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 C*ucurbita moschata*, 12 *CmaYABBYs* in *Cucurbita maxima*, and 11 *CpeYABBYs* in *Cucurbita pepo*) were identified and they were divided into five subfamilies (YAB1/YAB3, YAB2, INO, 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* (INO, 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 18889.49 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 (*CpeINOb*) 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 CpeYABBYs 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 YABBYs 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 YAB5 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 were 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 *CpeYABBYs* 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 suggestted 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*, *CmoYAB5a*, and *CmoYAB5a* 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 *CmoINOa* 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 *CmaYABBY* 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 naalysed. 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: PRJNA464060) 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 leaf 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 *AtYABBYs* [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 *CpeINO* 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 *Cucurbita*-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 effloression 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 C*ucurbita 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 (K_S) of *YABBY* gene was calculated by *KaKs* calculator, and the divergence time (T) of *YABBY* gene was calculated according to the formula: $T = K_S / 2\lambda \times 10^{-6}$ (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 $\log_2$ (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 ℃ / 16 ℃, 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 ℃ 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 H_2O. PCR was performed on Applied Biosystems 7500, and the reaction conditions were set as follows: 95 ℃ pre-denaturation for 30 s, 95 ℃ denaturation for 5 s, 60 ℃ degradation and extension for 34 s and 40 cycles. The melting curve is: 95 ℃ for 15 s, 60 ℃ for 60 s, 95 ℃ for 15 s. The $2^{-\Delta\Delta ct}$ 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

Acknowledgements

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	Chr[a]	Start[b]	End[c]	ORF length (bp)	*pI*	Molecular weight (Da)	Number of amino acid (aa)	Subcellular localization	Arab hom
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*	CmaINOa	Cma_Chr04	8746724	8752040	1179	8.19	43636.39	392	Nucleus.	AT1
CmaCh05G000220.1	*Cucurbita maxima*	CmaINOb	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
Cp4.1LG01g15370.1	*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 chromosom

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

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

Figures

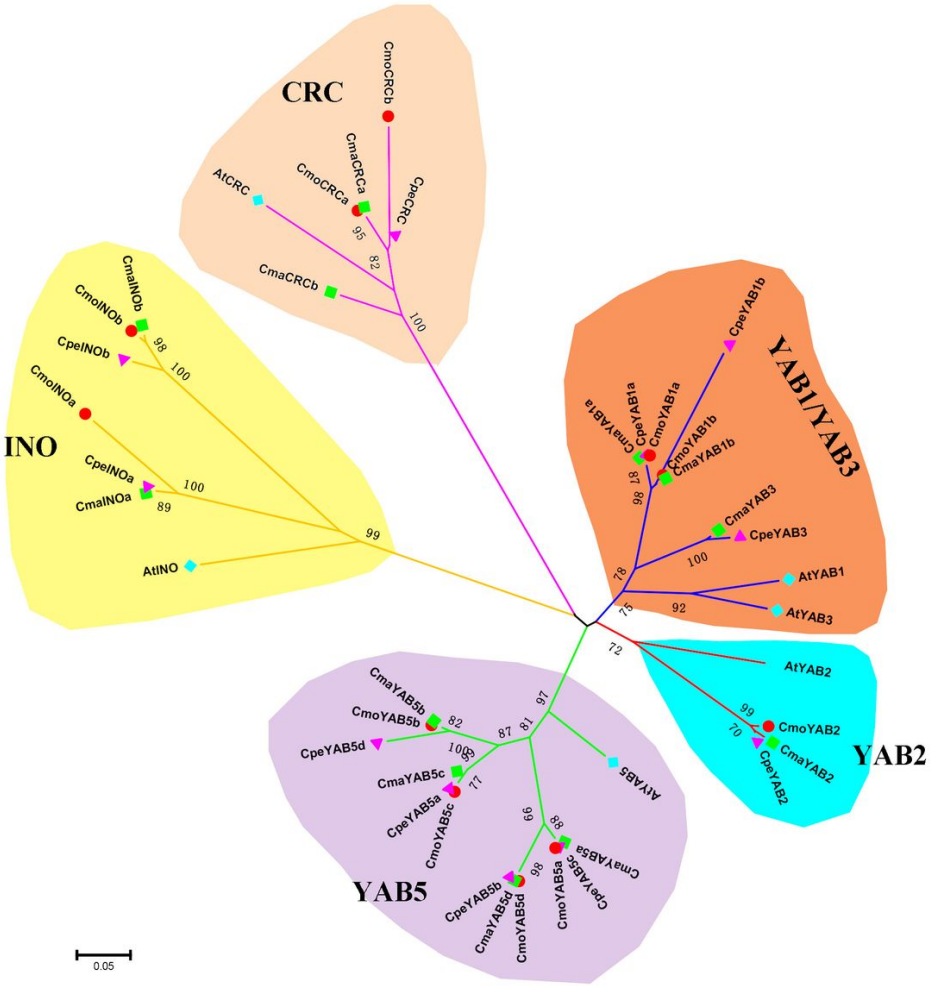

Figure 1

Phylogenetic trees of the YABBY gene family in Cucurbita moschata, Cucurbita maxima, Cucurbita pepo and Arabidopsis thaliana. Five different subfamilies (YAB1/YAB3, YAB2, INO, CRC, and YAB5) were displayed with different background colors.

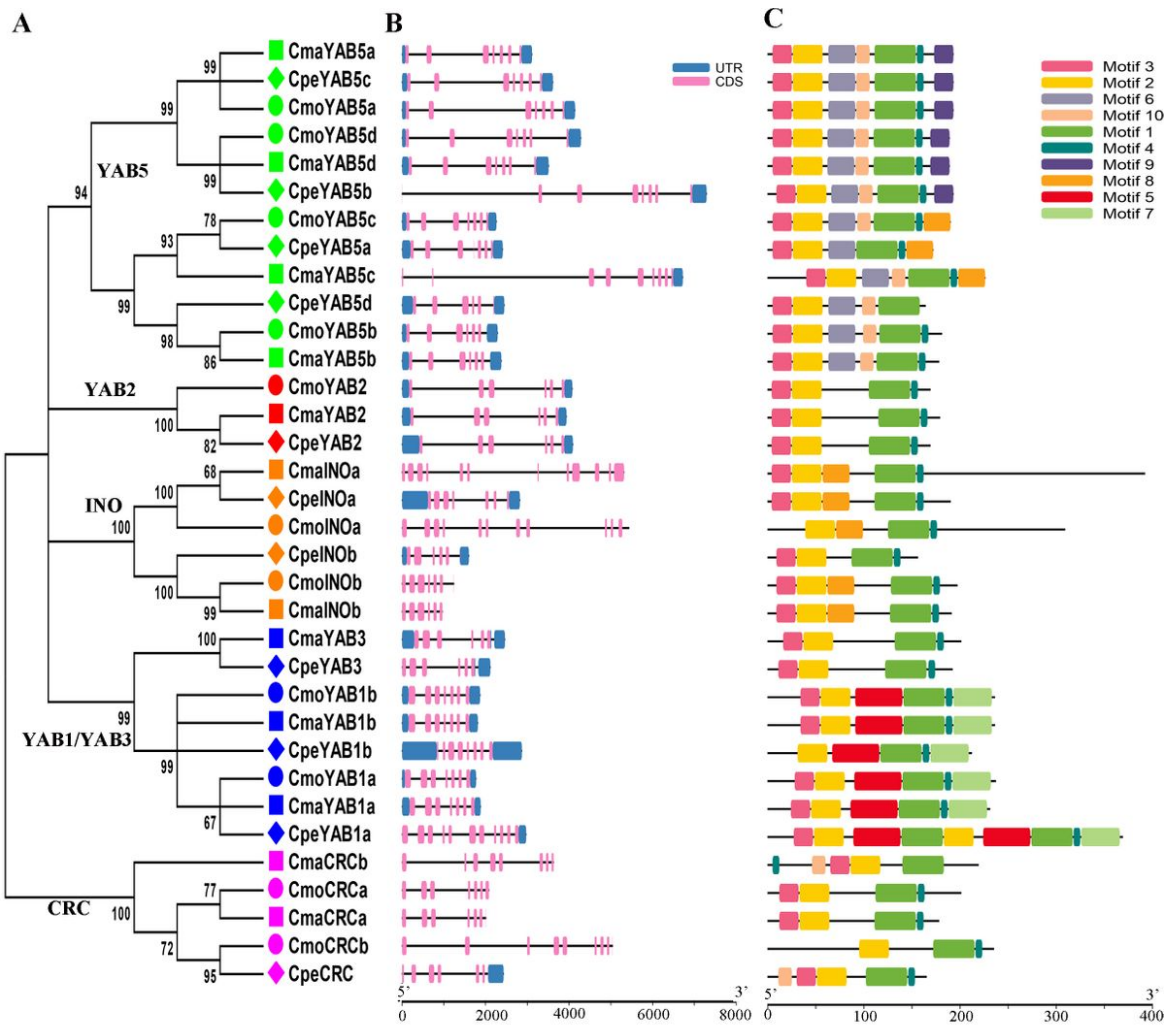

Figure 2

Gene structure and motif composition of YABBYs gene family in three Cucurbita Linn. cultivars. A. The phylogenetic tree of 34 YABBYs was constructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates, and a 65% cut-off value was used for the condensed tree. B. Exon-intron organization of 34 YABBYs in three cultivars of Cucurbita Linn.. The exons and introns were represented by pink boxes and black lines, respectively. Untranslated region (UTR) were indicated by blue boxes. The sizes of the exons and introns can be estimated using the scale at the bottom. C. Schematic representation of conserved motifs in 34 YABBYs. Each motif was represented by a numbered coloured box on the right. The same number in different proteins refers to the same motif.

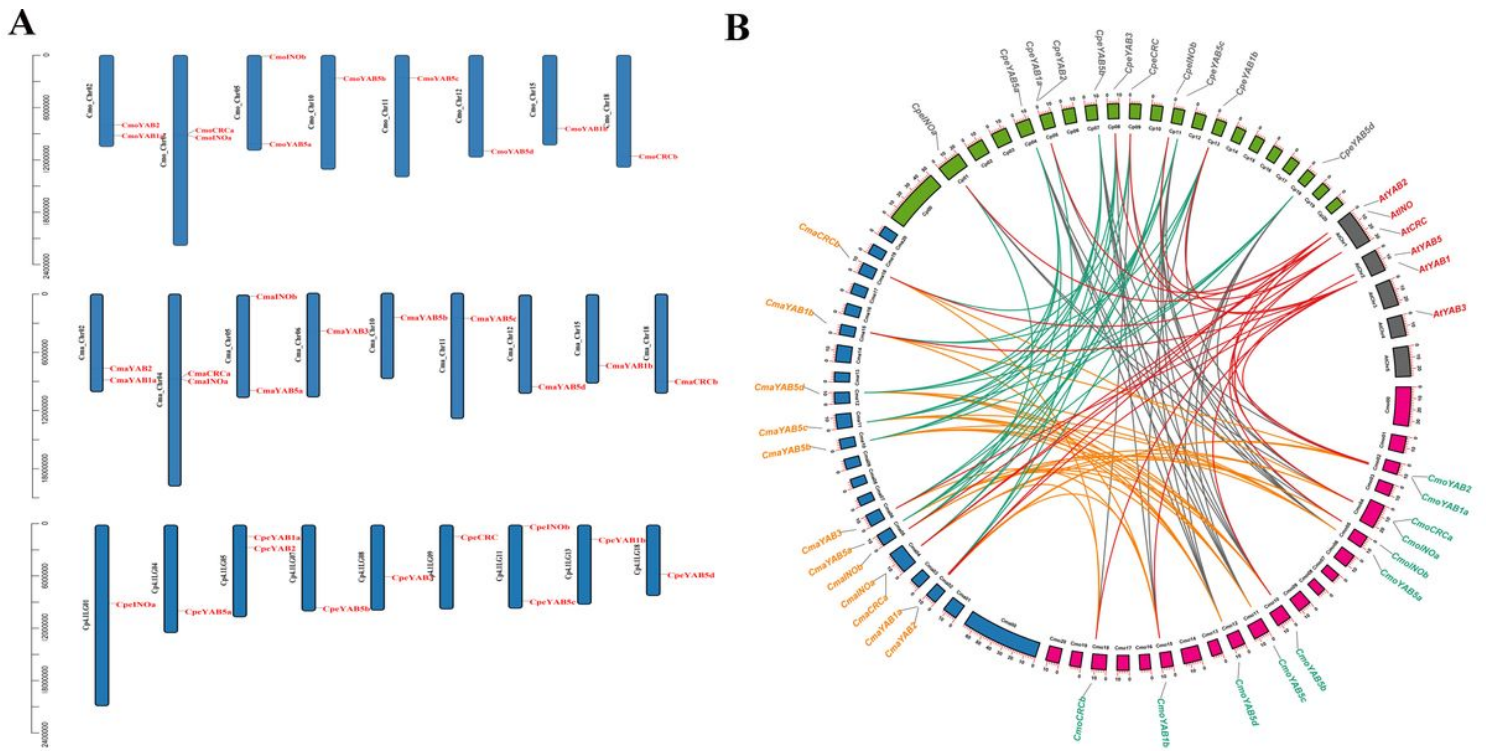

Figure 3

Distribution of YABBY family genes in three cultivars of Cucurbita Linn. and the collinear relationship between Cucurbita Linn. YABBY genes and AtYABBY genes. A. The distribution of YABBY genes from Cucurbita moschata, Cucurbita maxima, and Cucurbita pepo on a chromosome. B. Among the YABBY genes of Cucurbita moschata, Cucurbita maxima and Cucurbita pepo, the collinear genes of AtYABBY (represented by red lines), and the collinear genes between Cucurbita moschata, Cucurbita maxima, and Cucurbita pepo. The green lines indicated the collinearity between the CmaYABBY gene and the CpeYABBY gene; the orange lines indicated the collinearity between the CmaYABBY gene and CmoYABBY gene; the gray lines indicated the collinearity between CmoYABBY gene and CpeYABBY gene.

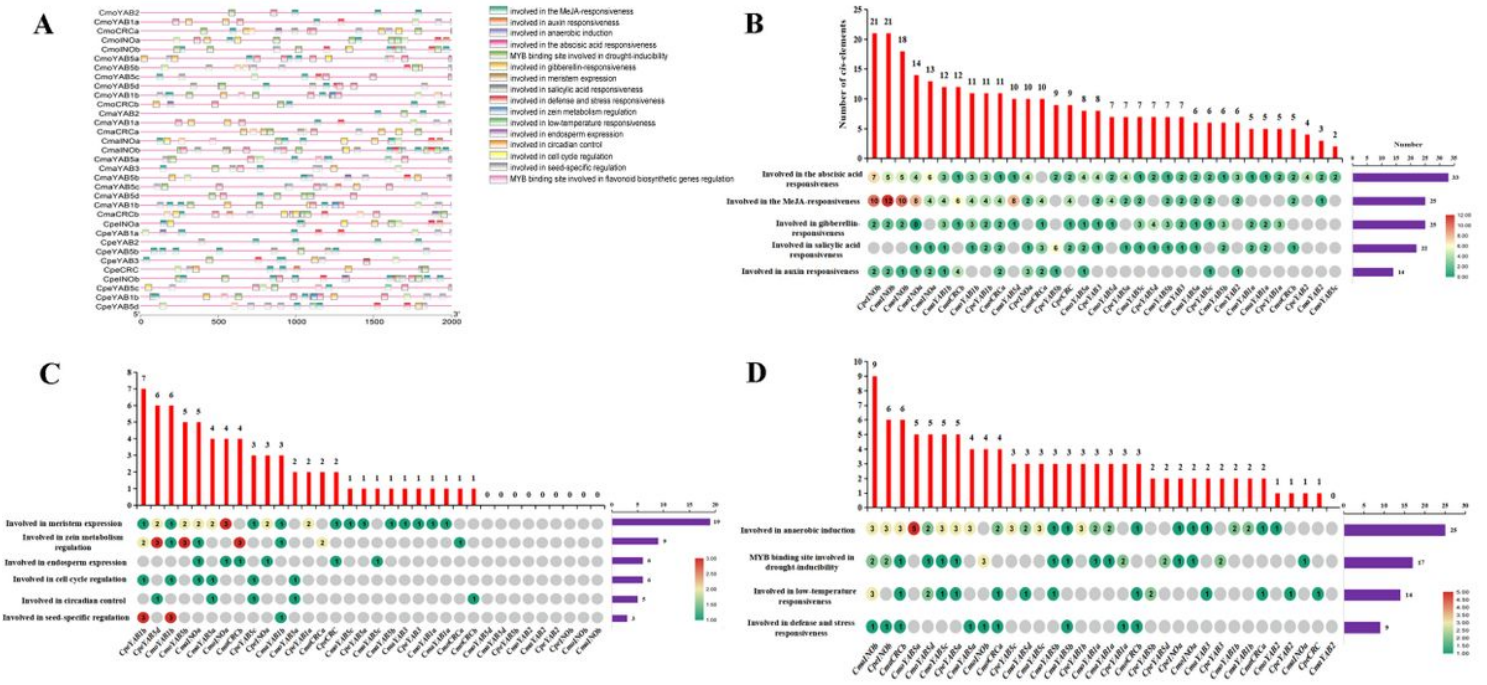

Figure 4

Distribution and statistics of cis-acting elements of YABBY gene promoter in three Cucurbitaceae cultivars. A, The schematic diagram of the cis-acting elements of the 34 YABBY gene promoters; B, Statistical analysis of the cis-acting elements of 34 YABBY genes involved in hormone response; C, Statistical

analysis of the cis-acting elements of 34 YABBY genes involved in plant growth and development; D, Statistical analysis of the cis-acting elements of 34 YABBY genes involved in abiotic stress response.

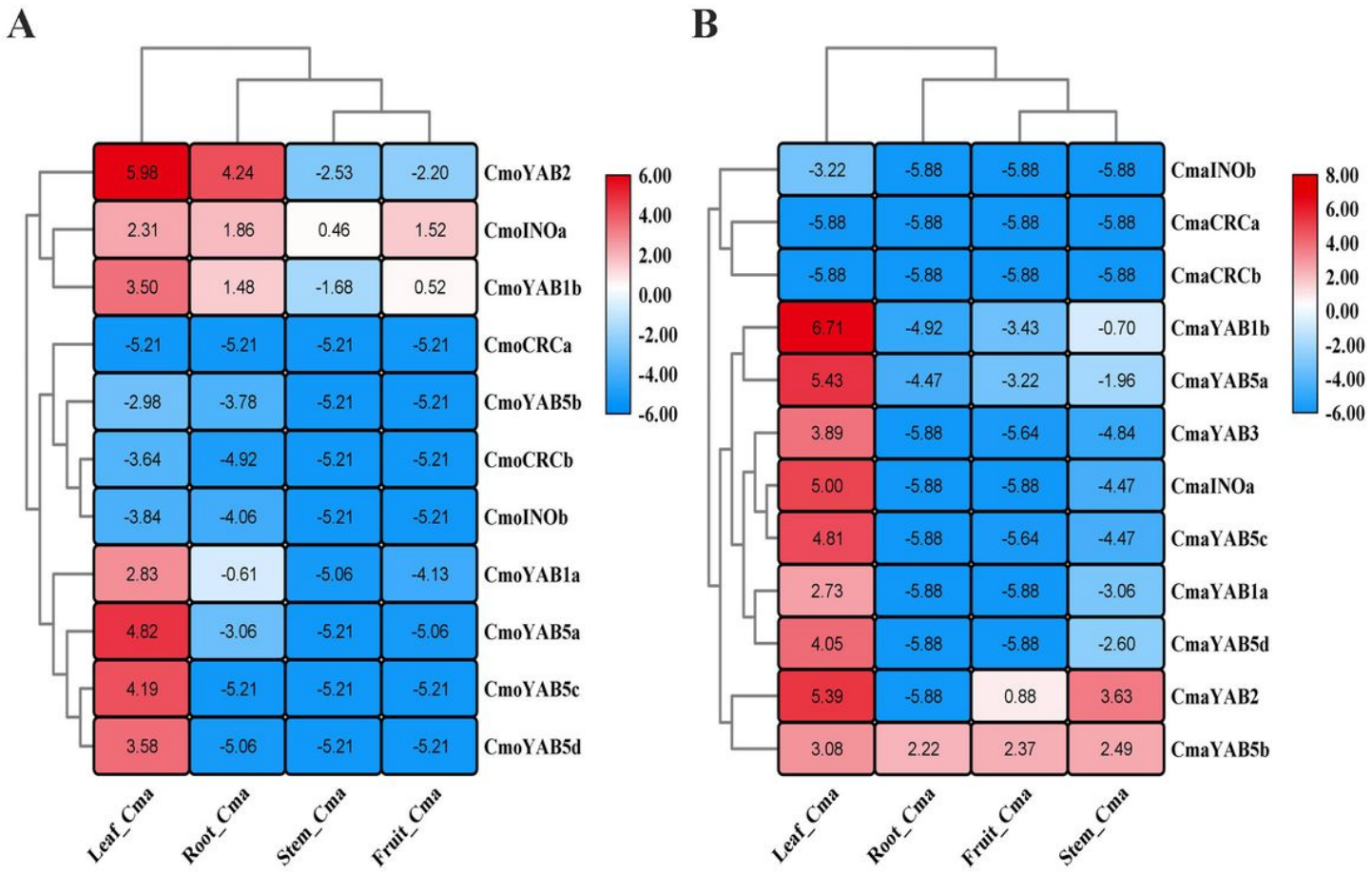

Figure 5

The relative expression levels of CmoYABBYs and CmaYABBYs in roots, stems, leaves, and fruits. A. The relative expression levels and clustering of CmoYABBYs in roots, stems, leaves, and fruits; B, The relative expression levels and clustering of CmaYABBYs in roots, stems, leaves, and fruits. The bar at the right of the heat map represented the relative expression values. The corresponding expression level was expressed as log2 (RPKM).

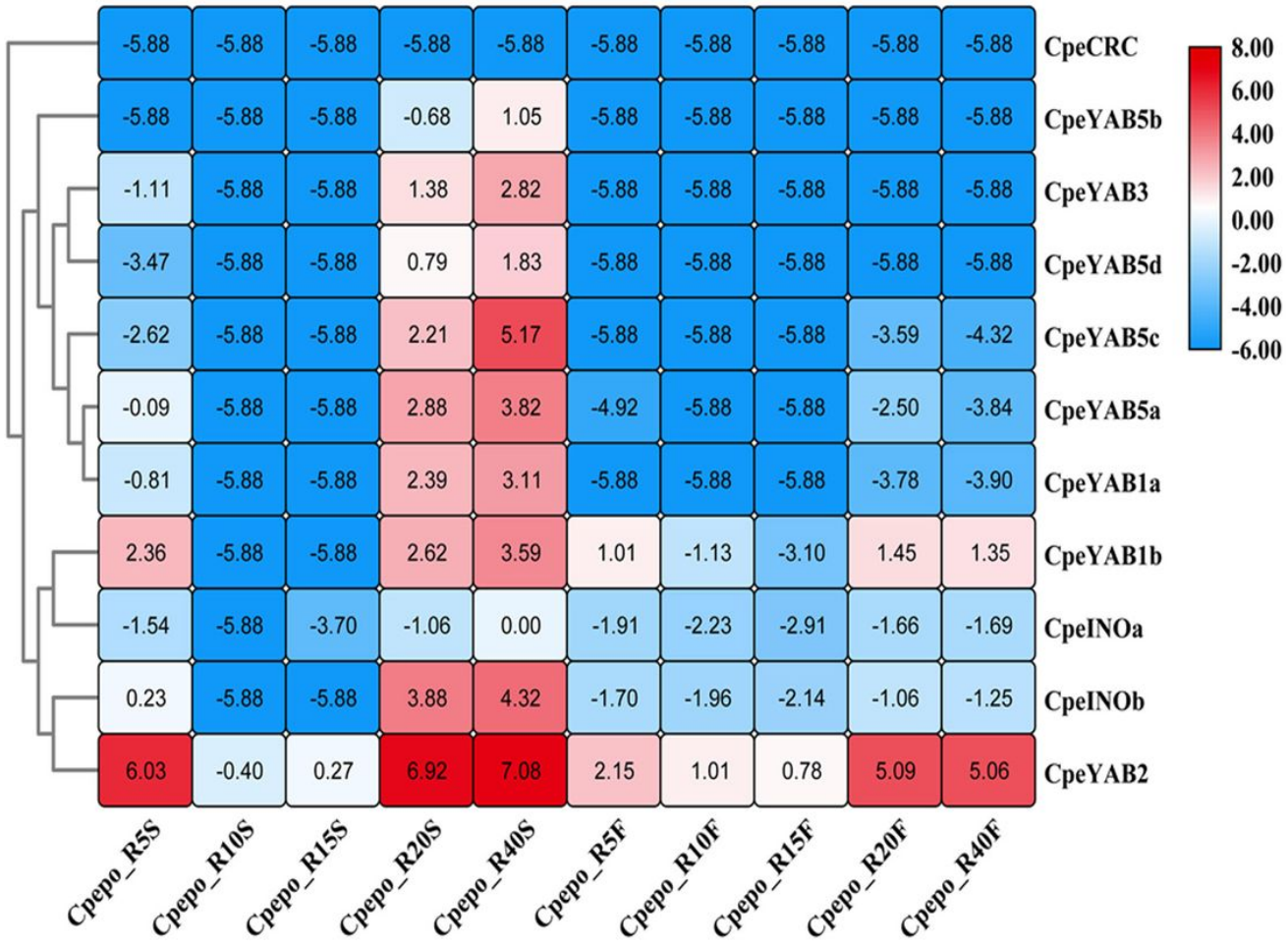

Figure 6

The relative expression levels and clustering of CpeYABBYs from "Sweet REBA" in seed and fruit mesocarp. The bar at the right of the heat map represented the relative expression values. The corresponding expression level was expressed as log2 (RPKM). R: Sweet REBA; S: seed; F: fruit mesocarp. 5, 10, 15, 20 , 40: 5 DAP , 10 DAP, 15 DAP, 20 DAP, 40 DAP; DAP, days after pollination.

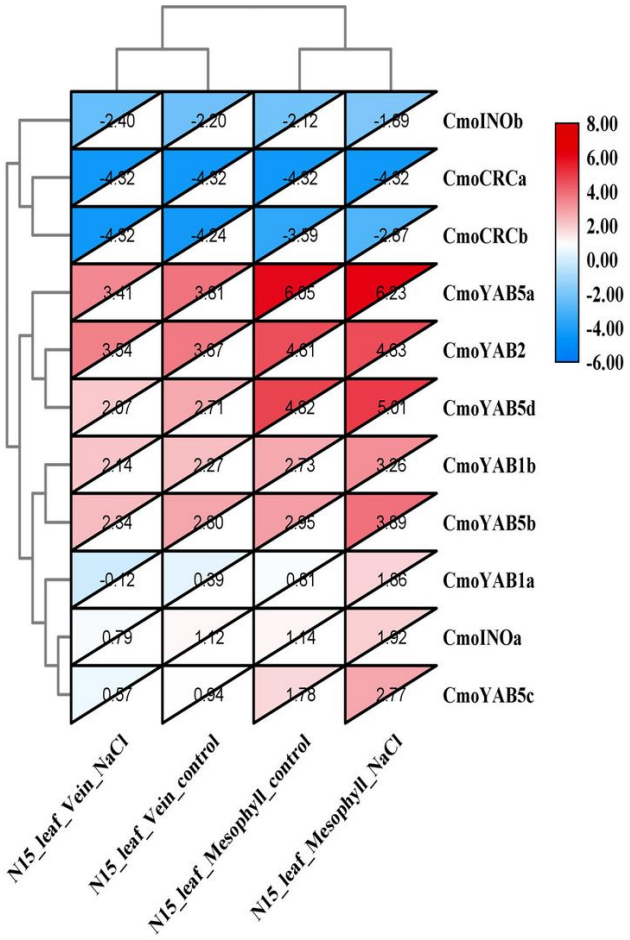

Figure 7

The relative expression level of the YABBY gene in leaf mesophyll and leaf vein of Cucurbita moschata under salt stress. The heatmap and cluster showed the relative expression level and clustering of CmoYABBY genes under NaCl treatment and control treatments. The bar at the right of the heat map represented the relative expression values. The corresponding expression level was expressed as log2 (RPKM). N15: Cucurbita moschata (“Rimu”).

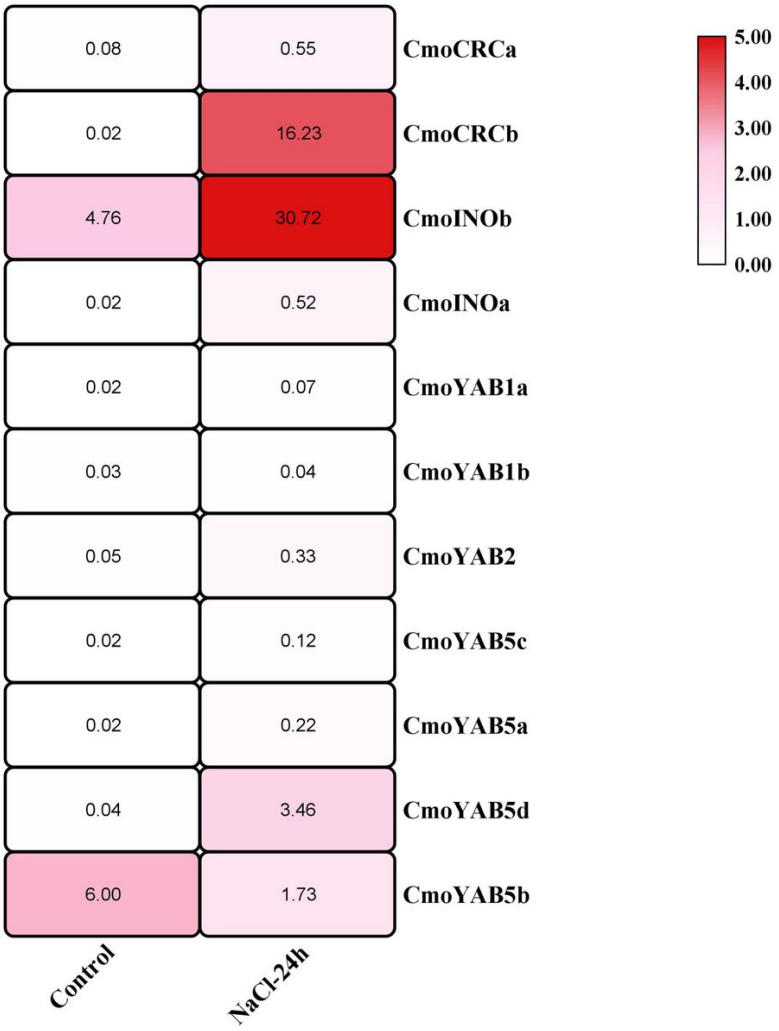

Figure 8

Heat map of the YABBY gene in Cucurbita moschata (“Baimi 9”) in root tip under salt stress. The results were calculated via the 2−ΔΔCt method, and the reference gene (β-Actin) was used to correct the expression level of target genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.tif
- Fig.S2.tif
- TableS1.docx
- TableS2.docx