Global analysis of SBP gene family in Brachypodium distachyon reveals its association with spike development

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SQUAMOSA-promoter binding like proteins (SBPs/SPLs) are plant specific transcription factors targeted by miR156 and involved in various biological pathways, playing multi-faceted developmental roles. This gene family is not well characterized in Brachypodium. We identified a total of 18 SBP genes in B. distachyon genome. Phylogenetic analysis revealed that SBP gene family in Brachypodium expanded through large scale duplication. A total of 10 BdSBP genes were identified as targets of miR156. Transcript cleavage analysis of selected BdSBPs by miR156 confirmed their antagonistic connection. Alternative splicing was observed playing an important role in BdSBPs and miR156 interaction. Characterization of T-DNA Bdsbp9 mutant showed reduced plant growth and spike length, reflecting its involvement in the spike development. Expression of a majority of BdSBPs elevated during spikelet initiation. Specifically, BdSBP1 and BdSBP3 differentially expressed in response to vernalization. Co-expression network, protein–protein interaction and biological pathway analysis indicate that BdSBP genes mainly regulate transcription, hormone, RNA and transport pathways. Our work reveals the multi-layered control of SBP genes and demonstrates their association with spike development and temperature sensitivity in Brachypodium.

Abbreviations

| SBP | SQUAMOSA-Promoter binding like transcription factor |
|-----|--------------------------------------------------|
| miR156 | MicroRNA156 |
| qRT-PCR | Quantitative real-time PCR |
| WT | Wild type |

Characterization of various transcription factors has revealed their organism-specific function and a particular class of these transcription factors has been discovered in animals, yeast and plants. SQUAMOSA-promoter binding like proteins (SBPs) form a major family of plant-specific transcription factors. SBPs were first identified in Antirrhinum majus interacting with the promoter sequence of floral meristem gene SQUAMOSA. SBP-box proteins share a highly conserved 76 amino-acids long DNA binding domain known as SBP domain, which contains two zinc ion binding motifs (Cys2HisCys and Cys3His) and a nuclear localization signal (NLS) sequence. The SBP domain of the SBP-box family members binds to TNCGTACAA consensus sequence present in the promoter regions with GTAC as a core motif. Phosphorylation of serine amino acid within the SBP domain has been recently shown to modify DNA binding affinity and immunity in rice. As a multigene family, SBP genes have been identified in green moss, algae, P. trichocarpa and angioseptic. There are 16 SBP genes in Arabidopsis thaliana, 19 in rice, 28 in Populus trichocarpa, 41 in soybean and 17 in barley. SBP genes play key roles in various plant developmental pathways such as flowering time, vegetative to reproductive phase transition, plant architecture, gibberellic acid biosynthesis, anthocyanin biosynthesis, and abiotic stresses.

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Another layer of gene regulation involves microRNAs (miRNAs), which are single-stranded non-coding RNA molecules of 20–22 nucleotides in length that bind to their complementary sequences present in the messenger RNAs (mRNAs) of their target genes\(^{25,26}\). Thus, both miRNAs and their target genes can be manipulated for crop improvement. Out of 16 SBP genes in *Arabidopsis*, 10 are known to be negatively regulated by a conserved miRNA, miR156\(^{27}\). The role of SBP/miR156 module has been observed in many plant developmental processes such as, vegetative to reproductive phase change, plant architecture and flowering time\(^{14,15}\). On the basis of SBP domain, these genes can be classified into five groups in *Arabidopsis* such as SPL3/4/5, SPL9/10/11, SPL6 and SPL13A/B\(^{28,29}\). The miR156 targeted SPLs accelerate the phase transition by positively regulating the expression of APETALA1 (API), FRUITFULL (FUL), and SUPPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1) and LEAFY genes\(^{28–30}\). In wheat and barley, VERNALIZATION 1 (VRN1) is the homolog of FUL/API and acts as both an activator and a target of VRN3 which is a homolog of FLOWERING LOCUS T (FT)\(^{31–33}\). Based on the gain or loss of functions, SPL genes in *Arabidopsis* can be classified into three groups\(^{30}\). Group 1 contains SPL2/9/10/11/13/15 genes which promote both the juvenile-to-adult transition (vegetative phase) and the vegetative-to-reproductive phase transition. The SPL9/13/15 genes are central players for these developments as compared to SPL2/10/11. Group 2 contains SPL3/4/5 genes, which play major roles to accelerate the transition of floral meristem identity. Group 3 contains only SPL6 which does not have a major role in shoot development but may be key to some other biological pathways.

In monocots, SBP/miR156 module has been anticipated as an important tool-box to genetically enhance crop productivity\(^{34}\). Interaction between miR156-SPLs and strigolactone signaling pathway regulates bread wheat panicle branching and grain yield\(^{18,36}\). Likewise, miR156 regulated using Ks values through the formula \(T = \frac{K_s}{2 \lambda} \times 10^{-6}\) (millions of year, Mya). The \(\lambda = 6.5 \times 10^{-9}\) substitutions per type and had aberrant flower development\(^{50}\). In the current study, we identified 18 SBP genes in *Brachypodium* and had aberrant flower development\(^{50}\). In switchgrass, miR156/SPL4 module regulates aerial auxillary bud formation; branching and biomass yield\(^{19}\).

*Brachypodium distachyon*, the small monocot plant, is an emerging model system ideal for functional genomics research to study complex monocot species, especially the triticaceae crops\(^{46,41}\). It is extensively being used to study the biology of flowering and vernalization response\(^{42,43}\). In *Arabidopsis*, FLOWERING LOCUS T (FT) gene is known as a key player of floral signaling processes\(^{34}\). In *Brachypodium*, FT undergoes age dependent alternative splicing and is regulated by miR5200 and FT/miR5200 module control photoperiod dependent flowering\(^{45–48}\). Homoeolog-specific transcriptome changes under heat stress conditions have been examined in *B. stacei*, *B. distachyon* and *B. hybridum*\(^{49}\). *Brachypodium auxin influx facilitator (AUX1)* T-DNA mutants showed dwarf phenotype and had aberrant flower development\(^{49}\). In the current study, we identified 18 SBP genes in *B. distachyon* genome and studied their phylogenetic relationship with barley, wheat, rice and *Arabidopsis*. Further, their gene structure, alternative splicing event, gene duplication event, miR156 mediated negative regulation, co-expression and protein–protein interaction network have been investigated systematically. Transcriptional changes of individual SBP genes in leaf and spike at different developmental stages and temperature regimes were critically examined. Moreover, SBP genes role in early (Bd21) and late (Bd1-1) flowering accessions of *Brachypodium* was verified. Further, Bdsbp9 T-DNA mutant was characterized to understand its function in spike development.

**Results**

**Identification and characterization of SBP-box genes in *B. distachyon***. In this study, we identified 18 SBP genes in *B. distachyon* and designated as BdsBP. BdsBP family members were named according to the closest homologs present in wheat, barley or rice. Details of SBP gene family in *Brachypodium* are given in Table 1. *Brachypodium* SBP genes encode proteins ranging from 177 (SPL7) to 1,110 (SPL14) amino acids (aa) in length and from 122 kda (SBP15) to 12 kda (SBP23A) in molecular weight. The number of exons ranged from 1 to 11 and isoelectric point (pI) was from 5 to 10. The 18 BdsBP genes were located on all 5 chromosomes (chr), with maximum number of BdsBP genes detected in chr 3 of *B. distachyon* (Table 1).

**Phylogenetic analysis and gene duplication in BdsBP genes**. A phylogenetic tree was constructed using conserved SBP domain sequences of SBP proteins from *Brachypodium*, wheat, barley, rice and *Arabidopsis* (Fig. 1A,B). A total of 79 SBP proteins from different plant species including 18 from rice, 10 from wheat, 17 from barley and 16 from *Arabidopsis* were used for phylogenetic analysis. SBPs clustered into 8 groups (G1–G8), with AtSBP3/4/5/6 as ungrouped members. Each group contained at least one SBP protein from *Brachypodium*. As anticipated, BdsBPs exhibited closer relationship with the SBP proteins from barley and wheat as compared to rice and *Arabidopsis*. Group 1 and group 5 contained maximum number of BdsBPs, where SBP proteins from barley and wheat were also grouped. Moreover, gene duplication analysis among BdsBP genes identified 9 putative paralogous gene pairs in the *Brachypodium* genome (Fig. 2A,B). Divergence time for duplicated BdsBP genes was estimated from Ka and Ks values and their ratios. The dates of duplication events (T) were calculated using Ks values through the formula \(T = \frac{K_a}{2\lambda} \times 10^{-6}\) (millions of year, Mya). The \(\lambda = 6.5 \times 10^{-9}\) substitutions per synonymous site per year was assumed as universal clock-like rate for *Brachypodium distachyon*. For BdsBP1 and BdsBP6 gene pair, Ka and Ks values were 0.60 and 2.14, respectively and their ratios 0.28 imply their evolution under purifying selection. Similarly, the ratio (0.30) of Ka and Ks values for BdsBP16 and BdsBP18 gene pair highlights purifying selection. Purifying selection also called negative selection, influence genomic diversity in natural populations. It eliminates the changes that produce deleterious effects on the fitness of the host. The frequency distributions indicate that SBP genes in *Brachypodium* went through a large-scale duplication event ranging from 55 to 164 million years ago (mya). SBP gene paralogs were located on same as well as different chromosomes, indicating that expansion of *Brachypodium* SBP genes was both, tandem as well as segmental/block duplication during evolution.
**Table 1. Characteristics of SBP Genes in Brachypodium distachyon. Asterisks * denotes miR156 Targeted BdSBPs.**

| Gene name | Gene symbol | CDS length (bp) | Domain | Length (aa) | MW (kDa) | pI | Chr | Position on genome | Exon No |
|-----------|-------------|----------------|--------|-------------|----------|----|-----|-------------------|---------|
| BdSBP18* | BRAD1G041250 | 1,278 | SBP | 425 | 44.35 | 7.11 | 3 | 3:43,201,676–43,206,673:1 | 3 |
| BdSBP14* | BRAD1G040030 | 1,176 | SBP | 391 | 40.50 | 9.16 | 3 | 3:42,259,872–42,263,157:–1 | 3 |
| BdSBP8 | BRAD1G24670 | 1,263 | SBP | 420 | 45.81 | 7.51 | 5 | 5:26,221,739–26,235,184:–1 | 3 |
| BdSBP3* | BRAD1G03510 | 1,458 | SBP | 485 | 52.24 | 8.34 | 3 | 3:2,279,990–2,284,027:–1 | 3 |
| BdSBP21 | BRAD1G05720 | 1,452 | SBP | 484 | 51.95 | 6.62 | 3 | 3:4,069,051–4,071,726:1 | 3 |
| BdSBP7 | BRAD1G17720 | 567 | SBP | 188 | 20.57 | 10.31 | 5 | 5:20,917,607–20,918,254:1 | 2 |
| BdSBP22 | BRAD1G31390 | 1,272 | SBP | 423 | 46.10 | 14.07 | 1 | 1:26,879,476–26,882,414:1 | 3 |
| BdSBP11* | BRAD1G05510 | 990 | SBP | 329 | 35.41 | 9.31 | 3 | 3:3,895,778–3,899,371:1 | 4 |
| BdSBP23* | BRAD1G59110 | 1,179 | SBP | 392 | 41.66 | 9.10 | 2 | 2:26,854,357–26,858,238:1 | 3 |
| BdSBP16* | BRAD1G34667 | 1,266 | SBP | 421 | 42.02 | 8.55 | 4 | 4:40,207,721–40,212,788:1 | 3 |
| BdSBP13* | BRAD1G26720 | 579 | SBP | 192 | 20.04 | 9.92 | 1 | 2:1,717,747–21,750,619:1 | 2 |
| BdSBP17* | BRAD1G33770 | 1,224 | SBP | 407 | 42.51 | 7.8 | 4 | 4:39,473,148–39,476,188:–1 | 3 |
| BdSBP6 | BRAD1G02760 | 2,889 | SBP, ANK | 962 | 105.09 | 5.44 | 1 | 1:1,858,041–1,863,792:1 | 11 |
| BdSBP15 | BRAD1G040240 | 3,381 | SBP, ANK | 1,126 | 122.96 | 6.92 | 3 | 3:42,427,821–42,432,953:1 | 10 |
| BdSBP1* | BRAD1G211240 | 2,664 | SBP, ANK | 887 | 84.73 | 9.18 | 2 | 2:9,490,127–9,495,150:1 | 11 |
| BdSBP9 | BRAD1G225580 | 2,550 | SBP, DEXDC | 849 | 92.84 | 5.69 | 2 | 2:23,666,234–23,676,756:1 | 10 |
| BdSBP23A | BRAD1G18890 | 360 | SBP | 119 | 12.54 | 9.12 | 4 | 4:21,459,257–21,462,340:1 | 1 |
| BdSBP13A* | BRAD1G18900 | 651 | SBP | 216 | 23.24 | 7.74 | 4 | 4:21,464,988–21,467,761:2 | 2 |

**BdSBP genes have diverse gene and protein structures.** Gene structure and genetic diversity analyses (Fig. 3A–C) in Brachypodium SBP gene family revealed that the BdSBP genes contain at least one intron; however genes in group 1–3 have the largest number (10–11) of exons (Fig. 3A). Other BdSBP genes possess only 2–4 exons. Interestingly, five sister gene pairs (SBP1/6; SBP14/17; SBP21/22; SBP3/11 and SBP16/18) have similar exon/intron numbers but intron phases with variable lengths. Conserved motif sequence database search identified a total of 10 motifs, which were designated as motif 1–10 (Fig. 3B). Gene pairs (SBP1/6/15; SBP14/17; SBP8/21/22 and SBP16/18) shared a similar type of motif structure. Some motifs were found to be specific to one or two groups of BdSBP proteins. Motif 6 that encodes miR156 target sequence was present in all miR156 targeted BdSBP proteins. Whereas motif 10 and motif 4 were found in group 1 BdSBP and group 3 and 4 BdSBP proteins respectively. To predict possible functions of BdSBP genes, we also performed gene ontology (GO) term enrichment analysis (Fig. 3C). Most of the BdSBP genes with similar gene structure and motifs (BdSBP1/6; BdSBP3/11; BdSBP13A/23A; BdSBP14/17; and BdSBP16/18) were predicted for their similar biological processes (BP), molecular function (MF) and cellular component (CC).

**Higher transcript abundance of BdSBP genes correlates with early inflorescence development.** RNA-seq data of B. distachyon acc. Bd21 (https://www.ebi.ac.uk/gxa/ experiments/E-MTAB-4401/ Results) from 9 different tissues and organs (leaf, early inflorescence, emerging inflorescence, anther, pistil, seed 5 days after pollination, seed 10 days after pollination, plant embryo and endosperm) was mined to understand the dynamics of BdSBP genes expression (Fig. 4). The expression profile of BdSBPs was grouped into three clusters. Higher expression of BdSBP genes of cluster 1 was observed in early inflorescence, emerging inflorescence and pistil tissues. Many BdSBP genes of cluster 1 were also expressed in anther, plant embryo, developing seeds (5 and 10 days after pollination), leaf, and endosperm tissues, implying their significant role throughout the Brachypodium plant development, especially in spike architecture. The BdSBP genes of cluster 2 (BdSBP7 and BdSBP1) either lacked expression in any tissue (BdSBP7) or poorly expressed (BdSBP1) in pistil, leaf, and developing seeds (seed 5 and 10 days after pollination), and endosperm. The BdSBP genes from cluster 3 were found to be expressed mainly in early and emerging inflorescence. Expression profiles of BdSBP genes indicate their involvement in the reproductive units of B. distachyon.

**Post-transcription of BdSBP genes is regulated by miR156 and alternative splicing (AS).** The cDNA sequences of BdSBP genes were searched for putative target sites of Brachypodium miRNAs (Fig. 5A). Ten
Figure 1. Evolutionary analysis of SBP domain transcription factors. (A) Phylogenetic tree of SBP proteins taken from Brachypodium, barley, wheat, rice and Arabidopsis. The amino acid sequences were aligned using MUSCLE tool and Interactive Tree of Life (iTOL) resource was used to annotate the phylogenetic tree. (B) Sequence logo of Brachypodium SBP domain. The height of amino acid residues shows level of conservation. Two zinc finger motif and nuclear localization signal (NLS) and joint peptide are shown.

Figure 2. Gene duplication of SBP genes in Brachypodium genome. (A) Segmental and tandem duplications gene pairs located on Brachypodium chromosome regions are marked in red and green colours. The gray lines on each chromosome represent the total number of SBP genes present in Brachypodium genome. (B) Summary of BdSBP duplicated gene pairs and type of duplication events in Brachypodium.
BdSBP genes are found to be the target of miR156. Out of these, 8 contain miR156 complementary sequences in their coding regions. However, in other two genes, BdSBP1 and BdSBP13, miR156 target site was found in their 3′-UTR and 5′-UTR regions, respectively. The BdSBP gene family undergoes AS which specifically targets miR156 regulated BdSBP genes (Fig. 5B). The number of splice isoforms for each BdSBP genes was derived from plant Ensembl database. Splice variants from Ensembl gene are compared to generate an inclusive list of elementary alternative splicing events. The range of splice isoforms produced by BdSBPs was between 2 and 7. Most of the splice variants of BdSBP genes possess miR156 target site except BdSBP1, which has 4 splice variants and only one contains miR156 target site.

**Organ specific differential accumulation of BdSBP genes is regulated by miR156.** Three BdSBP genes (BdSBP3, BdSBP17 and BdSBP23) were analyzed for miR156 mediated transcript degradation by 5′-RLM-RACE (Fig. 5C,D). Additionally, to observe miR156 mediated cleavage pattern, we also constructed cDNA libraries from leaf and spike. Interestingly, BdSBP genes were highly degraded by miR156 in leaf as compared to spike tissue. We hypothesized that this differential degradation of BdSBP genes in leaf and spike tissues might be connected with their expression patterns in these tissues. To validate this, we performed semi-quantitative RT-PCR of several potential BdSBP genes on the basis of in silico expression data (Fig. 5E). Our data indicate that miR156 targeted BdSBP genes indeed expressed poorly in the leaf and abundantly in the spike, confirming our hypothesis. The miR156 non-targeted genes (BdSBP9 and BdSBP15) expression was constant in both the leaf and the spike, suggesting no effect of miR156 on these genes. Furthermore, to map the miR156 cleavage site in BdSBPs transcript, the 5′-RLM-RACE products were cloned and sequenced. Data indicates that miR156 cleaves between 9 and 10th nucleotide of 5′ site of BdSBPs transcript, except BdSBP3 where cleavage site was found.
between 10 and 11th nucleotides. Collectively, our results suggest multilayered regulation of BdSBP genes at the post-transcriptional level.

**BdSBP genes are involved in complex regulatory network and pathways.** BdSBPs co-expressed genes were investigated using publicly available large-scale co-expression database (www.gene2function.de), and MapMan (https://mapman.gabipd.org) ontology term enrichment to study their roles in different biological pathways (Fig. 6A–B). Around 710 co-expressed genes were found to be associated with 15 members of BdSBP family (Supplementary Table S5). The MapMan ontology of the co-expressed genes suggests that 22 out of 35 of the major biological classes have at least one of the BdSBP family members (Fig. 6B). Cell, development, transport, hormone metabolism, secondary metabolism, stress, lipid metabolism, cell wall, DNA, RNA, protein and signaling were major biological processes in which co-expressed genes of BdSBP family members were involved. Some other BdSBP family members and their co-expressed genes were enriched in photosynthesis, major CHO metabolism, fermentation, oxidative pentose pathway, mitochondrial electron transport and amino acid metabolism. However, BdSBP co-expressed genes were not augmented in the C1-metabolism, microRNA, polyamine metabolism, nucleotide metabolism, 5-assimilation, N-metabolism, glycolysis and minor CHO metabolic pathways.

In addition, a network of protein–protein interaction of B. distachyon proteins was developed using STRING database. This database predicts interactions based on experimentally determined, predicted, text mining, co-expression, gene fusion, gene neighbourhood etc. A total of 39 interactive proteins were found (confidence value = 0.5) for 9 of BdSBP proteins, which were based on either predicted interactions or text mining (Fig. 7A, Supplementary Table S6). Protein annotation reveals that BdSBP proteins might interact with MYB33, PHABULOSA (PHB), Homeobox TF family, Growth regulating factor 5 (GRF5), Heat shock TF, ZnF C2H2, F-box TF, NBS-LRR, protein kinase family protein, ankyrin repeat protein, protein kinase family protein, chlorophyll a-b binding protein, DCL1, DCL2 and DCL3 proteins. The BdSBP7 was the only B. distachyon protein that interacts with DCL2 and DCL3 proteins. The MapMan term ontology of interactive protein partners of the BdSBPs indicates that 10 out of 35 proteins of the major biological terms were enriched by at least one of the BdSBP protein network (Fig. 7B). These biological processes were linked to development, RNA, photosynthesis, cell wall, protein, transport, signaling, cell cycle and stress.

**BdSBP genes express differentially during variable temperature conditions.** In order to advance our knowledge about the molecular mechanism controlling heat stress in Brachypodium, we examined the tran-
scriptional changes in *BdSBP* genes in the spike development under 22 °C and 42 °C in *B. distachyon*, *B. stacei* and *B. hybridum* (Fig. 7C). Transcript abundance of *BdSBP1*, *BdSBP14* and *BdSBP16* was higher at 42 °C among all the accessions, independent of ploidy level and it will be important to functionally validate these key genes in future. Negligible transcript abundance of *BdSBP8*, *BdSBP9* and *BdSBP23* was observed in *B. stacei*. However, transcript level of *BdSBP3*, *BdSBP9*, *BdSBP18* and *BdSBP23* was higher in *B. distachyon* and *B. hybridum* as compared to *B. stacei* under both conditions. We did not observe any temperature dependent specific expression pattern among miR156 targeted and non-targeted *BdSBP* genes. Our results imply important roles of *BdSBP* genes to beat the heat in the reproductive organs of *Brachypodium* spp. *BdSBP* genes regulate spike development and flowering. In silico expression analysis revealed higher expression of *BdSBP* genes during spike emergence and in early inflorescence development (Fig. 4). Therefore, the transcript abundance of 9 *BdSBPs* was examined during different developmental stages [7–24 Days after Heading (DAH) of *Brachypodium* spikelet] (Fig. 8A,B; Supplementary Fig. S3). Five genes including *BdSBP3*, *BdSBP16*, *BdSBP17*, *BdSBP18* and *BdSBP23* were highly abundant during early spikelet development (7 DAH), as compared to mid-phase (15–20 DAH) or maturation phase (24 DAH). However, the transcript level of *BdSBP1* was constant at 7, 15 and 20 DAHs except at 24 DAH. No change in transcript level of *BdSBP15* was observed at any of the above-mentioned developmental stages. Expression pattern of *BdSBP9/16/17/18* was confirmed by qPCR (Fig. 8B). Expression of *BdSBP9* was slightly lower at 15DAH as compared to 7 and 24DAHs.

To ensure the reproductive success, flowering is the critical stage of plant reproduction, which is mainly regulated by gibberellin, vernalization, photoperiod and autonomous pathways. Vernalization promotes the flowering in alpine species and its molecular mechanism has been investigated in *Arabis alpina* and *A. thaliana* plants (Bergonzí et al. 51). Therefore, to understand the genetic control of vernalization response in grasses, we analysed the expression pattern of *BdSBP* genes in *Brachypodium*. Transcript abundance of several *BdSBP* genes was compared in the rapid flowering (Bd21) and delayed flowering (Bd1-1) accessions of *Brachypodium* under vernalization and non-vernalization conditions (Fig. 8C,D; Supplementary Fig. S4). We observed that Bd21 accession flowered rapidly under non-vernalised condition, whereas Bd1-1 lacked flowering until maturity.
Figure 6. Co-expression and metabolic pathway analysis of BdSBP genes: (A) the co-expression neighbourhood was analysed using PlaNet tool. The green, oranges and red edge colours shows strong, medium and weak co-expression. Coloured shapes indicate label co-occurrences. The gene annotation of co-expressed genes is available in Supplementary Table S5. (B) The co-expressed genes of BdSBPs that are enriched for a biological pathway given by MapMan term are shown by red boxes.

Figure 7. Protein interaction network and pathway analysis of BdSBP proteins. (A) The potential interactors for 9 BdSBP proteins were predicted using STRING tool and are shown with different coloured connective lines. (B) The biological pathways enriched in MapMan terms in which the interacting partners of BdSBP proteins are involved. (C) Effect of heat stress on the expression of BdSBP genes in B. distachyon, B. stacei, and B. hybridum grown under normal (22 °C) and heat stress (42 °C) conditions.
sis in a species specific manner and underwent different gene duplication events. On the basis of phylogenetic analysis, SBP domain binds to the promoter regions of its target genes containing TNCGTACA A consensus nucleotide and comparison to soybean (41), moso bamboo (32) and P. gene pairs56. Additionally, gene duplication also assists organisms to cope up with different environmental conditions within the same phylogenetic groups (Fig. 3A). Additionally, most of the BdSBPs from the same phylogenetic group experienced positive selection, = 1 indicate neutral selection and < 1 indicates purifying selection or negative, respectively. Based on Ka/Ks ratio56; the BdSBP genes were divided into eight (G1–G8) groups (Fig. 1A). BdSBP genes grouped closely with HvSPLs and TaSPLs, suggesting that these SBP genes possibly diverged from a common ancestor. The DNA binding motif of BdSBP genes share similar gene structures within their same phylogenetic group as mentioned previously in barley13, rice11,54, and Arabidopsis17; but was smaller in comparison to soybean (41), moso bamboo (32) and P. trichocarpa (28), suggesting that SBP genes were evolved in a species specific manner and underwent different gene duplication events. On the basis of phylogenetic analysis, BdSBP genes were divided into eight (G1–G8) groups (Fig. 1A). BdSBP genes grouped closely with HvSPLs and TaSPLs, suggesting that these SBP genes possibly diverged from a common ancestor. The DNA binding motif of BdSBP genes share similar gene structures within their same phylogenetic group as mentioned previously in barley13, rice11,54, and tomato31. Gene duplication events are key to evolution and gene expansion which produce many paralogous gene pairs which were ranged from 0.2 to 0.7 suggesting that these genes were evolved under purifying selection. In order to study gene duplication, we estimated the Ka/Ks ratio for each duplicated genes using Plant Genome Duplication Database and PLAZA 4.0 (Fig. 2A,B), which suggests that BdSBP genes underwent duplication event ~ 74 to 164 mya. The Ka/Ks ratio of > 1 shows that the gene has experienced positive selection, = 1 indicate neutral selection and < 1 indicates purifying selection or negative, respectively. Based on Ka/Ks ratio46; the BdSBP gene pairs which were ranged from 0.2 to 0.7 suggesting that these genes were duplicated under purifying selection. Also, BdSBP genes shared similar intron/exon structures within the same phylogenetic groups (Fig. 3A). Additionally, most of the BdSBPs from the same phylogenetic groups possess similar motifs (Fig. 3B). Consequently, the genes in the same phylogenetic group might have similar roles in Brachypodium, which have been supported by gene ontology terms of BdSBP genes (Fig. 3C).

In addition to conserved BdSBPs motifs, several unique group-specific motifs were observed, such as motif 4, 9.

However, both the accessions produced flowers with 6-weeks vernalisation at 4 °C. BdSBP1 and BdSBP3 expressed differentially in these accessions following vernalization or non-vernialization. The expression of BdSBP1 and BdSBP3 were found to be lower in Bd1-1 as compared to Bd21 under non-vernalized condition. Whereas, under vernalized condition, no change in the transcript level was observed suggesting their possible role in flowering time and spikelet development. However, transcript level of BdSBP9/16/17/21/23 was not altered significantly under vernalization condition.

To further confirm the function, T-DNA mutant for BdSBP9 gene was obtained from JGI (Fig. 9A–C). The BdSBP9 mutant has a T-DNA insertion in the first exon of BdSBP9. Electron microscopy indicated that different patterns of lignification in the wild type as compared to Bdsp9. Wild-type patterns were straighter, with no circular patches whereas, Bdsp9 patterns are less uniform, with some circular patches. Further, we investigated the promoter region (1000 bp upstream of initiation codon) of the co-expressed genes of BdSBP9 (Fig. 9D).

Figure 8. Expression pattern of BdSBP genes during spikelet development and flowering. (A,B) Semi-quantitative RT-PCR and quantitative real time PCR (qRT-PCR) analysis of BdSBP genes at 7, 14, 20, and 24 DAH stages of spikelet development. *miR156 targeted BdSBP genes. (C) Brachypodium accessions Bd21 (early flowering) and Bd1-1 (delayed flowering) under vernalization and non-vernalization time course. Bar represents 1 cm. (D) Semi-quantitative RT-PCR analysis of BdSBP genes in vernalized and non-vernalized Brachypodium accessions Bd21 and Bd1-1. BdUBC18 was loaded as internal control.

Discussion

SBP/miR156 genetic circuit controls the transition of vegetative to reproductive phase change in Arabidopsis14,52. Owing to the importance of SBP genes, we conducted the first-ever genome-wide identification of this gene family in Brachypodium and discovered 18 BdSBP genes (Table 1). The number of SBP genes in Brachypodium were similar to the SBP genes in barley (17), B. luminifera (18), rice (19) and Arabidopsis (17), but was smaller in comparison to soybean (41), moso bamboo (32) and P. trichocarpa (28), suggesting that SBP genes were evolved in a species specific manner and underwent different gene duplication events. On the basis of phylogenetic analysis, BdSBP genes were divided into eight (G1–G8) groups (Fig. 1A). BdSBP genes grouped closely with HvSPLs and TaSPLs, suggesting that these SBP genes possibly diverged from a common ancestor. The DNA binding motif of BdSBP genes share similar gene structures within their same phylogenetic group as mentioned previously in barley13, rice11,54, and tomato31. Two zinc ion binding motifs Cys3His1 and Cys2His1Cys1 at N terminus and a nuclear localization signal (NLS) at C terminus were found in BdSBP proteins (Fig. 1B).
and 10 in group 1 and motif 8 in group 8. These specific motifs might be important for specified roles of BdSBP genes, and their functional differentiation could arise during evolution of different lineages.

All BdSBP genes expressed substantially during early and emerging inflorescence development except, BdSBP1 and BdSBP7, implying their role in inflorescence development of Brachypodium (Fig. 4). Most of the BdSBP genes except, BdSBP7/8/17/21, expressed significantly in pistil whereas BdSBP3/6/11/14/15/16 expressed highly in the anther, suggesting their role during reproduction. However, BdSBP6/9/15 were constitutively expressed in all the 9 tissues. Previously, differences in expression profiles of miR156 targeted and non-targeted SBP genes have been reported in barley13, Brassica napus58, Betula59 and soybean12. Importantly, miR156 targeted BdSBP genes showed differential expression pattern and most of the miR156 non-targeted BdSBPs showed constitutive expression profiles in Brachypodium. Post-transcriptional regulation of SBP genes through miR156 has been considered as the key process for the functionality of these genes27,29,60. A total, 11 of 19 SBP genes in rice11, 10 of 17 SBPs in Arabidopsis52, 7 of 17 SBPs in barley13, and 18 of 28 SBPs in Populus8 have been identified as targets of miR156. In our study, miRNA target prediction revealed that 10 BdSBP genes are regulated by miR156 (Fig. 5A).

A total 8 (BdSBP3,-11,-13A,-14,-16,-17,-18, and 23) of 10 miR156 targeted BdSBP genes contained miR156 complementary sequence in their coding region whereas, BdSBP1 and BdSBP13 contained target site in 5′ and 3′ UTRs, respectively. Thus, miR156 targets BdSBP1 and BdSBP13 along with other BdSBP genes will be unable to perform downstream roles. This phenomenon of binding of miRNAs to their complementary sequences in the coding sequences or un-translated regions of target genes to inhibit gene function either by transcript cleavage or deadenylating has also been reported elsewhere (Rhoades et al.61).

In humans ~ 95% and in Arabidopsis > 60% of multi-exonic genes undergo AS62. Meanwhile, we noticed that miR156 targeted BdSBP genes produced different splice variants via AS (Fig. 5B). AS generally produces transcripts with premature stop codons which are degraded in cytoplasm by non-sense-mediated decay (NMD) pathway63. Splice variants produced by AS generally exhibit spatiotemporal or environmental condition-specific expression patterns63. Our experiments in Brachypodium showed that BdSBP gene products are degraded at higher level by miR156 in the leaf as compared to the spike (Fig. 5C,D), resulting into higher transcript abundance of miR156 targeted BdSBP genes in young spike as compared to leaf (Fig. 5E; Supplementary Fig. S1). However, expression of miR156 non-targeted BdSBP9 and BdSBP15 genes was constitutive in these tissues. This confirms that miR156 negatively regulates SBP genes in Brachypodium and is consistent with previous findings in Arabidopsis, rice, tomato and wheat11,29,35,52,64. Taken together, these results suggest that miR156 in conjunction with AS regulates the transcriptome dynamics.
SBP-correlated gene network and interactome analysis revealed that SBP genes function by regulating other families of transcription factors and membrane transport proteins, and are involved in the metabolism of glucose, inorganic salts and ATP production in *Arabidopsis*<sup>25</sup>. Therefore, considering the significance of *Brachypodium* as a model plant for developmental biology of triticale crops, we examined the co-expression and MapMan biological pathways (Fig. 6A,B; Supplementary Table S5). MapMan terms enrichment analysis showed that in BdSBP genes perform their function by regulating transcription, protein, signalling, transport and development related biological pathways (Fig. 6B). The co-expression network contains mainly transcription factors, hormones (auxin, brassinosteroid, ethylene and gibberellin) responsive genes, cell wall biogenesis related genes and transporters, implying their roles in development as well as cell wall biogenesis of *Brachypodium*. Existence of CSLF3 and MYB TF indicate that BdSBP genes might be involved in secondary wall synthesis in *Brachypodium*<sup>66</sup>. Studying the protein–protein interaction network represents gene functions crucial to plant physiology, pathology, and growth<sup>67</sup>. Protein–protein interaction at the molecular level might be important in transcription regulation, post-transcriptional modification, cytoskeleton assembly, phosphorylation, acetylation, transporter activation and others<sup>48</sup>. Previously, it was found that IPA1 (OsSPL14), an important factor which controls plant architecture interacts with D53 protein (DWARF53) in-vivo and in-vitro<sup>69</sup>. Recently, OsSPL14 protein has been shown to be associated with disease and yield in rice by phosphorylation and non-phosphorylation of Ser<sup>183</sup> amino acid respectively during *Magnaporthe oryzae* fungal infection<sup>6</sup>. In our study 39 interacting proteins with 9 BdSBP proteins were identified (Fig. 7A,B; Supplementary Table S6). These interacting proteins mainly belonged to bZIP, Homeobox, MYB33, ZnF_C2H2, F-box and heat shock transcription factor families, Dicer-like proteins and protein kinases. These interacting protein partners have been involved in the regulation of the biological pathways including development, RNA, protein, stress, photosynthesis and cell wall, implying the diverse roles of BdSBP proteins in *Brachypodium* growth and development.

The grain development and filling of *Brachypodium* spikelet are completed (dry) in 50 days and has been classified into three stages namely-embryo and endosperm development [0–14 days after fertilization (DAF)]; maturation (14–36 DAF) and desiccation (36–50 DAF) stages<sup>70</sup>. Higher expression of BdSBP1/-3/-16/-17/-18/-21 at spikelet initiation stage as compared to the maturation stage, might be key to early spikelet development in *Brachypodium* (Fig. 8A,B). Further, BdSBP9 and BdSBP15 genes exhibited constitutive expression pattern during embryogenesis and maturation stages, suggesting their importance for these stages. Plants bear flowers at a certain time of reproductive phase which is mainly regulated by SBP/miR156 pathway<sup>29</sup>. As plants grow older, the level of SBP genes increases while miR156 abundance declines. Previously, it was reported that higher production of SBP genes ensures flowering in response to cold in the model perennial *Arabis alpina* accession Pajares<sup>54</sup>. It has been reported in *Cardamine flexuosa* that SBP/miR156 pathway plays a key role in flowering through integrating age and vernalization pathway<sup>71</sup>. The SPL/miR156 module has been known to be a key component for flowering phases<sup>32,33</sup>. Involvement of SBP genes in the control of flowering time of *B. distachyon* accessions Bd21 and Bd1-1 under vernalization condition (Fig. 8C,D) suggest that BdSBP1 and BdSBP3 potentially involved in this. This result positively supports the previous study about sensitivity of certain SBP genes to vernalization in older plants<sup>55</sup>. Cereals inflorescence (spike) architecture is one of the main determinants of their yield. In rice, SBP genes have been reported as an important regulator of plant architecture. Overexpression of OsSPL14, present on the IPA1 (ideal plant architecture)/WFP (wealthy farmer's panicle) QTL, decreased tiller branching but increased panicle branching and grain weight<sup>18,36</sup>. Likewise, OsSPL7, OsSPL13 OsSPL16 and OsSPL17 also regulate grain size, shape and yield in rice<sup>37,38</sup>. In our study, the *BdSbp9* mutant showed abnormal spike and delayed flowering, implying its role in spike development (Fig. 9A–E). In *Arabidopsis*, miR156/SPL module confers thermotolerance at reproductive stage<sup>72,73</sup>. Our study also indicates that BdSBP genes contribute thermotolerance during spike development in *Brachypodium*. Interestingly, differential expression of BdSBP genes in the developing spike under variable temperatures was not been associated with ploidy level in *Brachypodium* genome as described previously<sup>49</sup>. However, specific expression of these genes in response to high temperature in tetraploid genome, *B. hybridum*, probably induced by interaction of *B. distachyon* and *B. stacei* genomes (Fig. 7C; Supplementary Fig. S2). Overall, our study revealed that altering the expression pattern of BdSBP genes may provide an important tool-box for the genetic improvement of the cereal crops.

**Materials and methods**

**Identification and annotation of SBP genes in *Brachypodium distachyon***. To identify SBP genes in *Brachypodium distachyon* genome, PHMMER search was performed on EnsemblPlants database (https://plants.ensembl.org/Brachypodium_di/Info/Index) using *A. thaliana* SBP domain (Pfam: PF03110) sequence as the query<sup>35,37</sup>. Additionally, phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Bdiul gare_erp) database was also mined through TBLASTN using SBP domain amino acid sequences. The accession numbers of putative BdSBP genes were taken from databases and were named based on their closest homologs present in barley, wheat and rice. Further, EnsemblPlants database (https://plants.ensembl.org/Brachypodi um_di/Info/Index) was used to obtain the genomic sequences (Table S1), coding sequences (Supplementary Table S2) and protein sequences (Supplementary Table S3) of BdSBP genes.

**Gene structure and phylogenetic analysis of BdSBP genes**. The exon/intron structure of each BdSBP gene was predicted through gene structure display server program (https://gscs.cbi.pku.edu.cn/index.php) by comparing their coding and genomic sequences. The TAIR (https://www.arabidopsis.org/index.jsp) was used to obtain the *Arabidopsis* SBP sequences and rice genome annotation project database was used to obtain the rice SBP gene sequences. SBP sequences of wheat were obtained from a previous study<sup>34</sup>. SMART tool was used to identify SBP domain sequences from *Brachypodium*, rice, wheat, and *A. thaliana* which are presented in Supplementary Table S4. A phylogenetic tree was annotated using the Interactive Tree of Life resource.
Motif identification, miR156 target site prediction and alternative splicing event analysis. The MEME 4.11.0 tool (https://meme-suite.org/tools/meme/) was used to search for conserved motifs within BdSBP proteins by using default settings, except that the maximum number of motifs to find was 10, the maximum width was 50 and the minimum width was 6. The sequence logo of the Brachypodium SBP domain was created with an online available WebLogo3 platform (https://weblogo.threeplusone.com/). The cDNA sequences of BdSBPs were subjected to psRNA.Target tool (https://gtrnadb.ucsc.edu/targetsite) to predict the putative target sites of miR156. The Ensemble database (https://plantsensembl.org/Brachypodium_distachyon/Info/Index) was used to obtain the information on alternative splice events for each BdSBP gene (Supplementary Table S5).

Gene expression analysis of BdSBPs. The log2-transformed fragments per kilobase per million fragments measured (FPKM) values were used to study the expression of BdSBPs in nine tissues as described35,74. A heat map of the expression of BdSBPs was generated by the average hierarchical clustering method using the MeV tool (https://www.tm4.org/mev.html).

Co-expression, protein–protein interaction and gene duplication analysis. The co-expressed genes for BdSBP members were identified using online PlaNet (https://aranet.mpimp-golm.mpg.de/index.html) tool76. PlaNet uses the Pearson correlation coefficient (PCC) and constructs a co-expression network, with PCC cut-off to 0.777. Further, a highest reciprocal rank (HRR) co-expression network with standard edge cutoff of 30 was used. Additionally, a heuristic cluster chiseling algorithm (HCCA), which is optimized for HRR-based networks, was used with standard parameters (stepsize = 3). The protein–protein interaction network was identified using STRING database (https://stringdb.org/cgi,input.pl?sessionId=A92xEG0hsQEk&input_page_show_search=on), which contained information from various datasets such as; gene coexistence, protein–protein inter-actions, gene fusion and co-expressed genes to calculate the semantic links between proteins77. The genome-wide genomic duplication files of B. distachyon were retrieved from the plant genome duplication database (PGDD) (https://chibba.agtec.uga.edu/duplication) and PLAZA4.0 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_dicots). The synonymous substitution (Ks) and non-synonymous substitution (Ka) rates were obtained from PGDD and the ratios of Ka/Ks were used to assess the selection pressure for duplicated gene events.

Plant material and sample preparation. Brachypodium seeds from Bd21-3, Bd21, B. hybrida, B. stacei and Bd1-1 accessions were obtained from Prof. John Vogel (DOE Joint Genome Institute, CA, 94598 USA). Seeds were imbibed in water overnight, dried and stratified at 4 °C in the dark for 1 week. The daily temperature was 22 °C and the photoperiod was 16-h-light/8-h-dark (long day). The Bdxhp9 mutant line JJ12467 was obtained from a Brachypodium T-DNA insertion library36, Prof. John Vogel’s lab (JGI). The T1, Bdxhp9-mutant seeds were advanced for two further generations using conditions described above and according to method by81. For the vernalization experiment, Bd21 and Bd1-1 seeds were vernalized for 6 weeks at 4 °C. For heat stress study, B. distachyon, B. hybrida and B. stacei seeds were grown under 22 °C and 42 °C for 2 h. Further, immature spikes were collected from each accession to elucidate transcript abundance of BdSBP genes. To study the expression pattern of BdSBP genes in Brachypodium spike development, tissue samples were collected from leaf, and spike tissue at 7, 14, 21, and 25 days after heading (DAH).

Genomic DNA and RNA isolation. Leaves from Brachypodium distachyon plants were collected and DNA isolation was performed using cetyl-trimethyl-ammonium bromide-based (CTAB) extraction method as described elsewhere82. PCR-based genotyping was performed using primers following recommendations from Joint Genome Institute86. The spectrum plant total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) was used for RNA isolation following the manufacturer’s protocol. The RNA integrity and purity of all samples were verified on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). Prior to synthesizing cDNA, RNA samples were treated with DNase I to remove genomic DNA contamination (Invitrogen, USA). The reaction mixtures were incubated at 23 °C for 15 min and after that 1 μl of 25 mM EDTA was added to each sample.

First strand cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis. First strand cDNA was synthesized from 2 μg total RNA sample using AffinityScript QPCR cDNA Synthesis Kit (Agilent technology, Canada). The qRT-PCR was run on Mx3000p qPCR system (Stratagene, USA) in a 20-μl volume containing 5 μM gene-specific primers, 1 μl diluted cDNA, and 10 μl Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent, USA). Two biological and three technical replicates were used in all the experiments. The 2^-ΔΔCt method was used to quantify the relative level of gene expression (Livak and Schmittgen 2001). The gene-specific primers for BdSBP genes used in semi-quantitative RT-PCR and qRT-PCR are listed in Supplementary Table S7. PCR was performed in a 20 μl volume using GoTaq Green master mix (Promega, USA). BduBC18 (Ubiquitin-conjugating enzyme 18) was used as a reference gene for different developmental stages and SamDC (S-adenosyl methionine decarboxylase) was used for heat stress82.
Scanning electron microscopy (SEM). Immature spikes (14 days after anthesis) were collected from Bdsbp9-mutant and control plants. Samples were fixed in 25 mM phosphate buffer (pH 7.0) with 3% glacial acetic acid overnight. Dehydration of the tissue was carried out by increasing the ethanol concentration of the solution every hour (30%, 40%, 50%, 60%, 75%, 90%, 100%), keeping the samples in 100% ethanol for two days. Samples were critically point dried using the Leica EM CPD300 and coated with platinum using the Leica EM ACE200. The samples were visualized using Hitachi TM1000.

RNA ligase-mediated modified 5’ rapid amplification of cDNA ends (RLM-RACE). The miR156 mediated cleavage site in the BdsBP transcript was mapped by using First-choice RLM-RACE kit (Ambion, Austin, TX, USA). Total RNA was isolated from leaf and 7 days old spike (after heading). Without pre-treatment, 1 μg total RNA was ligated to the 5’ RACE RNA adapters. The M-MLV reverse transcriptase enzyme and 18mer oligo dT were used to reverse transcribe the adapter-ligated RNA. Primary and secondary nested PCR was carried out using 5’ RACE gene-specific outer and 5’-adapter outer primers and 5’ RACE gene-specific inner and 5’-adapter inner primers. The primer sequences used in nested PCR are listed in Supplementary Table S5. The 5’ RACE PCR amplified fragments were gel extracted and cloned into pGEMTeasy vector. Further, clones were confirmed by EcoRI restriction analysis and Sanger sequencing.

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**Author contributions**

R.K.T., J.S.: conceived and designed the experiments. R.K.T.; performed the experiments. W.O.; analysed the T-DNA mutant. R.K.T., J.S.: analysed the data. R.K.T., J.S. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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