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TRANSCRIPTOME-WIDE IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF BBX TRANSCRIPTION FACTOR FAMILY IN *TOONA SINENSIS*

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Abstract

The B-box proteins (BBXs) are a class of transcription factors that play important regulation roles in plant growth and development, and in the response to biotic and abiotic stresses as well. Currently there are few studies reporting *Toona sinensis* BBX transcription factors (TsBBX). In order to characterize the function of the TsBBX family, we used an array of bioinformatic tools to assess their types, nuclear localization, domains, phylogeny and advanced structures. Results showed that a total of 20 TsBBX protein sequences were identified from the *Toona sinensis* transcriptome database, with the number of amino acids ranging from 99 to 296 and the relative molecular mass ranging from 11293.3 to 31839.46 kD. Four TsBBX family members were identified to be stable proteins and 16 were unstable proteins; 15 family members were hydrophobic proteins and the remaining 5 family members were hydrophilic proteins. Most of the secondary structural elements of the proteins were irregular coils, followed by alpha helices and beta sheets. Subcellular localization analysis showed that 16 TsBBX proteins were predominantly distributed in the nucleus and 4 were distributed in the extracellular matrix. Phylogenetic analysis showed that 20 TsBBX proteins were classified into type IV transcription factors, indicating that they may have similar functions. The results provide clues for further functional studies of TsBBX proteins as well as genetic resources for agronomic trait improvement by molecular genetic breeding in *Toona sinensis* and other crops.

Key words: *Toona sinensis*; BBX transcription factor; Transcriptome; Bioinformatic analysis.

Introduction

Toona sinensis is a deciduous arbor plant of Toona Roem, member of family Meliaceae, which is a unique tree species in China mainly located in Henan, Shandong, Anhui and Hebei. *Toona sinensis* is widely used for wood, medicine, and also as a vegetable and ornamental tree. The trunk of *Toona sinensis* is straight, knotless and scar-free. The wood has beautiful patterns and is widely used in furniture making. It has a good mandate in the international market (Sui *et al*., 2018). *Toona sinensis* is bitter, warm and nontoxic; it is rich in flavonoids, anthraquinones, terpenoids, tannins, saponins and alkaloids, and it has high medicinal value. *Toona sinensis* leaves and young buds have a strong aroma and a unique flavor. They are rich in nutrition, and enjoy the status of being known as "tree vegetables". *Toona sinensis* generally has an open crown with pinnate compound leaves which are red in autumn, making it a good candidate for ornamental plants. Taihe *Toona sinensis* has a long history of cultivation and rich varieties, among which 'Heiyouchun' has the best quality. *Toona sinensis* grows more likely in a sunny, warm and humid environment, but has a high requirement for soil quality. The popularization and planting of *Toona sinensis* are restricted because of its high requirements on soil quality and poor tolerance to shade, drought and coldness (Sui *et al*., 2019).

Transcription factors (TFs), also known as trans-acting factors, usually interact with cis-acting elements in gene promoter regions, thereby activating or inhibiting the transcription of their target genes. BBX transcription factors belong to the zinc finger protein family, and their amino acid sequences contain one or two characteristic B-box motifs, some of which also contain a CCT domain (conserved carboxyterminal) (Gangappa & Botto, 2014). In *Arabidopsis thaliana*, BBX protein are divided into five categories: Class I and Class II both have two B-boxes and one additional CCT domain, the difference between them being that there are differences in the B-box2 domain; Class III only has one B-box and one CCT domain; Class IV contains two B-boxes, and Class V BBX only contains one B-box (Khanna *et al*., 2009). AtBBX1-AtBBX6 belong to Category I; AtBBX7-AtBBX13 to Category II; AtBBX14-AtBBX17 to Category III; AtBBX18-AtBBX25 to Category IV; and AtBBX26-AtBBX32 to Category V. The BBX transcription factors are not only involved in photomorphogenesis of plant seedlings (Khanna *et al*., 2009), but they also play key roles in flowering and shade (Gangappa & Botto, 2014), coping with biotic and abiotic stresses, and hormone signaling (Yang *et al*., 2020). Studies have shown that *Arabidopsis thaliana* BBX4 (Datta *et al*., 2006), BBX20, BBX21 (Datta *et al*., 2007), BBX22 and BBX23 (Datta *et al*., 2008) are positive regulators of photomorphogenesis and can promote root formation, while BBX18, BBX19 (Indorf *et al*., 2007), BBX24, BBX25 (Holtan *et al*., 2011) and BBX32 (Gangappa *et al*., 2013) are negative regulators of light signals. In *Arabidopsis thaliana*, most BBX family members are involved in flowering regulation. For example CO (BBX1) acts as an inducer, which promotes flowering by regulating the expression of the anthocyanin protein FT (Suárez-López *et al*., 2001), while BBX10 plays a role in delaying flowering by inhibiting the activity of the CO protein (Ordoez-Herrera *et al*., 2018). Also, overexpression of *Malus domestica* BBX10 can significantly enhance tolerance to salt stress and drought stress in *Arabidopsis thaliana* through ABA signaling (Liu *et al*., 2019). In Chrysanthemum, BBX19 interacts with ABF3, a major member of the ABA signaling pathway, to regulate drought stress in a BBX19-ABF3 modular manner (Xu *et al*., 2020), while heterologous expression of BBX22 delays leaf senescence and improves drought resistance in *Arabidopsis thaliana* (Liu *et al*., 2019).

At present, there are few studies on the molecular biology of *Toona sinensis*, and the systematic analysis of

the TsBBX transcription factor family has not been reported. In this study, 20 candidate gene sequences of TsBBX transcription factor family were obtained from transcriptome database. Using these sequences, the structural characteristics, classification and phylogenetic relationship of the TsBBX transcription factor family were systematically analyzed. Results will provide a basis for further functional studies of the TsBBX gene and provide excellent candidate genes for improving the quality and resistance of *Toona sinensis* through genetic control.

Materials and Method

Screening of TsBBX transcription factor genes: Thirty-four BBX protein sequences were screened from the *Toona sinensis* transcriptome data library. 20 sequences that could be translated into complete proteins were screened further by searching for open reading frames (ORF) using BioXM2.6 software. The plant material Taihe 'Heiyouchun' used in this study was harvested in the nursery of Taihe County, Anhui Province, China, in March 2018. Thirty-two amino acid sequences of the BBX transcription factor family were downloaded from the *Arabidopsis thaliana* database (TAIR).

Analysis of physicochemical properties of TsBBX family proteins: The complete amino acid sequences of the TsBBX family proteins were selected and the online software ExPASY (Http://web.expasy.org/compute_Pi/) was used to analyze the isoelectric point (PI) and molecular weight (MW) of the TsBBX protein sequences. ProtScale software was used to analyze the hydrophilicity of the proteins.

Prediction of secondary and tertiary structure of TsBBX family proteins: The secondary structure of the TsBBX family proteins was analyzed using SOPMA software provided by the Institute of Biology and Protein Chemistry, France. The online software Phyre2 (Http://www.sbg.bio.ic.ac.trk/phyre/index.egi) was used to predict the tertiary structure of TsBBX proteins.

Subcellular localization analysis of TsBBX family proteins: The amino acid sequences of 20 candidate BBX gene family members were analyzed for subcellular localization using Softberry (http://www.softberry.com/), and the transmembrane structures of proteins were analyzed using TMHMM software.

Phylogenetic tree construction and sequence analysis: To further investigate the evolution of the TsBBX protein family, the phylogenetic tree of 32 AtBBX transcription factors and 20 TsBBX transcription factors was constructed by Neighbor-joining using MEGA5.0 software. The sequences of 32 BBX transcription factors from the model plant *Arabidopsis thaliana* were obtained from the *Arabidopsis thaliana* database TAIR (Https://www.arabidopsis.org/). The gene sequences were saved in FASTA format in the same text document, imported into MEGA5.0 software, and ClustalW was used for multiple sequence alignment. The results were converted to *.meg format. The following parameters were defined: Neighbour-joining for tree building; model set to p-distance; boostrap set to 1000. All other parameters were left to default values.

Results and analysis

Identification of TsBBX transcription factors: In this study, 32 BBX transcription factor domain-related genes were screened from the *Toona sinensis* full-length transcriptome database by constructing a local BLAST database using the *Arabidopsis thaliana* BBX protein as a probe sequence. After DNAMAN software comparison, SMART, Pfam and CDD verification and identification, a total of 20 complete BBX gene sequences were obtained, which were named TsBBX1-20. By comparing these sequences with the homologous sequences of *Arabidopsis thaliana*, 10 TsBBX genes were shown to be homologous to AtBBX19, 1 TsBBX gene was homologous to AtBBX21, 3 TsBBX genes were homologous to AtBBX22, 1 TsBBX gene was homologous to AtBBX24, and 5 TsBBX genes were homologous to AtBBX25 (Table 1).

Table 1. The sequence information and physicochemical properties of BBX family protein in *Toona sinensis*.

Gene	Accession number	Isoelectric point	Molecular weight (kD)	Instability index (%)	Aliphatic index	Grand average of Hydropathy	Amino acids length (aa)	*AtBBX* Orthologs
TsBBX1	MW076204	6.59	22818.84 -	42.67	72.21	-0.53	208	*AtBBX19*
TsBBX2	MW076205	6.58	23 116.2	43.56	72.43	-0.524	210	*AtBBX19*
TsBBX3	MW076206	6.91	12 887.96	49.32	84.05	0.119	116	*AtBBX19*
TsBBX4	MW076207	6.98	23 135.25	40.94	73.33	-0.533	210	*AtBBX19*
TsBBX5	MW076208	6.91	12 871.97	49.32	84.91	0.141	116	*AtBBX19*
TsBBX6	MW076209	7.53	23 073.22	42.57	73.81	-0.512	210	*AtBBX19*
TsBBX7	MW076210	7.51	11 324.68	58.20	119.29	0.573	99	*AtBBX25*
TsBBX8	MW076211	8.16	21 455.9	50.05	81.55	-0.142	193	*AtBBX25*
TsBBX9	MW076212	7.51	11 296.63	50.57	117.27	0.567	99	*AtBBX25*
TsBBX10	MW076213	8.16	21 427.85	46.14	80.52	-0.145	193	*AtBBX25*
TsBBX11	MW076214	5.67	30 842.59	53.62	79.17	-0.318	277	*AtBBX21*
TsBBX12	MW076215	6.59	22 818.84	42.67	72.21	-0.530	208	*AtBBX19*
TsBBX13	MW076216	8.24	19 274.76	41.45	70.17	-0.069	175	*AtBBX19*
TsBBX14	MW076217	8.26	24 514.32	36.85	91.68	-0.111	220	*AtBBX19*
TsBBX15	MW076218	5.07	26 325.81	51.48	72.15	-0.427	237	*AtBBX25*
TsBBX16	MW076219	8.23	24 784.5	36.71	83.42	-0.234	222	*AtBBX19*
TsBBX17	MW076220	4.97	26 143.52	49.48	75.44	-0.365	237	*AtBBX24*
TsBBX18	MW076221	7.48	11 293.3	36.61	89.13	0.088	103	*AtBBX22*
TsBBX19	MW076222	4.96	30 254.4	39.33	81.40	-0.003	278	*AtBBX22*
TsBBX20	MW076223	5.07	31 839.46	53.81	67.94	-0.368	296	*AtBBX22*

Analysis of physicochemical properties of TsBBX family proteins: The 20 TsBBX proteins, identified in the sequence analysis, differed in size, with their relative predicted molecular mass ranging from 11293.3 to 31839.46 kD. The TsBBX proteins were composed of amino acids of various lengths ranging from 99 to 296 with an average length of 195 aa. Theoretical isoelectric point (pI) analysis revealed that the theoretical isoelectric points of 9 TsBBX proteins was greater than 7 while the remaining 11 TsBBX proteins had an isoelectric point of less than 7. The pI of TsBBX19 was the lowest (4.96) and the pI of TsBBX14 was the highest (8.26), with the average theoretical isoelectric points of all TsBBX proteins being 6.869. The coefficient of instability analysis found that the coefficients of TsBBX14, TsBBX16, TsBBX18 and TsBBX19 were less than 40 (with a value of 38.00), which predicts these proteins will be stable. The coefficients of instability for the remaining proteins were all greater than 40, which predicts these proteins will be unstable. The lipid solubility of the TsBBX transcription factors ranged from 67.94 to 82.27. The average hydrophilic index analysis showed that there were more hydrophilic proteins than hydrophobic proteins, among which 15 TsBBX proteins were predicted to be hydrophobic proteins, while the remaining 5 TsBBX proteins were predicted to be hydrophilic proteins; especially TsBBX7, which had the highest predicted water solubility, with a value of 0.573.

Table 2. Secondary structure of *Toona sinensis* BBX family protein.

Name	Alpha helix (%)	Extended strand (%)	β-turn (%)	Random coil (%)
TsBBX1	23.80	16.83	3.37	56.73
TsBBX2	21.90	17.62	3.30	57.14
TsBBX3	37.07	18.97	1.72	42.24
TsBBX4	21.43	18.57	2.86	57.14
TsBBX5	30.17	24.14	2.59	43.10
TsBBX6	20.00	19.52	2.86	57.62
TsBBX7	38.38	15.15	6.06	40.40
TsBBX8	35.23	18.65	4.15	41.97
TsBBX9	45.45	18.18	5.05	31.31
TsBBX10	40.41	18.13	4.15	37.31
TsBBX11	20.22	14.80	3.61	61.37
TsBBX12	23.08	16.83	3.37	56.73
TsBBX13	9.14	17.14	4.57	69.14
TsBBX14	34.09	18.18	1.82	45.91
TsBBX15	25.74	12.24	1.69	60.34
TsBBX16	27.03	19.82	4.45	48.20
TsBBX17	24.47	14.77	2.53	58.23
TsBBX18	66.99	8.74	0.97	23.30
TsBBX19	24.10	16.91	4.68	54.32
TsBBX20	20.61	12.84	2.36	64.19

Secondary structure analysis of TsBBX family proteins: The secondary structure analysis of the protein was carried out using the online software SOPMA. The results showed that all 20 TsBBX proteins constitute the secondary structure through alpha helices, extended strands, beta sheets and irregular coils. Among the secondary structural elements of the TsBBX proteins, alpha helices and irregular coils accounted for the highest proportion of structures, followed by extended strands and finally beta-sheets. The main components of secondary structure for 17 TsBBX proteins were irregular coils, while the remaining 3 TsBBX proteins displayed alpha helices (Table 2).

Hydrophobicity/hydrophilicity analysis and tertiary structure prediction of TsBBX proteins: The Phyre2 software was used to analyze the tertiary structure of the TsBBX protein, and a total of 20 simulated tertiary structures of TsBBX protein sequences were obtained. The tertiary structure models of TsBBX4, TsBBX7, TsBBX18 and TsBBX19 are shown here (Fig. 1). In the tertiary structure of the TsBBX protein, irregular coils are dominant, with alpha helices and beta sheets appearing most often at either end of the protein structure. It is presumed that this conformation forms the active site of the protein. Hydrophilic groups are predominantly distributed on the surface of TsBXX, while hydrophobic groups are located inside the protein. During the formation of the tertiary structure of TsBBX proteins, the hydrophobic part is directed internally via beta-sheets or alpha helices, thus stabilizing its conformation.

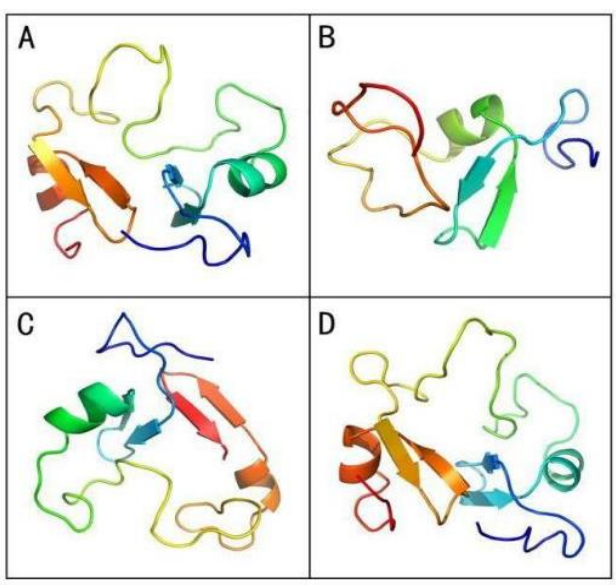

Fig. 1. The tertiary structure of *Toona sinensis* BBX protein.
Note: A: TsBBX4; B: TsBBX7; C: TsBBX18; D: TsBBX19

Subcellular localization analysis of TsBBX family proteins: The amino acid sequences of each member of the 20 TsBBX transcription factor family sequences were analyzed by online software SoftBerry to predict their subcellular localization (Table 3). The results showed that the TsBBX proteins were distributed in many cell locations, including the nucleus, cell membrane, extracellular matrix, microbody, mitochondria, endoplasmic reticulum, peroxisome, golgi apparatus, chloroplast and vacuole. However they were mainly located in the nucleus and extracellular matrix. A total of 14 (70%) of the 20 TsBBX proteins were predominantly distributed in the nucleus, indicating that these 14 TsBBX transcription factors play roles in the transcription processes. The remaining 6 (30%) BBX members were distributed extracellularly.

Table 3. Subcellular localization of *Toona sinensis* BBX family protein.

Gene	Nucleus	Plasma membrane	Extracellular	Cytosome	Mitochondrion	Endoplasmic reticulum	Peroxisome	Golgi apparatus	Chlorop last	Vacuole
TsBBX1	7.32	1.24	0.08	-	0.12	-	0.14	-	0.27	0.84
TsBBX2	-	-	2.43	1.60	1.52	0.03	2.15	1.40	0.87	-
TsBBX3	8.65	0.61	-	-	0.04	-	0.05	-	0.01	0.63
TsBBX4	-	-	2.49	1.39	1.11	0.16	2.25	1.30	1.29	-
TsBBX5	8.66	0.61	-	0.00	0.04	-	0.06	-	0.02	0.61
TsBBX6	-	-	2.73	1.67	0.64	0.20	2.41	1.34	1.02	-
TsBBX7	8.86	0.72	-	-	0.04	-	0.10	-	-	0.29
TsBBX8	8.87	0.70	-	-	-	-	0.06	-	0.02	0.35
TsBBX9	8.88	0.70	-	-	-	-	0.13	-	-	0.27
TsBBX10	8.93	0.70	-	-	-	-	0.07	-	-	0.30
TsBBX11	7.21	1.47	-	-	0.07	-	0.14	-	0.26	0.85
TsBBX12	7.32	1.21	0.08	-	0.13	-	0.13	-	0.26	0.84
TsBBX13	-	-	2.69	0.47	0.87	0.22	2.4	0.79	2.56	-
TsBBX14	-	-	2.56	1.73	1.22	-	2.25	1.19	1.06	-
TsBBX15	9.04	0.72	-	-	-	-	0.09	-	-	0.16
TsBBX16	-	-	2.85	2.14	0.67	-	2.47	1.21	0.66	-
TsBBX17	8.84	0.71	-	-	-	-	0.08	-	0.04	0.33
TsBBX18	8.87	0.68	0.02	-	-	-	0.15	-	-	0.28
TsBBX19	8.83	0.74	-	-	0.11	-	0.06	-	0.14	0.58
TsBBX20	7.27	1.32	0.06	-	0.15	-	0.12	-	0.28	0.79

Prediction and analysis of TsBBX transmembrane domain: The TMHMM (an online protein transmembrane domain prediction software) was used to predict the transmembrane domain of the TsBBX proteins. The results showed that only five TsBBX proteins (TsBBX7, TsBBX9, TsBBX14, TsBBX16 and TsBBX18) appeared to have transmembrane structures (partial protein transmembrane domain analysis, Fig. 2).

Motif analysis of TsBBX protein: Using the MEME program, 32 AtBBX protein sequences and 20 TsBBX protein sequences were analyzed online by motif software, and 8 motifs were selected. The results were shown in Figs. 3 and 4. All TsBBX proteins were predicted to have motif 1, as it is the core sequence of all the BBX proteins. In addition, TsBBX 1 and TsBBX14 have five identical motifs, TsBBX2, TsBBX4 and TsBBX6 have six identical motifs, TsBBX3 and TsBBX5 have two identical motifs, TsBBX7 and TsBBX9 have two identical motifs, TsBBX8 and TsBBX10 have three identical motifs, TsBBX19 and TsBBX20 have two identical motifs, and TsBBX18 has only one conserved motif.

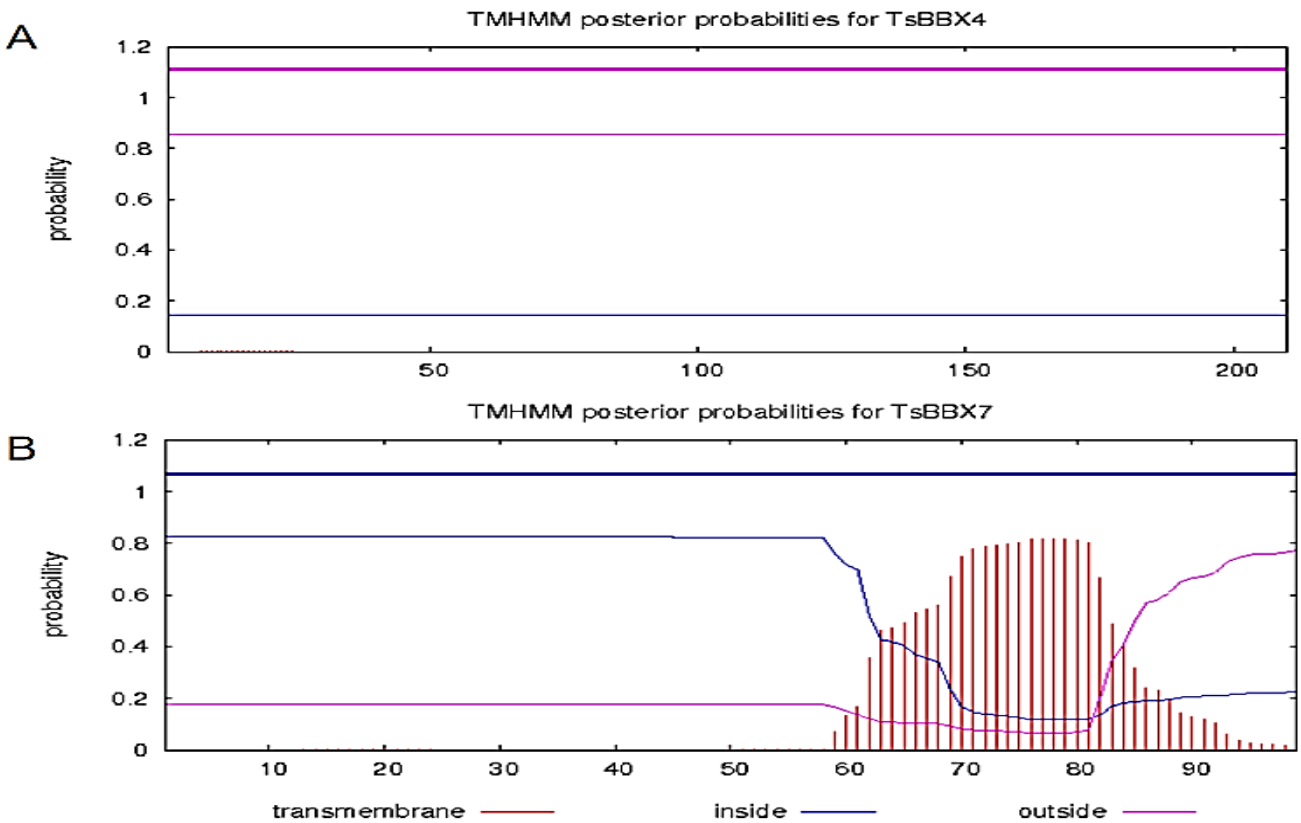

Fig. 2. Prediction of *Toona sinensis* BBX protein transmembrane domain.
Note: A: TsBBX4 protein without transmembrane domain in *Toona sinensis*; B: TsBBX7 protein with transmembrane domain in *Toona sinensis*

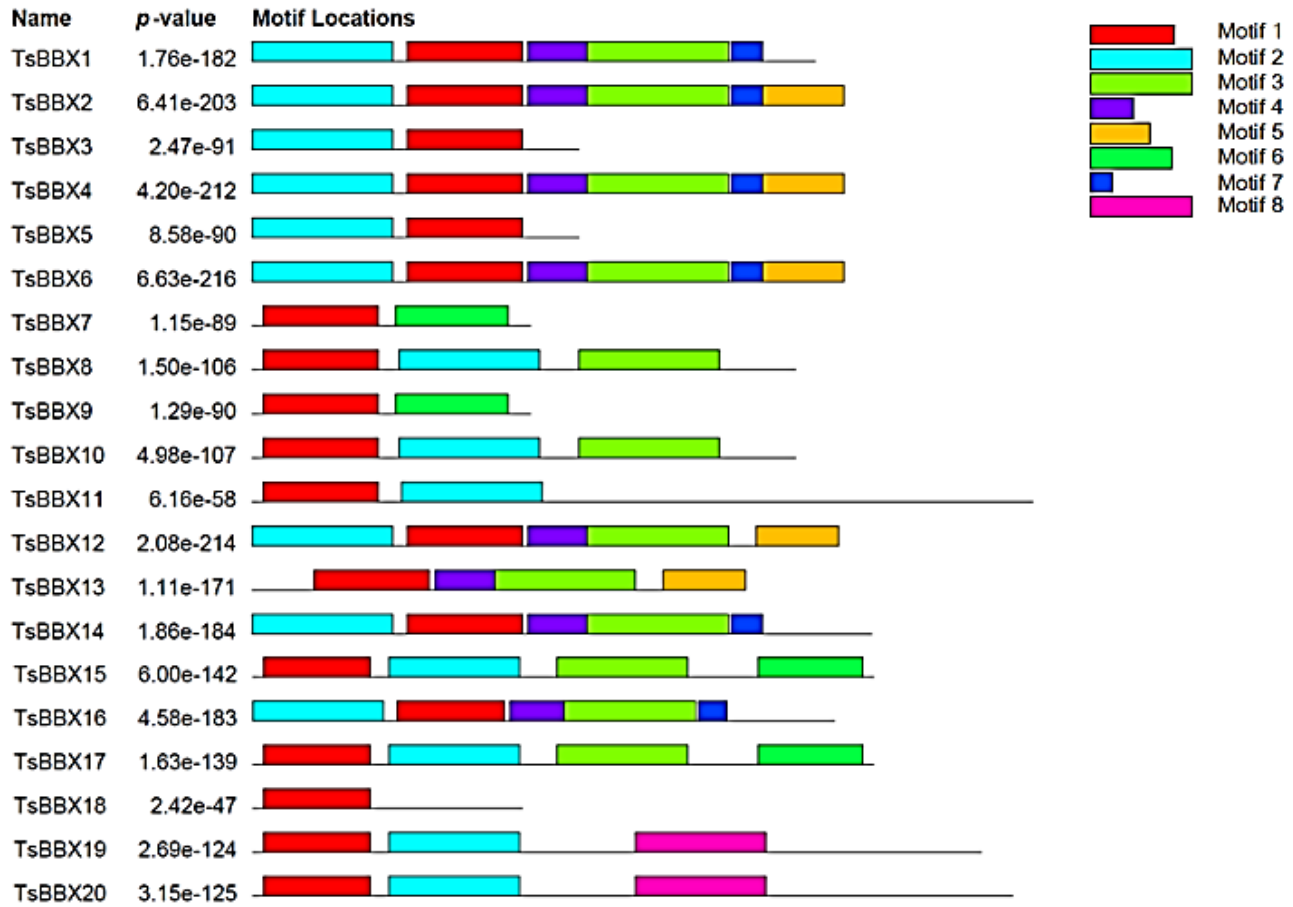

Fig. 3. Conserved motif analysis of BBX protein family.

Fig. 4. Conserved motif of BBX protein.

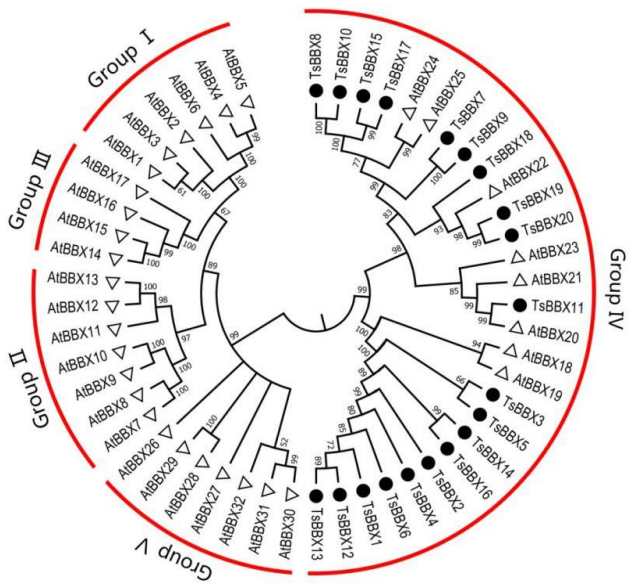

Fig. 5. Phylogenetic analysis of BBX protein in *Toona sinensis* and *Arabidopsis thaliana*.

Phylogenetic analysis of TsBBX protein: To further investigate the phylogenetic status of the TsBBX proteins, a phylogenetic tree was constructed and compared to *Arabidopsis thaliana* (Fig. 5). After clustering 20 TsBBX proteins and 32 AtBBX proteins, they were divided into five subfamilies: I, II, III, IV and V. The TsBBX protein was only found to be distributed in subfamily IV.

Discussion

With the advances in genomics and the availability of transcriptomes, bioinformatics now plays a larger role in protein prediction, laying a foundation for the functional study of genes. Bioinformatic studies have been used to identify 32, 30, 29 and 25 BBX family members in the genomes of *Arabidopsis thaliana* (Khanna *et al*., 2009), *Oryza sativa* L. (Huang *et al*., 2012), *Solanum lycopersicum* (Chu *et al*., 2016) and *Pyrus bretschneideri* (Cao *et al*., 2017), respectively. In this study, the BBX transcription factor family of *Toona sinensis* was screened using the *Toona sinensis* transcriptome. A total of 20 TsBBX gene sequences were isolated, with an average full length CDS of 594bp, coding for 195 amino acids. In comparison with the BBX proteins of *Arabidopsis thaliana*, it was found that the BBX family members of *Toona sinensis* had higher homology with the BBX19, BBX21, BBX22, BBX24, BBX25 of *Arabidopsis thaliana*, suggesting they belong exclusively to the type IV subclass, which was consistent with the results of the phylogenetic analysis. Studies have shown that members of subclass IV are extensively involved in abiotic stress. Overexpression of BBX24 in *Arabidopsis thaliana* can improve salt stress tolerance (Nagaoka & Takano, 2003) while the overexpression of *Chrysanthemum morifolium* BBX24 can significantly improve the tolerance of the plant to low temperatures and drought stress (Yang *et al*., 2014). Different BBX proteins have been shown to play different roles in photomorphogenesis, both synergistic and antagonistic. AtBBX24 and AtBBX25 have been shown to be negative regulators of photomorphogenesis (Gangappa & Botto, 2014). In this study, there are four TsBBX proteins having high homology with the AtBBX25 protein, and they are therefore predicted to play the same role as AtBBX25 in photomorphogenesis. In the hydrophilicity and hydrophobicity analysis, it was found that there were both hydrophilic and hydrophobic proteins in TsBBX, with some proteins displaying a total average hydrophilic coefficient close to 0. Analysis on the amino acid composition showed they contained almost the same number of hydrophilic and hydrophobic amino acids. In the analysis of protein secondary structure, it was found that the TsBBX protein was predominantly composed of irregular coils, along with additional alpha helices and beta sheets. The tertiary structure was found to be formed on the basis of the secondary structure. The prediction of the tertiary structure of the protein by the Phyre2 program verified the accuracy of the prediction of the secondary structural elements of TsBBX, with most of the amino acids constituting the tertiary structure forming irregular coils. The motif structure of the TsBBX protein is an important basis for its classification. The domain differences of 20 amino acids were shown using MEME analysis, which resulted in TsBBX being classified as a class IV subtype. The results of the phylogenetic analysis confirmed the accuracy of this classification.

Toona sinensis is a multi-functional tree species with edible, medicinal and timber uses, however little is known about the biology of the tree. The BBX gene family is an important family of transcription factors as they not only regulate gene expression, but they also play a role in regulating the function of other proteins via protein interactions. This regulation controls physiological and biochemical processes that are crucial for plant photomorphogenesis, flowering, breeding, hormone signaling and response to stress (Gangappa & Botto, 2014). In this paper, the BBX gene of *Toona sinensis* was analyzed using bioinformatics tools at the transcriptome level. Functional analysis was carried out which examined physical and chemical properties, domains, the phylogenetic evolution and protein structure. These results provide a reference for further study of BBX transcription factor function of *Toona sinensis* in the future.

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