Genome-wide Characterization and Expression Analysis of YABBY Gene Family in Three Cultivars of Cucurbita Linn. And Their Response of Salt Stress in Cucurbita Moschata

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Abstract

Background

Plant specific YABBY transcription factors have important biological roles in plant growth and abiotic stress. However, the identification of Cucurbita Linn. YABBY and their response to salt stress have not yet been reported. The gene number, gene distribution on chromosome, gene structure, protein conserved structure, protein motif and the cis-acting element of YABBY in three cultivars of Cucurbita Linn. were analyzed by bioinformatics tools, and their tissue expression patterns and expression profile under salt stress were analyzed.

Results

In this study, 34 YABBY genes (11 CmoYABBYs in Cucurbita moschata, 12 CmaYABBYs in Cucurbita maxima, and 11 CpeYABBYs in Cucurbita pepo) were identified and they were divided into five subfamilies (YAB1/YAB3, YAB2, INQ, CRC and YAB5). YABBYs in the same subfamily usually have similar gene structures (intron-exon distribution) and conserved domains. Chromosomal localization analysis showed that these CmoYABBYs, CmaYABBYs, and CpeYABBYs were unevenly distributed in 8, 9, and 9 chromosomes of 21 chromosomes, respectively. Total of 6 duplicated gene pairs, and they all experienced segmental duplication events. Cis-acting element analysis showed that some Cucurbita Linn. YABBYs were associated with at least one of plant hormone response, plant growth, and abiotic stress response. Transcriptional profiles of CmoYABBYs and CmaYABBYs in roots, stems, leaves, and fruits, and CpeYABBYs in seed and fruit mesocarp showed that YABBYs of Cucurbita Linn. had tissue specificity. Finally, the transcriptional profile of 11 CmoYABBYs in leaf and qRT-PCR analysis of CmoYABBYs in root under salt stress indicated that some genes may play an important role in salt stress.

Conclusions

Genome-wide identification and expression analysis of YABBYs revealed the characteristics of YABBY gene family in three cultivars of Cucurbita Linn.. Transcriptome and qRT-PCR analysis revealed the response of the CmoYABBYs to salt stress. This provides a theoretical basis for the functional research and utilization of YABBY genes in Cucurbita Linn..

Background

Plants are usually exposed to the extreme environment during their growth and development. Salt, drought, high temperature, low temperature, and other abiotic stresses have adverse effects on the growth of plants, resulting in loss of yield and quality [1]. In plants, transcription factors (TFs) play an important role in regulating gene expression of the plant defense system, and some of them are involved in abiotic stress response [2]. So far, there are about 30 common TFs, about half of which are deemed to be plant-specific TF families, such as AP2/ERF, WRKY, NAC, B3, SBP, and Dof families [3].

YABBY is a unique transcription factor in plants, belonging to the zinc finger protein superfamily. YABBY protein consists of two highly conserved domains: N-terminal C2C2 zinc finger motif and C-terminal YABBY domain [4-6]. Previous studies have shown that some zinc fingers have been observed as DNA-binding motifs, while many of them play a role in protein-protein interactions rather than binding to DNA [4].

YABBY transcription factors have been widely investigated in dicotyledons. There are six YABBY members in Arabidopsis thaliana, YABBY1 (FIL, filamentous lower), YABBY2 (YAB2), YABBY3 (YAB3), YABBY4 (INQ, inner no outer), YABBY5 (YAB5) and CRC (crabs claw). The main function of the YABBY in Arabidopsis thaliana is to specify the distal cell fate of lateral organs [7]. The evolutionary relationship has shown that YABBYs have different functions, including processes of plant growth and development, such as controlling the carpel number of tomato flowers [8]. Besides, YABBY is also affected by auxin, GA, and other hormones [9].

It is worth noting that YABBY plays a major role in the regulation of plant abiotic stresses, which is both an activator and an inhibitor [10]. In pineapple, RT-qPCR showed that the expression of AcYABBY2, AcYABBY3, AcYABBY6, and AcYABBY7 were highly susceptible to abiotic stress. Under NaCl stress, the overexpression of AcYABBY4 in Arabidopsis thaliana resulted in short roots, indicating that AcYABBY4 plays a negative regulatory role in salt tolerance [11]. GmYABBY10 in soybean involved in high salt and drought stresses [2]. At present, although 6 YABBYs are found in Arabidopsis, 8 YABBYs are found in rice, 1 YABBY is found in tomatoes [12], and YABBYs are also found in other species. However, there isn’t any previous work on role of YABBY transcription factors in modulating salt stress and other abiotic stresses in Cucurbita Linn..

The species of Cucurbita Linn. are one of the oldest crops cultivated by human beings. They have a long history, a wide range of varieties, strong adaptability, wide geographical distribution and high yield. The shape, size, and quality of the fruit are different, and the fruit color is colorful [13]. Cucurbitaceae plants are strongly affected by salt stress [14]. Under salt treatment, Cucurbitaceae plants are divided into Na⁺ overground accumulation type and Na⁺ underground accumulation type. The grafted cucumber seedlings with Na⁺ underground accumulation type as rootstock could significantly reduce the content of Na⁺ in overground and have stronger salt tolerance [15]. Three cultivars of Cucurbita Linn. (Cucurbita moschata, Cucurbita maxima, and Cucurbita pepo) are involved here. In this study, YABBY genes in three cultivars of Cucurbita Linn. were identified. Additionally, the distribution, physicochemical properties, phylogenetic relationship, structural conservation, gene duplication and tissue expression patterns of the YABBY gene were studied. Finally, the expression profile of Cucurbita moschata YABBY genes under salt stress was analyzed. The results have potential significance for the functional study and molecular mechanism of the YABBYs in Cucurbitaceae plants.

Results

Identification and characterization of Cucurbita Linn. YABBY transcription factor
YABBY proteins of *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo* were identified by the local BLASTp program using six AtYABBY protein sequences as query sequences. All candidate protein sequences were confirmed by the NCBI CD-search. Finally, 11, 12, and 11 YABBY proteins were identified in *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo*, respectively. The YABBY genes were named according to the naming of the AtYABBY genes and the position on the chromosome (from the first chromosome to the last chromosome, from the top position to the end position of one chromosome) (Table 1).

The amino acid sequence of YABBY was analyzed by the ExPASy proteomics server. The results showed that the coding regions of 11 CmoYABBYs ranged from 510 bp (CmoYAB2) to 930 bp (CmoINOa) (Table 1). The number of translated amino acids ranged from 169 aa to 309 aa, and the MW of protein ranged from 1889.49 Da to 35478.2 Da. The PI is between 6.29 (CmoYAB5b) and 9.99 (CmoCRCb). In *Cucurbita maxima*, the coding regions of 12 CmaYABBY genes ranged from 537 bp (CmaYAB5b) to 1179 bp (CmaINOa) (Table 1). The number of translated amino acids ranged from 178 aa to 392 aa, and the MW of protein ranged from 19479.12 Da to 43636.39 Da. The PI is between 6.37 (CmaINOb) and 9.32 (CmaYAB2). In *Cucurbita pepo*, the coding regions of 11 CpeYABBY genes ranged from 471 bp (CpeINOa) to 1110 bp (CpeYAB1a) (Table 1). The number of translated amino acids ranged from 156 aa to 369 aa, and the MW of protein ranged from 17044.7 Da to 40999.44 Da. The PI is between 6.06 (CpeYAB5a) and 9.76 (CpeYAB1b). Based on the physical and chemical characteristics of the YABBY gene in three cultivars of *Cucurbita Linn.*, it was found that they had similar characteristics, and they had the properties of basic protein (Table 1). The predicted subcellular localization results showed that 34 *Cucurbita Linn.* YABBY genes were all located to the nucleus (Table 1), which accorded with the characteristics of transcription factors.

**Phylogenetic relationship of YABBY in three cultivars of *Cucurbita Linn.***

In order to analyze the evolutionary relationship of YABBY proteins, we constructed a phylogenetic tree with 6 AtYABBYs, 11 CmoYABBYs, 12 CmaYABBYs and 11 CpeYABBYs (Fig. 1). All YABBYs were divided into five subfamilies (YAB1/YAB3, YAB2, INO, CRC and YAB5) according to the identity of amino acid sequences. Each subfamily contained AtYABBY, CmoYABBY, CmaYABBY and CpeYABBY proteins. The YAB1/YAB3 subfamily consisted of 2 CmoYABBYs, 3 CmaYABBYs, 3 CpeYABBYs, 2 AtYABBYs (AtYAB1 and AtYAB3), YAB2 subfamily contained 4 proteins: CmoYAB2, CmaYAB2, CpeYAB2 and AtYAB2; YAB5 subfamily contained 4 CmoYABBYs, 4 CmaYABBYs, 4 CpeYABBYs and AtYAB5; INO subfamily contained 2 CmoYABBYs, 2 CmaYABBYs, 2 CpeYABBYs and AtINO; CRC subfamily contained 2 CmoYABBYs, 2 CmaYABBYs, 1 CpeYABBY and AtCRC proteins.

**Gene structures and motif composition of YABBY gene family in three cultivars of *Cucurbita Linn.***

The gene structure can provide valuable information for the phylogenetic relationship of YABBY in three cultivars of *Cucurbita Linn.*. Based on the phylogenetic tree, the exon-intron structure and conserved motifs of 34 YABBYs were analyzed by TBtools (Fig. 2B). The classification pattern of exon-intron was consistent with the phylogenetic tree (Fig. 1; Fig. 2A). All YABBYs from three cultivars of *Cucurbita Linn.* contain introns, and the number of exons was 5-12. The number of exons and the length of introns in one branch were similar. For instance, in the YAB2 branch, all YABBY genes contained six exons. What's more, from the phylogenetic analysis, the closer the homology was, the more similar the structure was, such as CmoYAB1a_CmaYAB1a, CmoYAB5b_CmaYAB5b, and CmoYAB1b_CmaYAB1b (Fig. 2B). Through multiple sequence alignment, it was found that YABBY protein contained a conserved YABBY domain at the C-terminal, and most YABBY proteins contained a conserved C2C2 domain (Fig. S1). Ten conserved motifs of YABBYs were searched and identified by MEME online tools (Fig. 2C, Fig. S2). Motif analysis showed that motifs 1 and 2 existed in all YABBY proteins. In addition to CpeYAB1b protein, motif 3 existed in all other proteins. Motif 4 existed in all other proteins except CpeYAB5d. These results indicated that the domain and motif of YABBYs were highly conserved. What's more, motif 6 only existed in YABBY subfamily, motif 9 only existed in a branch of YAB5 subfamily; motif 8 only existed in some genes of INO and YAB5 subfamily, and motif 10 only existed in some genes of YAB5 and CRC subfamily (Fig. 2C), indicating that these genes may have special functions.

**Distribution, gene duplication and collinearity of YABBY transcription factors in three cultivars of *Cucurbita Linn.***

According to the genome sequence of *Cucurbita*, the chromosome position of YABBY in each cultivar of *Cucurbita Linn.* was determined (Fig. 3A). Eleven CmoYABBYs were located on 8 of the 21 chromosomes, and there were 2 CmoYABBYs on chromosomes Cmo_chr02, Cmo_chr04, and Cmo_chr05, respectively. Among the 12 CmaYABBYs, 11 CmaYABBYs were similar to CmoYABBYs, and CmaYB3 gene was located on chromosome Cma_chr06. Eleven CpeYABBYs were located on 9 of 21 chromosomes. Except for 2 CpeYABBYs on chromosomes CP4.1LG05 and CP4.1LG11, respectively, there was one CpeYABBY on the other chromosomes (CP4.1LG01, CP4.1LG04, CP4.1LG08, CP4.1LG09, CP4.1LG13, and CP4.1LG18), respectively (Fig. 3A).

According to the amino acid sequence identity > 80% and gene alignment coverage > 0.75, we found three duplicated gene pairs (CmoYAB1a-CmoYAB1b, CmoYAB5a-CmoYAB5d and CmoYAB5c-CmoYAB5b) in CmoYABBYs, two duplicated gene pairs (CmaYAB1b-CmaYAB1a and CmaYAB5d-CmaYAB5a) in CmaYABBYs, and one duplicated gene pairs (CpeYAB5b-CpeYAB5c) in CpeYABBYs, with all Ka/Ks < 1.0, indicating that these duplicated gene pairs mainly underwent purification selection, and the divergence time was 6.64–16.47 (MYA) (Table 2).

Synteny relationship of YABBY genes among *Arabidopsis* and three cultivars of *Cucurbita Linn.* was also analyzed. Eight, eight and six collinear genes of AtYABBYs were found in *Cucurbita moschata*, *Cucurbita maxima* and *Cucurbita pepo*, respectively (Fig. 3B). Based on the collinearity analysis of YABBYs among *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo*, it was found that there were 30 pairs of collinear genes between CmoYABBYs and CpeYABBYs, 29 pairs of collinear genes between CmoYABBYs and CmaYABBYs and 26 pairs of collinear genes between CmaYABBYs and CpeYABBYs (Fig. 3B, Table S1).

**Cis-acting elements in *Cucurbita Linn.* YABBY gene promoters**

To understand the transcriptional regulation of the YABBYs in three cultivars of *Cucurbita Linn.*, we extracted the 2.0 kb sequence before the translation initiation site (ATG) and predicted the cis-elements on the PlantCARE server. Fig. 4A showed the position of the cis-acting element on the promoter. It is worth
noting that five kinds of plant hormone-responsive cis-acting elements were identified, including abscisic acid response element, methyl jasmonate response element, gibberellin response element, salicylic acid response element and auxin response element (Fig. 4A; Fig. 4B). Thirty-four YABBY gene promoters contained at least two plant hormone response element. CpeINOb and CmaINOb contained the most (21) plant hormone response elements. Among them, there were 10 and 12 elements involved in methyl jasmonate, respectively (Fig. 4B). Additionally, we found that 74% (25) of the YABBYs contain elements that participate in the growth and development of plants, including 19 genes involved in meristem expression, and 3 genes (CmoYAB1b, CmaYAB1b, and CpeYAB1b) involved in seed-specific regulation (Fig. 4C). The YABBYs may also respond to abiotic stresses such as defense and stress, anaerobic, drought-induced and low temperature (Fig. 4D). CmaINOb contained the highest number (9) of abiotic stress elements, and CmoYAB5a contained 5 elements involved in anaerobic induction. These data suggested that YABBY may be involved in plant hormone response, abiotic stress, and plant growth and development through a complex mechanism.

The expression profiles of CmoYABBYs and CmaYABBYs in different tissues

According to RNA-seq data (BioProject: PRJNA385310), the expression profiles of CmoYABBYs and CmaYABBYs in root, stem, leaf and fruit were obtained. In general, CmoYABBY and CmaYABBY genes were mainly expressed in roots and leaves (Fig. 5). In Cucurbita maxima, CmoYAB1a, CmoYAB5a, CmoYAB5c, and CmoYAB5d were mainly expressed in leaves. The expression of CmoYAB1b in the root, leaf, and fruit was higher than that in the stem. The relative expression of CmoINOb in four tissues was higher than other genes. CmoYAB2 was mainly highly expressed in root and stem; The remaining CmoYABBY genes have a low expression (Fig. 5A). In Cucurbita maxima, CmaYAB1b, CmaYAB5a, CmaYAB3, CmaINOa, CmaYAB5c, and CmaYAB1a were mainly expressed in leaves. The expression of CmaYAB2 in stems, leaves, and fruits was higher than that in roots. CmaYAB5b was highly expressed in all tissues. The relative expression levels of other CmoYABBY genes were relatively low (Fig. 5B). Based on the above analysis, we speculated that CmoYABBYs and CmaYABBYs have tissue specificity.

Expression profiles of CpeYABBYs in fruit mesocarp and seed

To study the expression patterns of CpeYABBYs in seed and fruit mesocarp, the Cucurbita pepo cultivar, "Sweet REBA" was used and the published transcriptome data (BioProject: PRJNA339848) [16] was analyzed. It showed that all CpeYABBYs (except CpeCRC) were highly expressed in the seed at 20 days after pollination (DAP). Meanwhile, CpeYAB5c, CpeYAB5a, CpeYAB1a, CpeYAB1b, CpeINOa, CpeINOb and CpeYAB2 were highly expressed in the fruit mesocarp at 20 days after pollination (DAP). CpeYAB2 had a relatively high relative expression profile at each developmental stage in both seed and fruit mesocarp (Fig. 6).

Expression patterns of CmoYABBYs in leaf mesophyll and leaf vein under salt stress

To determine the expression pattern of the YABBY gene in Cucurbita moschata (salt-sensitive type) under salt stress, the RNA-seq data (BioProject: PRJNA646406) was analyzed. The results showed that the expression of all CmoYABBYs (except for CmoCRCa was unchanged) in leaf veins was inhibited under salt stress, while all CmoYABBYs in leaf mesophyll were induced. For instance, the expression level of CmoYAB1a in the leaf vein under salt stress was significantly reduced compared with the control treatment, while the relative expression of CmoYAB1a in mesophyll under salt stress was 2.29 times higher than the control treatment (Fig. 7).

Expression patterns of CmoYABBYs in root tip under salt stress

Tissue location analysis showed that all CmoYABBYs were mainly expressed in roots and leaves (Fig. 5A). In the leaves, the expression level of CmoYABBYs in the leaf vein was significantly inhibited under salt stress, while the expression level of CmoYABBYs in the leaf mesophyll was significantly induced under salt stress. However, the response of CmoYABBY in the root to salt stress was not clear. For further determine the response of CmoYABBYs in root to salt stress, the qRT-PCR data was analyzed. The results showed that most of the CmoYABBYs had low expression levels in root tips, but CmoCRCb, CmoINOb and CmoYAB5d were significantly induced under salt stress. For example, the relative expression profile of CmoINOb was 6.45 times that of the control. Therefore, we speculated that CmoINOb in root tips may play an important role in salt tolerance.

Discussion

Plant specific YABBY transcription factors play important biological roles in plant morphogenesis, growth and development, and abiotic stress response [17]. However, only a few species of YABBY gene family have been identified at the genome level, such as tomato [12], pineapple [11], soybean [2], grape [18] and cotton [19]. As far as we know, there was no systematic report on the YABBY gene family in Cucurbita Linn.. In this study, we explored 34 YABBY genes in three cultivars of Cucurbita Linn. by analyzing the phylogeny, chromosomal distribution, gene structure, conserved motifs, and cis-acting elements.

Based on phylogenetic tree analysis of YABBY proteins in three cultivars of Cucurbita Linn., it was found that they were divided into five subfamilies (YAB1/YAB3/YAB2/INO/CRC and YAB5), which was consistent with the previous classification of AYABBYs [17]. Besides, phylogenetic tree analysis found that each subfamily contains CmoYABBY, CmaYABBY, and CpeYABBY, indicating that they may have evolved from the same ancestor. In Arabidopsis thaliana, AtFIL, AtYAB2 and AtYAB3 are expressed in the adaxial region of all lateral organs, including cotyledons, leaves and flowers. However, AtINO is limited to the integument of ovule [20, 7]. Phylogenetic analysis showed that AtYAB1(FIL) and AtYAB3 had the closest homology with CmaYAB3 and CpeYAB3, AtYAB2 had the closest homology with CmoYAB2. Therefore, we speculated that CmaYAB3, CpeYAB3 and CmoYAB2 might participate in the expression of adaxial region in lateral organs. Besides, we found that AtINO had the closer homology with CmoINOb, CmaINOb and CpeINOb than other genes. Therefore, it is speculated that they play an important role in the integument of ovule. AtCRC participates in flower development [21], so the homologous genes CmaCRCb, CmaCRCb may have the same function as AtCRC.
Through the analysis of YABBY gene structure, it is found that most genes in the same subfamily have similar structural characteristics in terms of the number of exons or introns, which is similar to the structural characteristics of the YABBYs in other species [19, 22]. However, CmaINOa and CpeYAB1a have a different number of exons compared with other YABBYs in the same subfamily, which indicates the structural diversity of the YABBY gene family in Cucurbita Linn.. For a specific motif, high differences were also detected among different subfamilies. However, in one subfamily, most YABBY proteins of Cucurbita Linn. have conserved motifs, which suggests that the same subfamily may have similar functions.

Gene duplication plays an important role in biological evolution, including fragment duplication, tandem duplication and whole genome duplication [23]. Cucurbita experienced Cucurbit-common tetraploidization (CCT) events[13], and Cucurbita shared a recent Cucurbit-specific tetraploidization (CST) [24]. Six duplicated gene pairs were found in three cultivars of Cucurbita Linn.. Because these duplicated gene pairs were not on the same chromosome, we termed them fragment duplicated gene pairs. Ka / Ks < 1 indicated that these YABBY genes were in the process of purification and positive selection. The detailed analysis of collinear gene pairs and evolutionary relationship revealed the high complexity of chromosome evolution and rearrangement of three Cucurbita Linn. cultivars. Therefore, it is not surprising that the YABBY genes in Cucurbita moschata, Cucurbita maxima, and Cucurbita pepo are highly similar, and these YABBY genes may have similar functions.

Cis-acting elements can be bind specifically by transcription factors and can regulate gene transcription [25]. At least two cis-acting elements related to plant hormone response were found in 34 YABBY gene promoters, which means that the corresponding hormones may play an important role in its regulation. Besides, all YABBY genes (except for CmaYAB2) contain at least one cis-acting element related to stress response (Fig. 4C), indicating that these genes also play an important regulatory role in stress response. However, further research is needed to determine whether and how these cis-acting elements work in Cucurbita Linn..

The response of YABBYs in "Rifu" to salt stress showed that there was differential expression of YABBY in leaf mesophyll and leaf vein of Cucurbita moschata under salt stress. The expression of CmoYABB1a was inhibited in leaf veins under salt stress, and induced in leaf mesophyll, which may have an important relationship with sodium ion efflorescence of pumpkin under salt stress (Fig. 7). High concentration of Na⁺ in plants has a strong toxic effect on leaf photosynthetic organs. Under salt treatment, Cucurbita moschata were divided into Na⁺ above-ground accumulation type and Na⁺ underground accumulation type, and grafted cucumber seedlings with Na⁺ underground accumulation type as rootstock could significantly reduce the content of Na⁺ in aboveground and have stronger salt tolerance [15]. The main reason is that Cucurbita moschata rootstocks have strong Na⁺ storage capacity and strong Na⁺ root effluents, which further reduces the transport of Na⁺ to the overland part and the content of Na⁺ in the scion leaves [26]. To further verify the response of CmoYABBYs to salt stress, we also analyzed the root tips of Cucurbita moschata "Baimi 9". It was found that CmoINOb may play a key role in salt stress (Fig. 8), and the YABBY gene family of Cucurbita was may be involved in the response to salt stress.

Conclusions

In summary, we identified 34 YABBYs in three cultivars of Cucurbita Linn. based on a thorough analysis and provided genetic information such as chromosome locations and exon-intron structures, conserved domains, and duplicated genes. We specifically examined the expression profiles of these YABBYs in different tissues. At the same time, we examined the responses of CmoYABBYs to salt stress, and several key genes were found to regulate the resistance of three of Cucurbita Linn. cultivars.

Methods

Identification and characterization of Cucurbita Linn. YABBY transcription factor

Six Arabidopsis thaliana YABBYs (AtYABBYs) protein sequences from PlantTFDB database (http://planttfdb.cbi.pku.edu.cn/download_seq.php?Fam=YABBY) were used as query objects to conduct local BLAST [27] (E-value < 1e⁻⁵, identity > 50%) on the genome databases of Cucurbita moschata, Cucurbita maxima and Cucurbita pepo (http://cucurbitgenomics.org/). The HMM model of the YABBY domain (PF04690) in the Cucurbitaceae protein database was analyzed by using HMMER 3.0 software (E-value < 1e⁻⁵) [28]. Also, all YABBY protein sequences obtained were further analyzed on CDD (https://www.ncbi.nlm.nih.gov/cdd) [29] to verify whether C2C2 domain exists in the N-terminal and YABBY domain in C-terminal.

Using the ExPASY server (https://web.expasy.org/protparam/) to predict the physical and chemical properties of the YABBY protein, including the length of an amino acid sequence, molecular weight (MW), and isoelectric point (pI). The subcellular localization of YABBYs in three cultivars of Cucurbita Linn. was predicted by the Plant-mPloc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/#) [30]. To obtain the location information of YABBYs in three cultivars of Cucurbita Linn., the starting position, end position, and chromosome length of YABBYs were obtained from the Cucurbita database, and the distribution map of YABBYs was drawn by TBtools [31].

Structure analysis of Cucurbita Linn. YABBYs

The cDNA and DNA sequence of YABBYs were obtained from the Cucurbita database. The exon-intron structural map of YABBYs was drawn by TBtools software [31]. All YABBY protein sequences in three cultivars of Cucurbita Linn. were aligned with DNAMAN software and corrected manually [32]. At the same time, the online software MEME (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) [33] was utilized to predict the conserved motifs. The parameter was set as the number of motifs: 10, the length of motifs: 5-50.

Construction of phylogenetic tree
All *Cucurbita Linn.* YABBY proteins were aligned by ClustalW. The phylogenetic relationship between AtYABBY proteins and YABBY protein from three cultivars of *Cucurbita Linn.* was constructed by MEGA 7.0 [34] using the neighbor-joining (NJ) method. The parameters were as follows: completed deletion, poisson model, cut-off value for the condensed tree was 65%.

**Collinearity and gene duplicated of YABBY in Cucurbita Linn.**

The collinear genes of AtYABBYs in three *Cucurbita* cultivars were obtained by TBtools. Finally, the collinear relationship of YABBYs was drawn by Circos [35].

The CDS of YABBYs were blasted by the TBtools program, and the gene alignment coverage of YABBYs was calculated by the formula: gene alignment coverage = (alignment length - mismatches)/length of larger genes. The duplication of gene pairs is performed according to the previous indicators (the amino acid identity > 80%, the E-value < 1 × 10⁻10 and the gene alignment coverage > 0.75) in Chinese cabbage [36]. Besides, when the interval between the two genes is less than 100 kb, it is considered to be a tandem duplicated gene [37]. The synonymous substitution rate (Ks) of YABBY gene was calculated by KaKs calculator, and the divergence time (T) of YABBY gene was calculated according to the formula: T = Ks / 2λ × 10⁻8 (MYA , where “λ” is the neutral substitution rate, \( \lambda = 1.5 \times 10^{-8} \) [38].

**Extraction of YABBY promoter sequence and analysis of cis-acting elements**

To obtain the promoter sequence of the YABBY in three cultivars of *Cucurbita Linn.*, the 2000 bp before the start codon of YABBYs was obtained by TBtools [31]. On this basis, the cis-acting elements of the promoters of all genes were predicted by PLantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and finally, use TBtools to visualize the cis-acting elements.

**Expression profile of Cucurbita Linn. YABBYs in RNA-Seq**

To study the expression pattern of CmoYABBYs and CmaYABBYs in different organs, we downloaded the published transcriptome data (BioProject: PRJNA385310) [39]. Besides, to study the expression pattern of CpeYABBYs in seed and fruit mesocarp, we downloaded the published transcriptome data (BioProject: PRJNA339848) [16].

To determine the response of *Cucurbita moschata* YABBY gene family to salt stress, we analyzed the expression level of the *Cucurbita moschata* cultivar, "Rifu" in leaf vein and leaf mesophyll under salt and control treatments. We excavated the transcriptome data (BioProject: PRJNA464060) published in 2019 [40] and analyzed the transcription profile of YABBYs in the leaf vein and leaf mesophyll under salt stress.

All the published transcriptome data were represented by RPKM (Reads per kilobase of exon model per million mapped reads), which has been converted to log₂ (RPKM) when plotting heat map.

**Experimental materials and stress treatment**

To further clarify the differential expression of these genes in root, the *Cucurbita moschata* variety "Baimi 9" was used as the study material and the qRT-PCR was performed. Variety "Baimi 9" was provided by the pumpkin team of School of Horticulture and Landscape Architecture, Henan Institute of Science and Technology. The seeds were sown in a tray containing a matrix-meteorite (3:1) mixture and grown in a plant growth chamber. The artificial growth conditions were set to 25 °C / 16 °C, 16 h light / 8 h dark and 65% relative humidity. The two-month-old seedlings were cultured in 1/2 Hoagland solution, pH 6.5. After 5 days of adaptation, some of the seedlings were cultured with 75 mM NaCl. Root tips were collected at 24 h after the NaCl treatment. Three independent biological replications formed one sample. Control and salt-treated samples were frozen in liquid nitrogen and stored at -70 °C for further analysis.

**Quantitative real-time PCR (qRT-PCR) analysis**

The total RNA was extracted with RNA-Solv® reagent (Omega), and the first strand cDNA was prepared with PrimeScript™ RT Master Mix (TaKaRa). Using cDNA as a template, the expression of YABBY in *Cucurbita moschata* root tip under salt stress was analyzed. The specific quantitative primers (Table S2) were design by Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The pumpkin β-Actin was used as the internal reference gene. The reaction system was 20 μL, including 10 μL of SYBR Green I, 0.4 μL of ROX dye II, 0.4 μL of primers, 2 μL of cDNA template and 6.8 μL of dd H₂O. PCR was performed on Applied Biosystems 7500, and the reaction conditions were set as follows: 95 °C pre-denaturation for 30 s, 95 °C denaturation for 5 s, 60 °C degradation and extension for 34 s and 40 cycles. The melting curve is: 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. The 2^ΔΔct method [41] was used to quantitatively analyze the data. Each reaction was performed three times, and the data was presented by heatmap.

**Abbreviations**

TF: Transcription factor; qRT-PCR: Quantitative real-time polymerase chain reaction; MW: Molecular weight; pI: Isoelectric point; NJ: Neighbor-joining; GSDS: Gene structure display server; MEME: Multiple Expectation Maximization or Motif Elicitation; Ks: Synonymous substitution ratio; Mya: Million years ago; RPKM: Reads per kilobase of exon model per million mapped reads.

**Declarations**

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Not applicable.
**Author's contributions**

CWS conceived, designed and analyzed data; CWS and JPY wrote the manuscript; JJX and ZXL identified *Cucurbita Linn.* YABBYs and analyzed gene structure. XZL and JGZ studied chromosome distribution, gene duplication and syntenic analysis of *Cucurbita Linn.* YABBYs. JPY supervised the research. CWS and JPY revised the manuscript. All authors read and approved the manuscript.

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**Availability of data and materials**

The information on gene and protein sequence of *Cucurbita moschata* YABBY s was downloaded from the *Cucurbit* genomics database (CuGenDB, http://cucurbitgenomics.org/). The data and materials supporting our research findings were contained in the methods and additional files.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

There have no competing interests among authors.

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**Tables**

Table 1 Physical and chemical characteristics of the 34 YABBY genes identified in *Cucurbita maxima*, *Cucurbita moschata* and *Cucurbita pepo*. 
| Gene ID        | Cultivar        | Gene name | Chra | Startb | Endc   | ORF length (bp) | pI  | Molecular weight (Da) | Number of amino acid (aa) | Subcellular localization | Anci   |
|---------------|-----------------|-----------|------|--------|--------|-----------------|-----|------------------------|--------------------------|-------------------------|--------|
| CmoCh04G017950.1 | Cucurbita moschata | CmoCRCa   | Cmo_Chr04 | 9028899 | 9030980 | 606             | 9.08| 22132.26               | 201                      | Nucleus.                | AT1    |
| CmoCh18G011200.1 | Cucurbita moschata | CmoCRCb   | Cmo_Chr18 | 11567244 | 11572287 | 708             | 9.99| 25743.41               | 235                      | Nucleus.                | AT1    |
| CmoCh05G000290.1 | Cucurbita moschata | CmoINOb   | Cmo_Chr05 | 107469  | 108710  | 594             | 6.43| 21846.84               | 197                      | Nucleus.                | AT1    |
| CmoCh04G018270.1 | Cucurbita moschata | CmoINOa   | Cmo_Chr04 | 9186167 | 9191600 | 930             | 9.35| 35478.2                | 309                      | Nucleus.                | AT1    |
| CmoCh02G015970.1 | Cucurbita moschata | CmoYAB1a  | Cmo_Chr02 | 9202861 | 9204633 | 714             | 8.8 | 26238.55               | 237                      | Nucleus.                | AT2    |
| CmoCh15G012090.1 | Cucurbita moschata | CmoYAB1b  | Cmo_Chr15 | 8382484 | 8384352 | 711             | 8.8 | 26015.43               | 236                      | Nucleus.                | AT2    |
| CmoCh02G013370.1 | Cucurbita moschata | CmoYAB2   | Cmo_Chr02 | 7963763 | 7967840 | 510             | 9.06| 18889.49               | 169                      | Nucleus.                | AT1    |
| CmoCh11G005350.1 | Cucurbita moschata | CmoYAB5c  | Cmo_Chr11 | 2613770 | 2616030 | 573             | 7.56| 21429.38               | 190                      | Nucleus.                | AT2    |
| CmoCh05G013080.1 | Cucurbita moschata | CmoYAB5a  | Cmo_Chr05 | 10151693 | 10155834 | 582             | 8.95| 21726.63               | 193                      | Nucleus.                | AT2    |
| CmoCh12G012310.1 | Cucurbita moschata | CmoYAB5d  | Cmo_Chr12 | 10977446 | 10981728 | 570             | 8.98| 21268.06               | 189                      | Nucleus.                | AT2    |
| CmoCh10G005780.1 | Cucurbita moschata | CmoYAB5b  | Cmo_Chr10 | 2633020 | 2635308 | 546             | 6.29| 20280.73               | 181                      | Nucleus.                | AT2    |
| CmaCh04G017090.1 | Cucurbita maxima | CmaCRCa   | Cma_Chr04 | 8594676 | 8596684 | 537             | 8.81| 19479.12               | 178                      | Nucleus.                | AT1    |
| CmaCh18G010980.1 | Cucurbita maxima | CmaCRCb   | Cma_Chr18 | 8991641 | 8995269 | 660             | 9   | 23653.51               | 219                      | Nucleus.                | AT1    |
| CmaCh04G017410.1 | Cucurbita maxima | CmafNOa   | Cma_Chr04 | 8746724 | 8752040 | 1179            | 8.19| 43636.39               | 392                      | Nucleus.                | AT1    |
| CmaCh05G000220.1 | Cucurbita maxima | CmafNOb   | Cma_Chr05 | 105423  | 106388  | 576             | 6.37| 21366.42               | 191                      | Nucleus.                | AT1    |
| CmaCh02G015570.1 | Cucurbita maxima | CmaYAB1a  | Cma_Chr02 | 8833805 | 8835686 | 696             | 8.8 | 25570.87               | 231                      | Nucleus.                | AT2    |
| CmaCh15G011490.1 | Cucurbita maxima | CmaYAB1b  | Cma_Chr15 | 7308199 | 7310013 | 711             | 8.8 | 25929.29               | 236                      | Nucleus.                | AT2    |
| CmaCh02G012970.1 | Cucurbita maxima | CmaYAB2   | Cma_Chr02 | 7630829 | 7634766 | 540             | 9.32| 19978.84               | 179                      | Nucleus.                | AT1    |
| CmaCh11G005360.1 | Cucurbita maxima | CmaYAB5c  | Cma_Chr11 | 2583470 | 2590191 | 681             | 8.06| 25346.08               | 226                      | Nucleus.                | AT2    |
| CmaCh05G012870.1 | Cucurbita maxima | CmaYAB5a  | Cma_Chr05 | 9820965 | 9824076 | 582             | 8.95| 21715.6                | 193                      | Nucleus.                | AT2    |
| CmaCh12G012020.1 | Cucurbita maxima | CmaYAB5d  | Cma_Chr12 | 9427635 | 9431144 | 570             | 8.86| 21179.89               | 189                      | Nucleus.                | AT2    |
| CmaCh10G005410.1 | Cucurbita maxima | CmaYAB5b  | Cma_Chr10 | 2483897 | 2486274 | 537             | 6.8 | 19988.49               | 178                      | Nucleus.                | AT2    |
| CmaCh06G007630.1 | Cucurbita maxima | CmaYAB3   | Cma_Chr06 | 3875334 | 3877797 | 606             | 8.61| 22196.04               | 201                      | Nucleus.                | AT4    |
| Cp4.1LG09g02290.1 | Cucurbita pepo   | CpeCRC    | Cp4.1LG09 | 1313947 | 1316378 | 498             | 6.63| 17647.61               | 165                      | Nucleus.                | AT1    |
| Cp4.1LG09g15370.1 | Cucurbita pepo   | CpeINOa   | Cp4.1LG01 | 9207257 | 9210078 | 573             | 8.57| 21514.93               | 190                      | Nucleus.                | AT1    |
| Cp4.1LG11g00620.1 | Cucurbita pepo   | CpeINOb   | Cp4.1LG11 | 100544  | 102144  | 471             | 9.3 | 17044.70               | 156                      | Nucleus.                | AT1    |
| Cp4.1LG13g02080.1 | Cucurbita pepo   | CpeYAB1b  | Cp4.1LG13 | 1656187 | 1659056 | 639             | 9.76| 23823.32               | 212                      | Nucleus.                | AT2    |
Cp4.1LG05g02370.1  |  Cucurbita pepo | CpeYAB1a | Cp4.1LG05 | 1340984 | 1343955 | 1110 | 9.37 | 40999.44 | 369 | Nucleus. | AT2
---|---|---|---|---|---|---|---|---|---|---|---|---
Cp4.1LG05g04630.1  |  Cucurbita pepo | CpeYAB2 | Cp4.1LG05 | 2635623 | 2639712 | 510 | 9.21 | 18817.42 | 169 | Nucleus. | AT1
Cp4.1LG11g10670.1  |  Cucurbita pepo | CpeYAB5c | Cp4.1LG11 | 8932872 | 8936484 | 582 | 8.94 | 21738.64 | 193 | Nucleus. | AT2
Cp4.1LG07g11420.1  |  Cucurbita pepo | CpeYAB5b | Cp4.1LG07 | 9727251 | 9734543 | 582 | 8.86 | 21686.53 | 193 | Nucleus. | AT2
Cp4.1LG18g04950.1  |  Cucurbita pepo | CpeYAB5d | Cp4.1LG18 | 5778295 | 5780743 | 495 | 6.7 | 18255.67 | 164 | Nucleus. | AT2
Cp4.1LG04g09420.1  |  Cucurbita pepo | CpeYAB5a | Cp4.1LG04 | 10087072 | 10089482 | 519 | 6.06 | 19308.98 | 172 | Nucleus. | AT2
Cp4.1LG08g07580.1  |  Cucurbita pepo | CpeYAB3 | Cp4.1LG08 | 6069752 | 6071866 | 579 | 8.6 | 21203.08 | 192 | Nucleus. | AT4

a The chromosome in which the gene is located
b The start position of the gene on the chromosome
c The end position of the gene on the chromosome

| Gene ID | Identity (%) | Alignment length (bp) | Mismatches (bp) | length of the larger gene (bp) | Gene alignment coverage | Ka | Ks | Ka/Ks | Divergence time (MYA) |
|---------|--------------|----------------------|----------------|-------------------------------|------------------------|----|----|-------|----------------------|
| CmoYAB1a-CmoYAB1b | 84.92 | 723 | 68 | 714 | 0.92 | 0.06 | 0.42 | 0.14 | 14.12 |
| CmoYAB5a-CmoYAB5d | 89.71 | 583 | 46 | 582 | 0.92 | 0.06 | 0.22 | 0.28 | 7.26 |
| CmoYAB5c-CmoYAB5b | 85.58 | 541 | 57 | 573 | 0.84 | 0.10 | 0.49 | 0.20 | 16.47 |
| CmaYAB1b-CmaYAB1a | 87.04 | 702 | 60 | 711 | 0.90 | 0.04 | 0.32 | 0.13 | 10.72 |
| CmaYAB5d-CmaYAB5a | 90.38 | 582 | 44 | 582 | 0.92 | 0.06 | 0.20 | 0.30 | 6.64 |
| CpeYAB5b-CpeYAB5c | 89.71 | 583 | 46 | 582 | 0.92 | 0.06 | 0.24 | 0.23 | 8.15 |

Table 2 Ka/Ks calculation and estimated divergence time for the YABBY duplicated gene pairs