 almost simultaneously existed in all CnSBP proteins apart from CnSBP2 and CnSBP3. According to the gene and protein structures, 38 genes were divided into

four groups (GA-GD). Members of Group D owned 2 or 4 extra motifs, which hinted they may have relatively specific structure and function, and Group C didn't share any other motifs except motif 1 and 2 (Fig. 3B). In order to display the detailed information of the motifs intuitively, the motif 1 and 2 sequence logos were showed in Fig. 3C. On a basis of sequence alignments and domain analysis in above, it was clear that motif 2 corresponded to Zn1 and partial Zn2 finger-like domain, while motif 1 contained the complete NLS domain (Fig. 3B, Fig. 3C). The biological functions of other motifs remained unknown, so it could be predicted that some CnSBP proteins had unknown functions.

Gene duplication and evolution analysis of *CnSBPs*

Our analysis identified 10 putative paralogous gene pairs (Cn-Cn) in *C. nankingense* genome and 6 orthologous gene pairs (Cn-At) between the *CnSBP* and *AtSPL* genes using BLASTn sequence similarity alignments. The results of homologous gene comparison for fragment duplication were highly consistent with phylogenetic tree clustering scheme of the evolutionary group (Fig. 2). All of the paralogous and orthologous pairs are listed in Table S3. For every homologous gene pair, we calculated Ka, Ks and Ka/Ks values to further explore evolutionary selection pressure and investigate the divergence of *CnSBPs* (Table S4). Furthermore, the frequency distributions of the Ks and Ka/Ks values for the homologous gene pairs from *C. nankingense* and *Arabidopsis* were analysed (Fig. 4). The distribution of the calculated Ks values for the paralogous pairs in *C. nankingense* averaged ~ 0.3 (Fig. 4A), indicating that a large-scale duplication event occurred in *SBP-box* gene family in *C. nankingense* approximately 10 million years ago (Mya). Recent research has suggested that the most recent WGD event in *C. nankingense* occurred ~ 5.8 Mya, which was a persuasive evidence that the duplicate event of the *SBP-box* genes occurred earlier than whole-genome WGD event. Also, for the *Arabidopsis-C. nankingense* orthologous pairs, the average value at ~ 0.72 estimated that the divergence time of the *SBP-box* genes was 24 Mya (Fig. 4B). Significantly, the Ka/Ks peaks in the *C. nankingense* genome were distributed between 0.5-0.6 (Fig. 4C), while the Ka/Ks betweem *C. nankingense* and *Arabidopsis* genomes were 0.7-0.8 (Fig. 4D). On the basis of the value of Ka/Ks, it reflected that the *SBP-box* genes subjected to purification selection (Ka/Ks<1) for homologous gene pairs in *C. nankingense* genome as well as *C. nankingense* and *Arabidopsis* genomes, and tended to eliminate harmful mutations in the population.

Analysis of Cis-regulatory elements in the promoter regions of *CnSBPs*

The upstream promoter regions (2000 bp) of *CnSBP* genes were retrieved from the *C. nankingense* genome to identify *cis*-regulatory elements for further comprehending the gene regulation mechanism (Fig. 5A) and the critical corresponding *cis*-elements function descriptions were listed in Table S5. Light-responsiveness regulatory elements, including AE-box, 3-AF1, ACE, Box 4, G-box and others were distributed around most *CnSBPs* promoter regions (Fig. 5B). Besides, stress regulatory elements GC-motif, MBS, LTR, ARE, TC-rich and WUN-motif, which were separately involved in anoxic specific inducibility, drought-inducibility, low-

temperature responsiveness, anaerobic induction, defense and stress responsiveness and wound responsiveness were identified in 1, 11, 7, 17, 5 and 9 *CnSBP* genes, respectively. Likewise, 52 ARE elements occupied the major proportion of stress-responsive elements (Fig. 5B), providing an insight that *CnSBPs* may have a hand in anaerobic induction.

It hinted that 21 *CnSBP* genes may have an intense response to hormone signal with various hormone-responsive elements, such as, 83 abscisic acid responsive (ABRE, ABRE 2/3a/4), 58 MeJA-responsive (CGTCA motif and TGACG-motif), 14 salicylic acid responsive (TCA-element), 10 auxin-responsive (TGA-element and AuxRR-core) and 10 gibberellin-responsive (GARE-motif, TATC-box, and P-box). The percentage of various hormone-responsive *cis*-elements were showed in Fig. 5C. It was worth noting that all of the *CnSBP*s promoter regions contained at least one hormone-responsive elements. *CnSBP5* and *CnSBP4* only owned ABA-responsive elements and *CnSBP12* owned MeJA-responsive elements (Fig. 5C). Different types and numbers of hormone-responsive regulatory elements provided sufficient bases that specific *CnSBP* genes may respond to exogenous hormones and regulate to abiotic stresses.

MiR156-targeted sites prediction of *CnSBPs*

Target sites of *miR156* in all plants tend to be relatively conserved. Due to lack of miRNA sequencing of *C. nankingense*, we used five *miR156* family members (*Ath-miR156i/j/e/a-5p/f-5p*) in *Arabidopsis* to predict the mature *miR156*-targeted sites in 21 *CnSBP* genes initially. Multiple sequence alignments of the *CnSBP* genes and reverse complement sequences of *Ath-miR156* showed that 11 *CnSBPs* contained highly consistent sequences with *Ath-miR156* binding sites no more than one to three mismatches (Fig. 6). This result was consistent with previous research that had proven 11, 6, 12 and 19 *miR156*-targeted *SBP-box* genes in P. mume, melons, grape and walnut. It demonstrated the reliability and accuracy of the predictions of the *SBP-box* gene family. Interestingly, the *miR156*-targeted *SBP-box* genes in *Arabidopsis* and *C. nankingense* were distributed into only three of the subgroups (GV, GVI and GVIII) (Fig. 2) and they all shared common motif 6 (Fig. 3B), which may possess *miR156*-targeted gene sites in this motif. It suggested that *SBP-box* genes play a necessary and significant function in the evolution of various species due to the extremely conserved *miR156* recognition sites of the *SBP-box* genes.

Interaction prediction of CnSBP proteins

The STRING online website was used to conduct a preliminary prediction of protein interaction between the SBP-*box* gene family, and the software Cytoscan was used to visualizate the interactive network relationship (Fig. 7A). On the basis of AtSPL homologous proteins of CnSBP in SBP-*box* gene family, it may have functional similarities to further predict the protein function. For example, AtSPL5, homologous protein of CnSBP1, converged many interacting proteins, such as SNZ, SMZ, AGL8, AGL20 and TOE2 (Fig. 7B). SNZ and SMZ were AP2-like ethylene-responsive transcription factor and might be involved in the regulation of gene expression by stress factors and by components of stress transduction pathways. It provided an insight that CnSBP5 might play a critical regulation role in hormone signal transduced pathway

and abiotic stresses. AtSPL7 (homologous protein of CnSBP13 and CnSBP20) interacted with SIZ1 which involved in the regulation of plant growth, drought responses, freezing tolerance and salicylic acid (SA) accumulation (Fig. 7C). Besides, SPL8 interacted with AGL8, AGL18, AGL20 and AP1 (*MAD-box* gene family), indicating that the homologous protein, CnSBP7, largely involved in promoting flowering and inflorescence meristem identity and regulating flowering time (Fig. 7D). In summary, the CnSBP proteins may be related to expression patterns of genes in response to biological and abiotic stress, regulation to phytohormone pathway as well as growth and development in plants.

Tissue-specific expression profiles of *CnSBP* genes

The patterns of gene tissue-specific expression often have a correlation with its encoded protein function. Publicly available transcriptome data of six tissues (root, stem, leaf, bud, ligulate flower and tubular flower) showed expression patterns and cluster analysis (G a-e) of 21 *CnSBP* genes (Fig. 8, File S1). It showed that more than two-thirds of *CnSBP* genes significantly expressed in floral tissues by comparison with one-third in root, stem and leaf tissues. Among these, *CnSBP3* and *CnSBP7* only showed a high expression level in the stage of flower development, and *CnSBP4* expressed evidently in roots. Overall, eight *CnSBP* genes (*CnSBP5*/*9*/*11*/*14*/*17*/*18*) in group e shown constitutive expression pattern in all six tissues/organs, while group c and d showed a lower levels across the nutritive organs than reproduction organs examined. For instance, the expression of *CnSBP3* and *CnSBP7* in ligulate flower and tubular flower was obvious, but inapparent in root, stem and leaf. *CnSBP9*/*14*/*17*/*18* had relatively high expression levels in leaf and *CnSBP8* and *CnSBP21* nearly expressed in all tissues. With regard to tissue-specific expression patterns, the majority of *miR156*-targeted *CnSBP* genes showed a higher expression levels in floral tissues instead of non-targeted *CnSBP* genes. For example, *miR156*-targeted *CnSBP5*/*9*/*11*/*17*/*18* (members of G e) genes significantly expressed in all tissues, and *miR156*-targeted *CnSBP13*/*19* genes tended to exhibit higher transcript levels in floral tissues. In terms of single *CnSBP* gene, group a, *CnSBP8* expressed a ultrahigh transcript levels in all six tissues, and *CnSBP21* similarly exhibited expression trend but almost no expression in roots (Fig. 8).

Expression profiles of *CnSBP* genes under plant hormone and abiotic stresses

The expression trends of *CnSBP* genes under plant hormones treatments were examined to investigate the responsive profiles and regulation functions of *CnSBPs* by qRT-PCR (Fig. 9). The raw datas of 21 CnSBP genes with ABA, GA, MeJA, SA and ETH treatments were placed in (File S2, S3, S4, S5 and S6). Oligonucleotide primers used in qRT-PCR assays for all 21 *CnSBP* and actin genes listed in Table S1.

Vast majority of the *CnSBP* genes could be induced or downregulated subjected to GA_3 phytohormones. *CnSBP5*, *CnSBP8*, *CnSBP13* and *CnSBP19* were evidently upregulated by nearly 2.47-, 3.24-, 2.81- and 3.18- fold during 12 h treatment, among these, *CnSBP3*/*5*/*13*/*14*/*15*/*19* increased in expression gradually at all stages, but *CnSBP4*/*8*/*9*/*12*

induced a peak at 12 h and had a downward trend from 24 h to 48 h (Fig. 9). Under ABA treatment, most *CnSBP* genes experienced a downregulated trend from 3 h to 6 h, but gradually upregulated during the follow-up periods or reached a maximum peak at 12 h. All the remaining *CnSBP* genes displayed a less obvious expression fluctuation, for instance, *CnSBP12/14/19* increased after slightly decreased in expression levels. *CnSBP2/4/7/12* showed an obvious increase trend in response to MeJA treatment before 12 h , *CnSBP6/19/20* owned a explosive increase in transcript levels from 24h to 48h. *CnSBP14/17/18/21* exhibited slightly decreases patterns at various point of time. Following SA treatment, most *CnSBP* genes presented a decreased trend, except *CnSBP9*/17 prominently increased. Additionally, other *CnSBP* genes displayed slightly up- and downregulated fluctuations during processing of SA treatment. Finally, it occurred that most *CnSBP* gene expressions upregulated at apex of 12 h or 24 h, but descended from 24 h to 48 h, affected by ETH treatment. In general, *CnSBP1/7/11/14/16/18* continuously upregulated during the whole process , and tandem duplicated genes (*CnSBP1/16* and *CnSBP9/17*) showed a similar expression pattern throughout various hormone treatments (Fig. 9). We also observed that the same subgroup *CnSBPs* showed a distinct expression trend, such as *CnSBP10, CnSBP11* and *CnSBP20* in GVII (Fig. 2, Fig. 9). It suggested that specific *CnSBP* genes may play multiple roles in hormone signal pathway and activate the adaptive regulatory response in plants and participate in the regulatory response under abiotic stress.

In order to investigate the relationship of resisting stress conditions in plants by regulating the expression of *SBP-box* gene dependent on hormone signal pathway. The expression profiles and raw data of 21 *CnSBP* genes in response to salty and drought stresses were examined by qRT-PCR (Fig. 10, File S7 and S8). It showed that most *CnSBP* genes more or less affected by salt and drought treatments, implying that *CnSBP* genes may play a pivotal role in abiotic stress processes. In detail, *CnSBP5/12/13* (2.35, 1.50 and 2.05 fold), *CnSBP2/7/20* (2.14, 2.29 and 1.43 fold) and *CnSBP1/3/6/11/15/16/17/21* (1.50, 1.91, 1.63, 1.61, 1.66, 1.62, 1.54 and 2.47 fold compared to 0h) were significantly upregulated by salt stress at early (0h-6h), medium (6h-12h) and late (12h-48h) responsive periods, respectively (Fig. 10). It exhibited an expression trend that increased and then decreased with the passage of time in CnSBP5/7/8/12/13/20 and a continuous decline in CnSBP14. Under drought treatment, *CnSBP12/13/15/18* performed decreased expression levels (0.63, 0.47, 0.69 and 0.49 fold at 48h) during the whole stage of time; *CnSBP7/9//10/14/17/19* showed initially increase before then decreasing trend and *CnSBP5* continuously increased in expression levels (Fig. 10). Interestingly, the vast majority of *CnSBP* genes had no large multiple differentially induced or downregulated under salinity and drought stresses. In comparison, *CnSBP* genes showed a more representative expression patterns in response to phytohormone signal rather than abiotic stresses, indicating that a complex regulatory network covered the process of plant resistance to stresses.

Discussion

Chrysanthemum, chinese traditional flowers, famous for various floral characters and colors were called the four gentlemen of flowers with plum, orchid and bamboo as well as four important cut-flower with rose, carnation and gladiolus in plants. Previously, 12 *CmSPL* genes

and expression patterns in response to stresses had identified and discussed on the basis of transcriptomic data (Song et al. 2016). *C. nankingense*, a close relative of *C. morifolium*, has been considered as a convenient genomic model owing to its simple diploid nature (Song et al. 2018). *SBP-box* gene family, a class of plant-specific transcription factor, which evolved before the divergence of green algae and the ancestor of land plants and widely involved in life processes such as regulation of plant growth and development, hub in flower development, spores, fruits development, stress responses and hormone signal transduction . In this study, a comprehensive analysis of 21 SBP transcription factor family genes were identified in *C. nankingense* genome (Fig. 1). It provided new insights for enriching the understanding of the *SBP-box* genes in non-model plants. Compared with cotton (83 *GhSBPs*), maize (42 *ZmSBPs*), rape (58 *BnaSBP*) and wheat (50 *TaSBPs*), *C. nankingense* contained much less *SBP-box* genes (Zhang et al. 2015; Cheng et al. 2016; Peng et al. 2019; Li et al. 2020), but resembled the model plant *Arabidopsis* (17 *AtSPLs*), flowing plants petunia (21 *PhSPLs*) and rose (17 *RcSPLs*), indicating that the *SBP-box* family genes were species-specific endowed with more diversified and complicated functions.

Studying the conserved domains of *CnSBP* genes was conducive to highlight the cognition of the SBP-box structure. All of the CnSBP proteins were composed of a complete SBP domain, which contained with two zinc finger-like structures (Zn1 and Zn2) and a nuclear localization signal region (NLS) (Fig. 1). It was unique that the Zn2 and NLS regions shared the common four amino acid residues (KRSC). Unlike other zinc finger structures owned a staggered binding mode, Zn^{2+} and NLS region were necessary for binding to *cis*-elements in the promoter of nuclear genes. Moreover, CnSBP8 possessed an extra ANK domain in the C-terminal (742-843 aa) of protein, which had a bearing on protein-protein interaction in plant cells (Lee et al. 2016). It was clear that the ANK domain corresponded to motif 4 and motif 8 and encoded correlative exon sequences (Fig. 3). In like wise, CsSBP12 and CsSBP10b in sweet orange and AtSPL14 in *Arabidopsis* with the same ANK domain, were separately in sensitivity to pathogen *Diaporthe citri* and fungal toxin Fumonisin B1 (FB1) (Stone et al. 2005; Song et al. 2021). It perhaps indicated that *CnSBP8* played a significant role in resisting pathogen fungal infection.

Based on phylogenetic tree and gene structure analysis, 21 *CnSBPs* were clustered into 8 groups (GI - GVIII) from 4 species and exhibited a relative close homology with *Arabidopsis* (17 *AtSPLs*) and *A. annua* (12 *AaSBPs*) rather than rice (*OsSPLs*) suggesting that conservative evolution and common ancestor shared in compositae and dicots plants away from the lineage leading to monocots (Fig. 2). The exon-intron structures and motif analysis also supported significant determinants to cluster phylogenetic tree to a point. Within the same group shared similar structures, such as the members of GVI, CnSBP12/13/18/19 contained motif 1/2/6/7 and 3 exon distributions (Fig. 3B), indicating that the evolution and gene structures may be interrelated. Besides, separate branch members in GI and GIII owned more complex motifs and gene structures heralding that *CnSBP8* and *CnSBP14* may perform additional functions and independent evolution similar to *CsSBP11* of Group IV in sweet orange (Fig. 3) (Song et al. 2021). Intriguingly, on the basis of amino acid sequence alignments, it seemed that CnSBP8

owned a comparable AHA-like motif outside the N-terminal and a IRPGC motif outside the C-terminal of the SBP-domain, which was characteristic of many transcriptional activation domains and also found *C. reinhardtii* CRR1 (Fig. S2) (Riese et al. 2007). The sequence logos of AHA-like and IRPGC motif were showed in (Fig. S4). The same structures were also found in AtSPLs and OsSPLs clustered with CnSBP8 in group III, complex motifs and intron-exon distributions hinted regulated functions combined with gene structures (Fig. 3, Fig. S2, S3). Furthermore, there was a conserved IRPGC aa residues existing in downstream of the SBP domain, which was also found in CRR1 in *C. reinhardtii* (Fig. S3, S4) (Kropat et al. 2005). It was reported that *SPL7* (homologous gene of *CnSBP8*) played a central role in regulating of Cu^{2+} and transmembrane transporter activity and *SPL12* (homologous gene of *CnSBP14*) regulated root tip and embryonic meristem development, nitrogen metabolism and plant thermotolerance at reproductive stage in *Arabidopsis* (Chao et al. 2017; Kastoori Ramamurthy et al. 2018).

SBP-box genes had underwent several duplication events leading to the formation and preservation of multiple *SBP* paralogs and clades . As evident from the phylogenetic tree, 4 pairs of duplicated genes (*CnSBP2/3*, *CnSBP1/16*, *CnSBP9/17* and *CnSBP13/19*) were identified (Fig. 2) consistent with *Arabidopsis* and rice, indicating that duplicate genes might result in amplified *SBP-box* gene family in *C. nankingense* (Yang et al. 2008). Additionally, evolution analysis of the *CnSBP* genes confirmed that species experienced a purification selection and adaptively grew in various environments (Fig. 4). As discussed, the Ks values of the paralogous (*Cn-Cn*) and orthologous (*Cn-At*) gene pairs confirmed that the *CnSBP* genes approximately occurred duplication events ~10 and ~24 Mya ago earlier than the recent whole genome duplication (WGD) event between *C. nankingense* and *Arabidopsis*, indicating that the *SBP-box* gene family experienced an earlier divergence than the separation of the two most recent species (Fig. 4). In accordance with moso bamboo, *SBP-box* genes family occurred a positive and neutral selection in CnSBPs and PeSPLs (Pan et al. 2017).

Remarkably, recent research found that 11 out of 17 *AtSPL* and 11 out of 19 *OsSPL* genes were targeted by *miR156/157*, here, the miRNA response element (MRE) with speculative *miR156/157*-targeted sites was located downstream of the SBP domain and part of the last exon (Fig. S3) (Xie et al. 2006; Riese et al. 2007; Xing et al. 2010). In this study, 11 out of 21 *miR156*-targeted *CnSBP* genes were calculated and all clustered in clades of GV, GVI and GVII with recognition region in motif 6 (Fig. 2, Fig. 3B). A large amount of data indicated that miRNAs carried out diverse functions by targeting *SBP-box* genes, which may be a major determinant of their performed functions and the *miR156*-targeted sites sequences presented a highly conserved in evolution. The *miR156b* regulated two paralogous genes, *SPL9* and *SPL15*, to control shoot maturation and the temporal initiation of rosette leaves (Schwarz et al. 2008). TaSPL3/17 interacted with DWARF53 to reveal potential association in SL signaling pathways during bread wheat tillering and spikelet development by *miR156* targeted (Liu et al. 2017a). *MiR156*-targeted *CnSBP5/10/11/13/17/18/19* highly expressed in floral organ (Fig. 7), demonstrating that *CnSBP* genes, as well as their regulators *miR156* remained to regulate flower morphological characteristics. It would be relevant that *SPL3* (clustered with *CnSBP9* and

CnSBP17) regulated by *miR156* integrated endogenous signals into flowering pathway (Gandikota et al. 2007).

Tissue-specific analysis showed that most *CnSBP* genes highly expressed in floral organs possibly on account of SBP proteins interacting with the *SQUAMOSA* (a *MADS-box*) promoter, a floral meristem gene correlated with the origin and evolution of reproductive organs such as flowers and ovules. Eight members (*CnSBP5/8/9/11/14/17/21*) showed high levels expression in all tissues regarded as significant regulatory factors in plant growth process (Fig. 8). In group b, six *CnSBP* genes exhibited relatively lower expression levels in six tissues compared with other members. Interestingly, paralogous genes *CnSBP2* and *CnSBP3*, performed differentially organizational expression levels in floral organs, it perhaps associated that the expanded *CnSBP* genes occurred functional divergence resulting in novel biological function. In group c, same subgroup members *CnSBP13* and *CnSBP19* expressed in floral organs and leaves. Likely, homologous gene *AtSPL13A/B* participated in the formation of leaf shape and reproductive stages. Furthermore, *AtSPL3* (clustered with *CnSBP4*) regulated flowering time and activated downstream gene expression during flowering morphological development (Jung et al. 2012). OsSPL9 (clustered with *CnSBP14*) regulated grain number and yield as well as Cu accumulation and metabolismin in rice, suggesting its potential roles in *CnSBP14* (Tang et al. 2016). In general, the tissue-specific expression analysis of *SBP-box* genes provided a profound impact on chrysanthemum.

During the lengthy evolution of organisms, plants have obtained complex genic regulatory mechanisms to mitigate effects from adverse environments. Both enzymes and hormones were crucial means by which plant affected a series of physiological or biochemical changes to gain adaptive capacity to resist the stress (Sah et al. 2016). In the study, a further finding was that numerous of hormone-responsive elements, such as ABA (ABRE), GA_3 (P-box, TATC-box, GARE-motif), MeJA (CGTCA-motif, TGACG-motif), SA (TCA-element) and ethylene (ERE) as well as stress-responsive elements were exhibited in *CnSBP* promoters (Fig. 5). Therefore, in line with the ideas that differentially expression of genes response to abiotic stresses and exogenous induction was related to *cis*-regulatory elements in promoters of genes, some *CnSBPs* were considered as candidate genes to involve in hormone response and abiotic stresses. Consequently, we researched the expression profiles of the *CnSBP* genes under ABA, SA, MeJA, GA_3 and Eth hormone treatments. Exogenous spraying induction can not only activate the expression of defense-related genes, but also interconnect hormonal signal network with defense responses. Expression analyses showed that 12 out of 21 members were significantly induced by ABA treatment at 12 h with a high proportion of ABA-responsive elements (Fig. 5, Fig. 9). ABA, regarded as a positive signal of stress, can improve plant tolerance to variable environment by inducing the production of H_2O_2 and establish ROS balance (Mittler & Blumwald 2015). Research showed *OsSPL7* (orthologous gene of *CnSBP15* and *CnSBP21*) in rice was proved to play a critical role in ROS balance response to biotic and abiotic stresses (Hoang et al. 2019), indicating that *CnSBP15* and *CnSBP21* may involve in stress responses via ABA signaling pathway along with up-regulated expression. 10 out of 21 members were markedly induced by

598 GA_3 treatment with polytype GA-responsive elements, which may represent more complex
599 expression and regulation patterns (Fig. 5, Fig. 9). An example was *AtSPL3*, clustered with
600 *CnSBP4*, had been proved integrated photoperiod and GA signals to regulate flowering via
601 SOC1-SPL module (Jung et al. 2012). In chinese chestnut, *CmSPL6*/*CmSPL9*/*CmSPL16* highly
602 and *CmmiR156* lowly expressed during flowering development by exogenous GA_3 spraying
603 (Chen et al. 2019). Moreover, we revealed *CnSBP6*/*9*/*17* and *CnSBP6*/*7*/*12* prominently induced
604 by SA and MeJA, respectively (Fig. 9). It confirmed that plants induced trans-activating factors
605 to activate promoter of defense genes related to SA pathway to improve disease resistance in
606 *Arabidopsis* (Dong 1998). In grape, *VvSBP17* was upregulated response to SA and pathogen
607 infection treatment the same as homologous gene, *AtSPL14*, in sensitivity to fumonisin B1 (FB1)
608 (Hou et al. 2013). Previous studies have proved miR156-resistant *SPL13* involved in ethylene
609 biosynthesis by upregulating the expression of *ACC* oxidase gene in accordance with the same
610 subgroup members, *CnSBP12*/*13*/*18*/*19*, inductively expression patterns. Similarly 12 *MdSPLs*
611 upregulated and one *MdSPL* downregulated in apple by exogenous ethylene spraying (Li et al.
612 2013).
613 Although the dominant roles of *SBP-box* genes have been explored in processes of plant
614 growth and development, the combined analysis between various stresses and hormonal response
615 were also worthy to attention. SA and MeJA can active multiple defense strategies and converge
616 complex signaling networks to enhance the stress resistance capacity in plants, such as salinity
617 stress (Qiu et al. 2014; Kim et al. 2018). In grape, *VvSBP9*/*14*/*16* were downregulated expression
618 in response to SA and MeJA and salt stress (Hou et al. 2013). *CsSBP3*/*4*/*8*/*13* genes in tea plant
619 (*Camellia sinensis*) significantly up-regulated expressed under MeJA and drought treatments
620 (Zhang et al. 2020). DELLAs and some components in GA and ABA signaling pathway
621 participate in the regulation of tolerance response to abiotic stress in plants. Special *PeSPL* genes
622 induced by GA treatment but inhibited by drought stress in moso bamboo (Pan et al. 2017).
623 In our study, most *CnSBP* genes exhibited inapparently transcript levels but regulated more
624 or less due to complex stress-responsive mechanism and regulation network. QRT-PCR analysis
625 showed *CnSBP1*/*3*/*5*/*16* were upregulated and *CnSBP8*/*12*/*13*/*15*/*18* were downregulated under
626 drought stress (Fig. 10). Among these, most *CnSBP* genes were prominently induced by at least
627 one hormone and coordinated by MBS (drought-inducibility) *cis*-elements in promoter regions.
628 The expression levels of *CnSBP1*/*3*/*9*/*13*/*15*/*18*/*21* were variable under salty stress combining
629 that it may be integrated with TC-rich repeats (Fig. 5, Fig. 10), which regarded as defense and
630 stress responsiveness *cis*-element. In rice, overexpression-*OsSPL10* (clustered with *CnSBP7* and
631 *AtSPL8*) weakened salt tolerance and regulated trichome formation (Lan et al. 2019). In Alfalfa,
632 *MsamiR156-MsSPL* module partially improving drought tolerance by *MsamiR156*
633 overexpression to silence *MsSPL13* (Arshad et al. 2017).
634 In addition, lots of evidence indicated that *miR156*/*SBP*(*SPL*) modules regulated a variety of
635 developmental processes and abiotic stress response in plants (Jerome Jeyakumar et al. 2020),
636 for instance, upregulated expression levels of *miR156* then inhibited targeted SPL2/9/11 genes to
637 balance and adapt the adverse bearing on heat stress during plant growth and development (Stief

et al. 2014). Besides, it was reported that *MdWRKY100* gene expression was upregulated by *miR156/SPL* module to regulate salt tolerance in apple (Ma et al. 2021). With sequence alignments of *miR156*-target genes sites, it was preliminarily clear that specific *CnSBP* genes were core factors in phytohormone crosstalk and abiotic stresses, which need to verify by further experiments in future.

Conclusions

In this study, the conclusion followed the fact that 21 *SBP-box* genes were identified in *C. nankingense* genome and provided a comprehensive overview of SBP transcription factor family in chrysanthemum. 21 *CnSBPs* were classified into eight groups with other *SBPs* (*SPLs*) in *Arabidopsis*, rice and *A. annua*. Further studies analysed the alignments of conserved domain, protein motifs, gene structures, gene duplication and evolutionary. Subsequently, predicted analysis of protein physiochemical properties, secondary and tertiary structures, promoter *cis*-regulator elements, *miR156*-targeted sites and protein-protein interaction contributed to in depth functional analysis. Tissue-specific expression profiles revealed that *CnSBPs* may play a pivotal role in floral organ growth and development. *CnSBPs* also responded to exogenous hormone induction and abiotic stresses. The expression patterns with same clustering pattern tended to be consistent. Taken together, our results helped shed light on *SBP-box* gene basic information in *C. nankingense* and provided an experimental basis on the functions of *CnSBP* genes in plant growth regulation. Candidate *CnSBP* genes should further functionally demonstrated for comprehensive understanding of the co-related regulatory patterns of hormone responses and abiotic stresses. It laid a theoretical foundation for the subsequent study of *miR156*/*SBP*(*SPL*) modules regulatory mechanism and the improvement of chrysanthemum breeding.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

All authors declare that they have no known competing financial interests.

Author Contributions

- Ziwei Li conceived and designed the projects, performed the experiments, authored drafts of the paper and prepared figures and tables.
- Yujia Yang prepared figures and tables, performed the experiments and drafted the work.
- Bin Chen analyzed the data and revised it critically for important content.
- Bin Xia analyzed the data and revised it critically for important content.
- Hongyao Li analyzed the data and prepared figures and tables.
- Miao He conceived and designed the projects, reviewed drafts of the paper and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplementary Files.

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