GENOME-WIDE IDENTIFICATION AND CHARACTERIZATION OF THE SBP GENE FAMILY IN *EUCALYPTUS GRANDIS*

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Abstract. SQUAMOSA promoter binding proteins (SBP) are a group of plant transcription factor (TF) families that play an important role in plant development and defence response. Genome-wide investigations of *SBP* genes have been carried out in many plant species, however this is the first comprehensive report on the identification and characterization of *SBP* genes in *Eucalyptus grandis* (*E. grandis*). According to the results herein, 16 *SBP* genes from *E. grandis* were identified and SBP proteins were clustered in eight clades. 16 *SBP* genes were presented to distribute 7 out of 11 chromosomes and three segmental duplicated gene couple were found throughout the entire genome of *E. grandis*. A total of 15 conserved motifs were described and 3 out of 15 were found to be conserved among all Egrandis_SBP proteins, due to forming of SBP domain which is essential for the function of SBP proteins. Gene structures of all *Egrandis_SBP* genes were investigated and the estimated number of exons among all genes extended from 2 to 11. According to the synteny analysis, it was seen that homologs of *E. grandis* genes were found in corresponding syntenic blocks of *Arabidopsis thaliana* and *Vitis vinifera*. Digital gene expression analyses showed that most of the *Egrandis_SBP* genes were highly expressed in shoot tips, young leaf, xylem, mature leaf, immature xylem and phloem tissues of *E. grandis*. Taken all together, the results of this study will provide an important source for literature and further works.
Keywords: *bioinformatic approach, phylogeny, RNAseq, digital gene expression*

Introduction

Squamosa promotor binding proteins (SBPs) are transcription factor (TF) genes that are only found in plants, their presence have not been verified in animals and prokaryotic organisms thus far (Klein et al., 1996; Hou et al., 2013). One common feature in members of the SBP TF family is the presence of 76 aa DNA-binding domain which have been called: SBP domain containing two zinc fingers (Cys-Cys-His-Cys and Cys-Cys-Cys-His) (Klein et al., 1996; Yamasaki et al., 2004; Pan et al., 2017).

They were first discovered in *Antirrhinum majus* (Klein et al., 1996) and their first comprehensive characterization was performed in *A. thaliana* (Cardon et al., 1997) in order to explore their exact function in plants. Like all TFs that have been discovered up to date, SBP proteins were indicated to play crucial roles in the expressional regulation of genes that are involved in many important processes related to plant development and defence response (Unte et al., 2003; Zhang et al., 2007; Wu and Poethig, 2006).

Genome-wide characterization and expression studies of *SBP* genes have been conducted on a number of plant species including *Betula pendula, Chlamydomonas, Oryza sativa, Zea mays, Populus trichocarpa, Lycopersicon esculentum* L., *Malus* × *domestica* Borkh., *Salvia miltiorrhiza, Gossypium hirsutum, Arachis hypogaea* L., *Petunia, Capsicum annuum* L., and *Phyllostachys edulis* (Chuck et al., 2010; Kropat et al., 2005; Lannenpaa et al., 2004; Li et al., 2013, 2016; Lu et al., 2011; Pan et al., 2017; Preston et al., 2016; Salinas et al., 2012; Xie et al., 2006; Zhang et al., 2016, 2014; Zhang et al., 2015). Nonetheless, the SBP proteins in *Eucalyptus grandis,* a tall forest tree in the *Myrtaceae* known as the flooded gum or rose gum, have not been identified and characterized despite the fact its genome was sequenced in 2014 (Myburg et al., 2014).

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Eucalyptus grandis (*E. grandis*) is a plantation crop which spreads widely in tropics and subtropics areas. It is commonly found in nature in Australia, and it is considered an important plant for plant scientist because, of its extreme ability to adapt to both climate and soil (Robinson et al., 2006). Moreover, there is a wide range of uses for *E. grandis* in farm; specialty products (flowers generate nectar for honey production), urban (used for ornamental purposes), wildlife value (its flowers attracts birds) and wood products including: pulpwood, boat building, boxes, flooring, heavy construction, fuelwood, industrial charcoal, panelling and timber for furniture etc (dos Santos et al., 2004; Nautiyal and Couto, 1984; Hardie and Wood, 1973; Dye, 2013).

Due to its importance in a wide range of use for the industry, and its ability to adapt to extreme soil and climate conditions, our aim was to identify and characterize SBP proteins in *E. grandis* which are important TF family for the plant developmental processes. Accordingly, 16 Egrandis_SBP members were identified and comprehensive analyses of the sequence phylogeny, genomic organization, exon-intron region of gene, conserved protein motifs, gene duplication events, and expression analysis were performed.

Materials and methods

Identification of SBP genes in E. grandis genome

E. grandis of SBP protein sequences were retrieved from Phytozome database v12.1 (www.phytozome.net) using keywords in the search with Pfam Accession Number (PF03110) obtained from Pfam Database (http://pfam.xfam.org). BLASTP and BLASTX searches (National Center for Biotechnology Information [NCBI]: http://www.ncbi.nlm.nih.gov) were used to confirm Egrandis_SBP proteins. Non-redundant sequences were obtained using decrease redundancy tool (http://web.expasy.org/decrease_redundancy/). SBP domains in non-redundant sequences were checked by HMMER (http://www.ebi.ac.uk). The solid and chemical traits of SBP proteins in *E. grandis* were identified using the ProtParam tools (http://web.expasy.org/protparam/) such as: the theoretical isoelectric point (pI), number of amino acids, and molecular weight (Da).

Phylogenetic analysis, physical location, conserved motifs of Egrandis_SBP genes, gene structure and gene duplication events

Chromosomal locations and CDS sizes (bp) were identified by using Phytozome database v12.1. The *Egrandis_SBP* genes were mapped with MapChart (Voorrips, 2002). Multiple sequence alignment of aminoacid sequences of Egrandis_SBP proteins was conducted with ClustalW Phylogenetic analysis were performed using MEGA v7 (Tamura et al., 2013; Buyuk and Aras, 2016) and Neighbor-joining (NJ) algorithm with 1000 replicated-bootstrap value.

Egrandis_SBP protein sequences of the conserved motifs were identified using MEME (Multiple Expectation Maximization for Motif Elucidation; http://meme-suite.org/) (Bailey et al., 2006; Buyuk et al., 2016; Inal et al., 2017; Ilhan et al., 2018). The limits for maximum number of motifs and minimum/maximum width were adjusted to; 20 and 2, 50, respectively. Motif sites were among 2 and 300. Site distribution was set as any number of repetitions. The described conserved motifs were examined in InterProscan with default adjusting (Quevillon et al., 2005).

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Gene Structure Display Server program tool (GSDS; http://gsds.cbi.pku.edu.cn/) was used (Guo et al., 2007) to predict the exon/intron organization of the *Egrandis_SBP* genes. Genomic DNA sequences and coding sequences of *Egrandis_SBP* genes were utilized.

Segmental duplicate gene pairs were examined on the Plant Genome Duplication Database server (http://chibba.agtec.uga.edu/duplication/index/locus), with a display range of 100 kb. The nonsynonymous rates (Ka), synonymous rates (Ks) and developmental constraints (Ka/Ks) with the duplicated pairs of *Egrandis_SBP*s were evaluated using CODEML program in PAML (Yang, 2007).

Synteny analysis

E. grandis and *A. thaliana*, *E. grandis* and *V. vinifera* of orthologue *SBP* genes were identified with Plant Genome Duplication Database (PGDD; http://chibba.agtec.uga.edu/duplication/) (Lee et al., 2013). Then, the protein sequences of orthologue were retrieved from Phytozome v12.1. The obtained synteny map was created using iTAK - Plant Transcription factor & Protein Kinase Identifier and Classifier (http://itak.feilab.net/cgi-bin/itak/index.cgi) (Zheng et al., 2016).

Gene expression analysis in silico

The expression levels of *Egrandis_SBP* genes were examined in special tissue libraries of plants at different stages of; shoot tips, young leaf, xylem, mature leaf, immature xylem and phloem. They were retrieved from Phytozome Database v12.1 (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvulgaris). FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced) units were used for the expression levels in silico. FPKM values were log2 transformed and the heatmap was produced with the algorithm CIMMiner (http://discover.nci.nih.gov/cimminer).

Results and discussion

Identification of SBP gene family in E. grandis

Sequences of *SBP* proteins in the *E. grandis* genome were downloaded from Phytozome database v12.1 (www.phytozome.net) using keywords in the search with Pfam Accession Number (PF03110) retrieved from Pfam Database (http://pfam.xfam.org/). Subsequently, SBP domains were analyzed by performing a search in the HMMER and Pfam databases in candidate Egrandis_SBP proteins, and the redundant sequences were discarded after obtaining confirmation. A total of 16 candidate *SBP* genes in *E. grandis* genome were discovered and given in *Table 1* which includes information about chromosomal location, amino acid (length), molecular weight, number of isoelectric point (pI) and instability index.

As shown in *Table 1* and *Figure 1*, all of the non-redundant *Egrandis_SBP* genes were distributed on 7 out of 11 chromosomes of *E. grandis*. While the lowest number of *Egrandis_SBP* genes was observed on chromosome 4 (one *Egrandis_SBP* gene), the highest number was found on chromosome 11 (4 *Egrandis_SBP* genes) (*Fig. 1*). The length of Egrandis_SBP proteins extended from 147 (Egrandis_SBP_8) to 1078 (Egrandis_SBP_10) amino acids (aa). PI values of Egrandis_SBP proteins were among 5.77 (Egrandis_SBP_9) and 9.50 (Egrandis_SBP_5) ranging from acidic to alkaline

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while the molecular weight of Egrandis_SBPs was between 21.277 kDa (Egrandis_SBP_11) and 119.285 kDa (Egrandis_SBP_10) (*Table 1*). SBP, which is a plant specific transcription gene family, was detected and classified in various species thus far, such species include: *A. thaliana* (Rhoades et al., 2002), *Orzya sativa* (Xie et al., 2006), *Lycopersicon esculentum L.* (Salinas et al., 2012), *V. vinifera* (Hou et al., 2013), *Citrus* (Shalom et al., 2015), *Triticum L.* (Wang et al., 2015), *Zea mays* (Mao et al., 2016), *Gossypium raimondii* (Ali et al., 2017), *Petunia* (Zhou et al., 2018), *Fragaria vesca*, *Pyrus bretschneideri*, *Prunus persica* and *Prunus mume* (Abdullah et al., 2018). Nevertheless, this is the first comprehensive report on identification and characterization of *SBP* genes in *E. grandis.*

Table 1. *Information of 16 Egrandis_SBP proteins*

Gene ID	Gene name	Locations	aa	mw (kDa)	pI	Instability index	Classifies
Egrandis_SBP_1	Eucgr.A01019	Chr01:30283917..30289641 forward	984	109.125	5.79	56.75	Unstable
Egrandis_SBP_2	Eucgr.A02441	Chr01:39752248..39757521 reverse	390	41.273	9.12	54.92	Unstable
Egrandis_SBP_3	Eucgr.B00631	Chr02:6573009..6576407 reverse	524	57.179	8.74	55.34	Unstable
Egrandis_SBP_4	Eucgr.B03500	Chr02:54998894..55003285 reverse	488	53.532	8.67	53.61	Unstable
Egrandis_SBP_5	Eucgr.B03518	Chr02:55118387..55120136 reverse	318	34.533	9.50	53.22	Unstable
Egrandis_SBP_6	Eucgr.D02505	Chr04:39040236..39042737 forward	348	38.354	8.45	64.98	Unstable
Egrandis_SBP_7	Eucgr.E01600	Chr05:18228156..18230663 forward	367	38.759	9.07	72.79	Unstable
Egrandis_SBP_8	Eucgr.E03260	Chr05:53739864..53743190 reverse	147	16.977	6.00	88.37	Unstable
Egrandis_SBP_9	Eucgr.B01228	Chr06:24482430..24488179 forward	821	91.221	5.77	55.35	Unstable
Egrandis_SBP_10	Eucgr.F01828	Chr06:24482430..24488179 forward	1078	119.285	7.11	58.38	Unstable
Egrandis_SBP_11	Eucgr.F03303	Chr06:44594386..44595751 reverse	186	21.277	8.86	69.09	Unstable
Egrandis_SBP_12	Eucgr.H04114	Chr08:55717528..55724988 reverse	1005	112.303	6.39	49.39	Unstable
Egrandis_SBP_13	Eucgr.K01046	Chr11:13456169..13458136 forward	236	26.056	8.66	64.51	Unstable
Egrandis_SBP_14	Eucgr.K01828	Chr11:23807022..23809835 reverse	376	39.879	9.12	59.39	Unstable
Egrandis_SBP_15	Eucgr.K02545	Chr11:33320448..33325312 reverse	551	59.583	6.83	50.07	Unstable
Egrandis_SBP_16	Eucgr.K02708	Chr11:34626111..34629250 forward	363	39.804	8.96	55.21	Unstable

Based on the importance of gene duplications in the evolution of gene families in plants, the gene duplication events of putative *SBP* genes in *E. grandis* genome have been examined in this study. Our analysis identified three duplicated gene couple (Egrandis_SBP_2/ Egrandis_SBP_4, Egrandis_SBP_8/ Egrandis_SBP_13, Egrandis_SBP_11/ Egrandis_SBP_13 and Egrandis_SBP_3/ Egrandis_SBP_15) among eight identified *Egrandis_SBP* genes (*Fig. 1*). The Ka/Ks ratios between these duplication gene couples were found to be lower than '1' which suggests that: natural selection has occurred during segmental duplication events (Juretic et al., 2005).

Likewise, many segmental duplications of SBP genes have been detected in *A. thaliana* (*AtSPL10*/*AtSPL11*, *AtSPL4*/*AtSPL5*, *AtSPL1*/*AtSPL12*) and *O. sativa* (*OsSBP10*/*OsSBP5*, *OsSBP11*/*OsSBP4*, *OsSBP12*/*OsSBP3*) (Blanc and Wolfe, 2004; Bowers et al., 2003; Paterson et al., 2004; Wang et al., 2005). Besides segmental duplication events, some plant species including *Malus* × *domestica Borkh* (Li et al., 2013), *V. vinifera* (Hou et al., 2013), *F. vesca, Pyrus bretschneideri, P. persica* and *P. mume* (Abdullah et al., 2018) have demonstrated to have tandem duplication events either.

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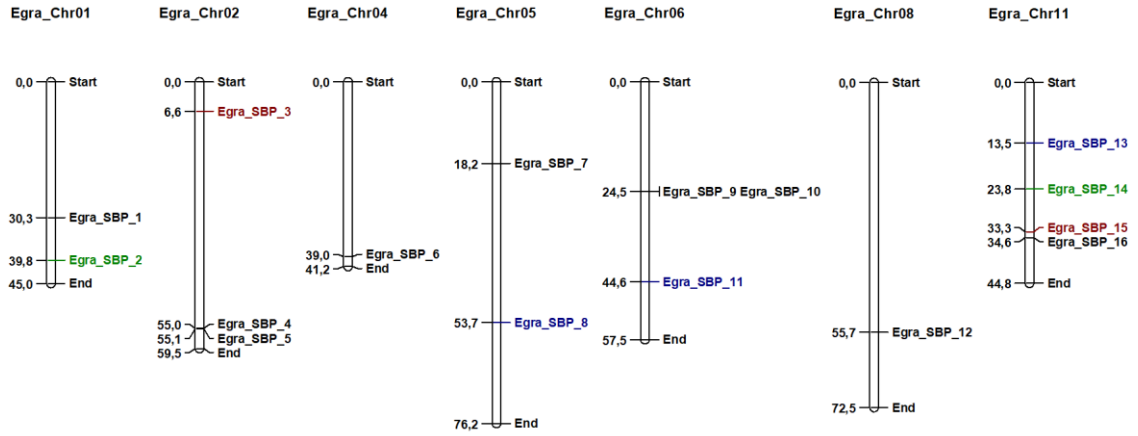

Figure 1. *Distribution of Egrandis_SBP genes on chromosomes of Eucalyptus grandis*

Phylogenetic analysis, gene structure and conserved motifs of Egrandis_SBPs

To discover the relationships between Egrandis_SBP proteins, a phylogenetic tree of SBP proteins in *E. grandis*, *A. thaliana* and *V. vinifera* was constructed using neighbor-joining (NJ) method with bootstrapping 1000 times (*Fig. 2*). Egrandis_SBPs were clustered into eight groups from A to D4 (*Fig. 2*). Group C2 was the largest group containing 18 SBP members, that is almost 34% of the total SBP proteins in the tree. And a minimum of one member of Egrandis_SBP proteins have made a contribution to each group, except for the Group A which has only one SBP protein from *V. vinifera* (*Fig. 2*). Egrandis_SBP proteins were clustered with the same number (7 from each) of *A. thaliana* and *V. vinifera* SBP proteins in Group C2. In contrast, any of *A. thaliana* were clustered with SBP proteins of *E. grandis* and *V. vinifera* in Group C1 suggesting that these genes may have been lost during evolution in *A. thaliana*. As previously declared by Abdullah et al. (2018), this kind of loss and birth of SBP genes which are specific to species might cause divergence in these genes in terms of functionality (Abdullah et al., 2018). In addition, similar coding and exon-intron sequences were observed in *SBP* genes which were found in the same subgroup of the phylogenetic tree (*Fig. 2*).

Similarly, phylogenetic trees of SBP proteins from several plant species including rice (Xie et al., 2006), *A. thaliana* (Guo et al., 2008), *Z. mays* (Chuck et al., 2010), *L. esculentum* (Salinas et al., 2012), *Malus slyvestris* (Li et al., 2013) and *Phyllostachys edulis* (Pan et al., 2017) were also divided into eight groups in accordance with the phylogenetic tree of SBP proteins from *E. grandis.*

To investigate conserved motifs in Egrandis_SBP proteins, MEME (v4.12.0) were used, and a total of 15 conserved motifs were described (*Fig. 3* and *Table A1* in the *Appendix*). The lengths of identified motifs were between 11 and 50 amino acids and all identified Egrandis_SBP proteins were found to have Motif 1, 2 and 3, which constitute the SBP domain (*Fig. 3*). Some of the other SBP motifs were only found in some Egrandis_SBP proteins, suggesting that these motifs may be provide by a specific function of these proteins (*Fig. 3*).

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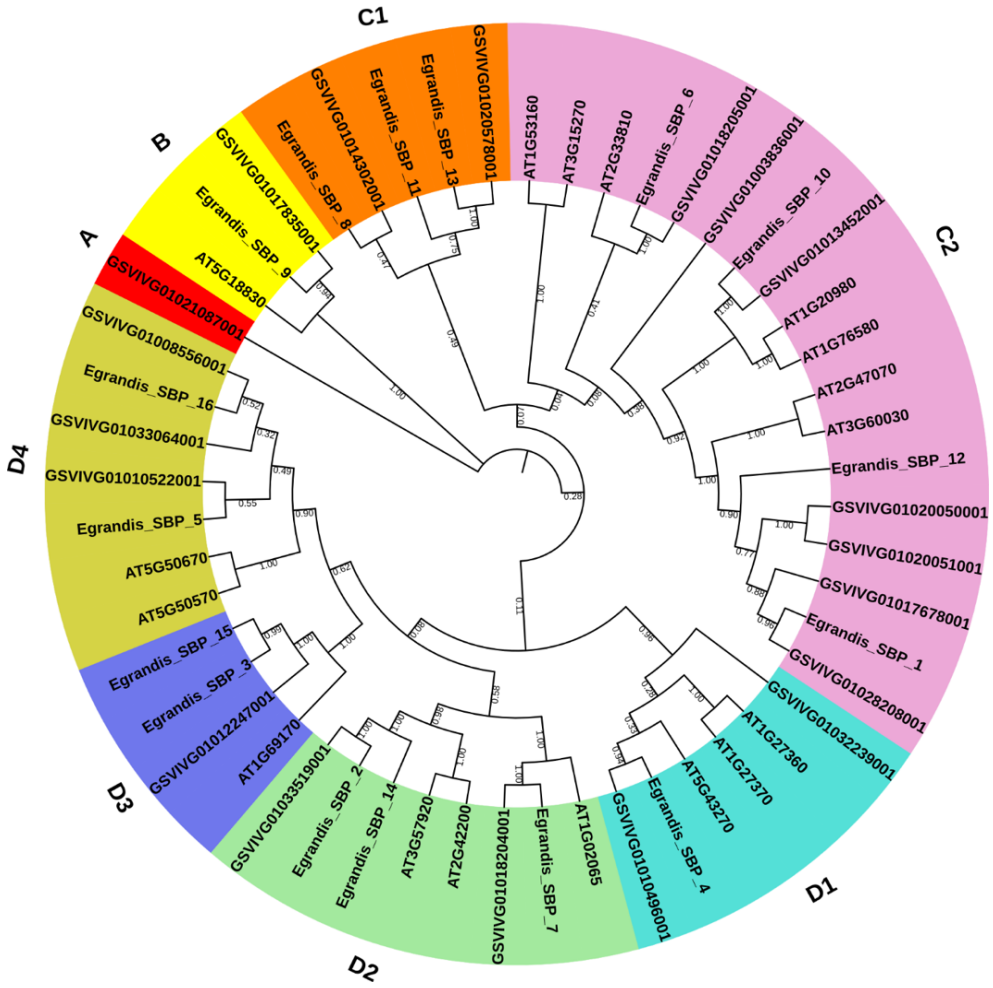

Figure 2. *Phylogenetic tree based on the conserved SBP domains alignment of 16 Egrandis_SBP predicted proteins, 17 A. thaliana SBP proteins and 19 Vitis vinifera SBP proteins, respectively. The tree was generated with MEGA v7.0 software, using the Neighbor-joining (NJ) method, and bootstrap values were calculated with 1000 replicates. Identified groups are shown on the outside of the circle*

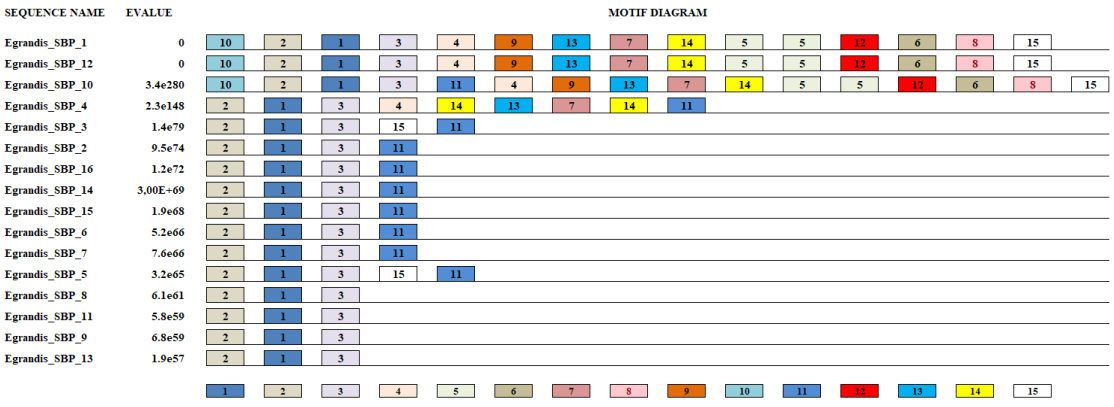

Figure 3. *Distribution of conserved motif of SBP genes in E. grandis. Full explanation of the motifs were given as seen in Table A1 in the Appendix*

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The SBP domain were shown to form two zinc fingers in all *Egrandis_SBP* proteins and zinc finger 1 (Cys-Cys-His-Cys) was composed of Motif 1 while Motif 2 and 3 were involved in the construction of zinc finger 2 (Cys-Cys-Cys-His) as shown in *Figure 4*. These zinc binding sites were shown to be essential for SBP domain however they have been reported as having different structures than other zinc binding structures by Yamasaki et al. (2004) and thus, they are known as a novel zinc-binding motif (Yamasaki et al., 2004).

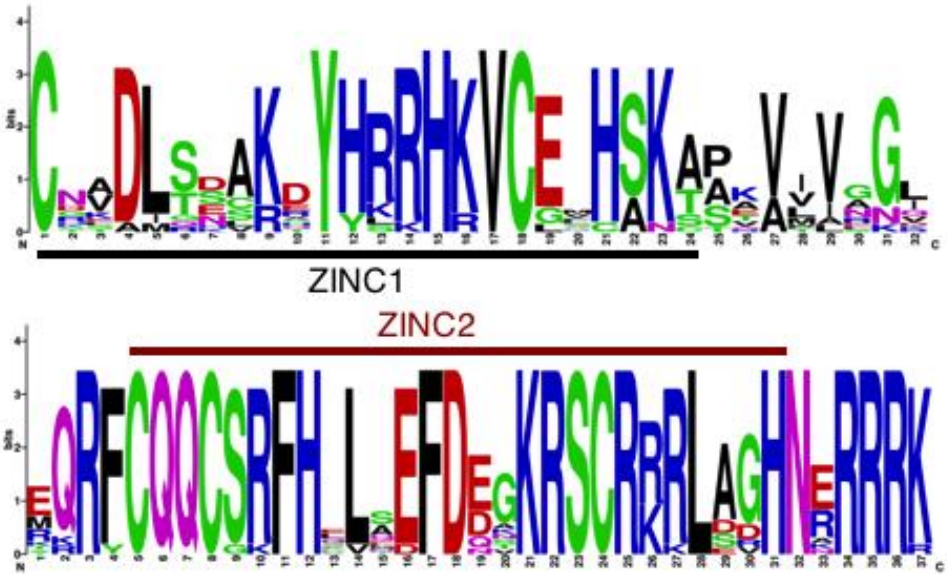

Figure 4. *Domain compositions of Egrandis_SBP genes. Thirteen Egrandis_SBP genes have both the characteristics of ZINC1 (Cys-Cys-His-Cys) and ZINC2 (Cys-Cys-Cys-His). The bit score represents the information content for each position in the sequence*

Gene structures of 16 *E. grandis* SBP gene were investigated and the estimated number of exons among all genes extended from 2 (*Egrandis_ SBP_8*) to 11 (*Egrandis_ SBP_1*) (*Fig. 5*). This varied numbers of exons in *E. grandis SBP* family genes by gain or loss of exon(s)/intron(s) might have occurred during evolution of *Egrandis_SBP* genes.

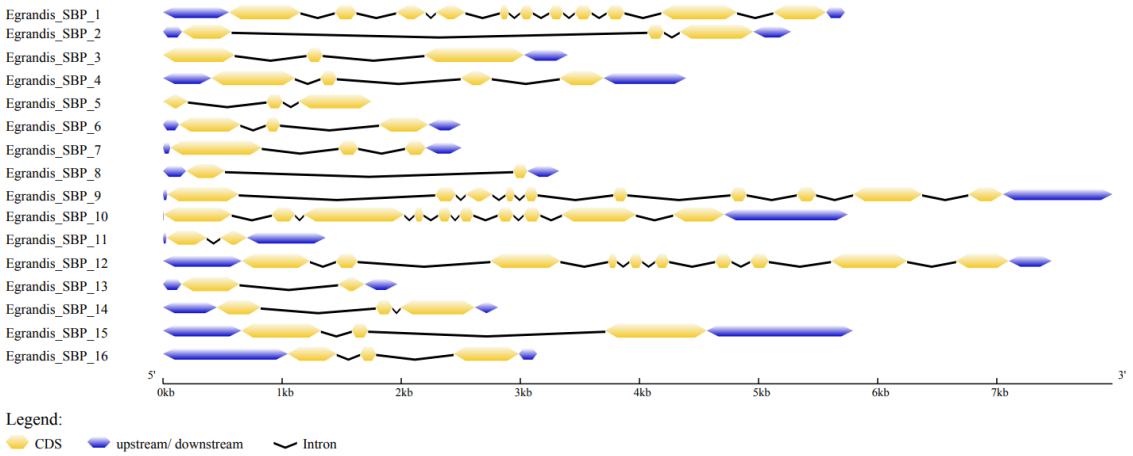

Figure 5. *Gene structure of SBP genes in E. grandis*

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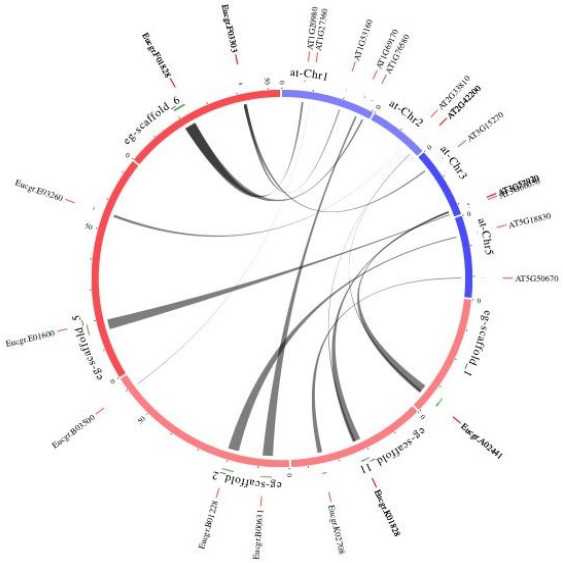
Comparative and synteny events among SBP genes of E. grandis, A. thaliana and V. vinifera

SBP protein sequences from *E. grandis*, *A. thaliana* and *Vitis vinifera* were subjected to comparative synteny analysis, to calculate evolutionary relationship of the SBP gene family between these species (*Fig. 6*). A total of 16 SBP proteins from *E. grandis*, 12 from *A. thaliana*, and 10 from *V. vinifera* was used to perform the synteny analysis. According to the synteny analysis results, homologs of *E. grandis* genes were found in corresponding syntenic blocks of *A. thaliana* and *V. vinifera* (*Fig. 6*). In terms of co-evaluation of gene duplications and synteny analyses of *Egrandis_SBP* genes, we can conclude from here, that both of these events might contribute to the evolutionary expansion of SBP genes in *E. grandis* genome (*Figs. 1* and *6*).



A

B

Figure 6. *Genome wide synteny analysis of SBP genes.* ***A.*** *Comparative map between E. grandis and A. thaliana.* ***B.*** *Comparative map between E. grandis and V. vinifera*

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Expression profiles of Egrandis_SBPs in different tissues

In this study, a common mRNA analysis of *Egrandis_SBP* genes were performed via publicly available expression data in Phytozome v12.1 online plant genomics resource (https://phytozome.jgi.doe.gov). The heatmap shows the expression variance of identified 16 *Egrandis_SBP* genes in different plant tissues (*Fig. 7*).

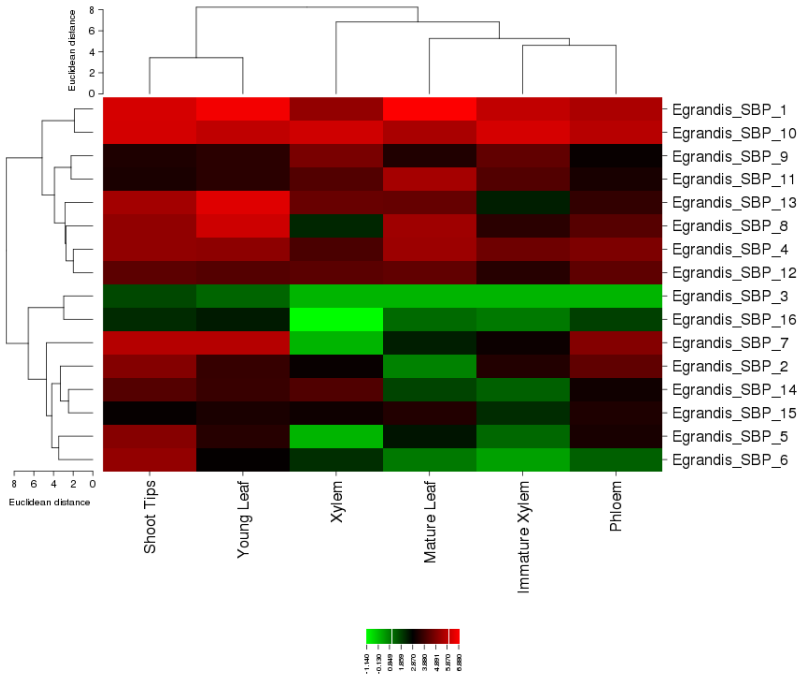

Figure 7. *Expression profiling of E. grandis SBP genes in shoot tips, young leaf, xylem, mature leaf, immature leaf and phloem tissues. Green and red colors indicate low-expression and high-expression, respectively*

As seen in the *Figure 7, Egrandis_SBP_1, -10, -9, -11, -13, -8, -4* and *-12* genes revealed relatively high expression levels in almost all tissues including shoot tips, young leaf, xylem, mature leaf, immature xylem and phloem. However, *Egrandis_SBP_2, -3, -5, -6, -7, -14, -15* and *-16* genes constituted relatively low expression levels in all tissues except for: shoot tips and young leaf (*Fig. 7*). In short, a conclusion can be drawn from the heatmap, that most of the *Egrandis_SBP* genes were highly expressed in all examined tissues of *E. grandis.*

Conclusions

The study herein, provides a comprehensive genome-wide identification of SBP genes in *E. grandis.* A total of 16 SBP genes were identified and were labelled as: *Egrandis_1* to *Egrandis_16.* They were sorted based on their chromosomal locations. To get insight into their biological functions in the genome of *E. grandis,* several analyses were conducted using many online and offline bioinformatic tools and genome databases. This study being the first study regarding the identification of SBP genes in *E. grandis,* it can be considered as a useful resource for the future studies regarding SBP genes in either in *E. grandis* or comparative different plant species.

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APPENDIX

Table A1. *Sequence information of predicted motifs in Egrandis_SBP proteins*

Motif #	Width	Best possible match
#1	50	YHRRHKVCEVHSKAPKVIVGGLEQRFCQQCSRFHELSEFDEGKRSCRRRL
#2	21	GAQPPRCQVEGCNADLSDAKD
#3	13	AGHNERRRKPPPE
#4	50	RTGRIVFKLFGKDPNDFPLALRTQIFNWLSHSPTEMESYIRPGCIILTVY
#5	24	KWLLHFAVERDCRALVKKLLDYJF
#6	41	NDPQLVGIEAWKSARDASGQTPEDYAVLRGHYSYIHLVQKK
#7	50	DELQFLKFPCSIPKVCGRGFIEVENQGLPGSFFPFIVAEEEVCSEIRMLE
#8	28	YRPAMLSMVAIAAVCVCVALLFKSLPEV
#9	50	AWEELHGNLGSSLRKLLDVSDDDFWRTGWIYVRVQDKLAFVYNGQIVLGT
#10	21	VEWDPNDWKWDGDLFVAKPLN
#11	19	VSESSRALSLLSSQSQDSS
#12	21	NFLFKPNAVGPAGLTPLHIAA
#13	48	HNSPRJLSIRPIAISAGQSTEFVVKGFNLFQPATRLLCALEGKYLAQE
#14	11	FINEIGWLLKR
#15	14	GCLQPFRWEALDYG

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