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Genome-wide investigation of the nuclear factor Y gene family in Ginger (*Zingiber officinale* Roscoe): evolution and expression profiling during development and abiotic stresses

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Abstract

Background Nuclear factor Y (NF-Y) plays a vital role in numerous biological processes as well as responses to biotic and abiotic stresses. However, its function in ginger (*Zingiber officinale* Roscoe), a significant medicinal and dietary vegetable, remains largely unexplored. Although the NF-Y family has been thoroughly identified in many plant species, and the function of individual NF-Y TFs has been characterized, there is a paucity of knowledge concerning this family in ginger.

Methods We identified the largest number of NF-Y genes in the ginger genome using two BLASTP methods as part of our ginger genome research project. The conserved motifs of NF-Y proteins were analyzed through this process. To examine gene duplication events, we employed the Multiple Collinearity Scan toolkit (MCScanX). Syntenic relationships of NF-Y genes were mapped using the Dual Synteny Plotter software. Multiple sequence alignments were performed with MUSCLE under default parameters, and the resulting alignments were used to generate a maximum likelihood (ML) phylogenetic tree with the MEGA X program. RNA-seq analysis was conducted on collected samples, and statistical analyses were performed using Sigma Plot v14.0 (SYSTAT Software, USA).

Results In this study, the ginger genome was utilized to identify 36 NF-Y genes (10 *ZoNF-YAs*, 16 *ZoNF-YBs*, and 10 *ZoNF-YCs*), which were renamed based on their chromosomal distribution. Ten distinct motifs were identified within the ZoNF-Y genes, with certain unique motifs being vital for gene function. By analyzing their chromosomal location, gene structure, conserved protein motifs, and gene duplication events, we gained a deeper understanding of the evolutionary characteristics of these ZoNF-Y genes. Detailed analysis of ZoNF-Y gene expression patterns across various tissues, performed through RNA-seq and qRT-PCR, revealed their significant role in regulating ginger rhizome

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and flower growth and development. Additionally, we identified the ZoNF-Y family genes that responded to abiotic stresses.

Conclusion This study represents the first identification of the ZoNF-Y family in ginger. Our findings contribute to research on evolutionary characteristics and provide a better understanding of the molecular basis for development and abiotic stress response. Furthermore, it lays the foundation for further functional characterization of ZoNF-Y genes with an aim of ginger crop improvement.

Keywords Ginger, ZoNF-Ys, Inflorescence/Rhizome development, Abiotic stress

Background

Ginger (*Zingiber officinale* Roscoe), a widely used spice and herbal remedy, holds significant importance due to its numerous health benefits and culinary versatility. Rich in bioactive compounds like gingerol, shogaol, and paradol, ginger exhibits potent anti-inflammatory and antioxidant properties. It effectively alleviates nausea and vomiting, especially in pregnancy and chemotherapy patients, and aids in digestion by promoting gastrointestinal motility [1]. However, ginger faces significant challenges in growth and development due to various stress factors such as high temperature, salinity, and dehydration. For instance, while ginger's antioxidant system is activated under high temperature and intense light stress to enhance its stress tolerance, it still struggles to maintain optimal growth conditions [2]. Additionally, although certain stress-responsive genes can improve its growth and photosynthetic efficiency under salinity and drought stress, these conditions still pose significant threats to its overall development [3]. These challenges underscore the resilience and adaptability of ginger, highlighting the importance of ongoing research to improve its cultivation and stress management.

Transcription factors have been shown to play critical roles in regulating plant growth, development, and stress response. Specifically, through the release of the ginger genome sequence [4], several transcription factor families, such as AP2/ERF, GRAS, and CYP [5–7], have been identified as involved in regulating rhizome development. However, this represents only a fraction of the complex transcriptional network. Nuclear factor Y (NF-Y), also referred to as Heme Activator Protein (HAP) and CCAAT-box Binding Factor (CBF), is a heterotrimeric complex composed of three unique subunits: NF-YA (also known as HAP2 or CBF-B), NF-YB (HAP3 or CBF-A), and NF-YC (HAP5 or CBF-C). While NF-Y is widespread among eukaryotes, in plants, each subunit is encoded by multiple genes, whereas in animals and yeast, only one or two genes encode each subunit [8, 9]. The subunits of NF-Y can be identified by their conserved domains. For example, NF-YA contains a core region consisting of two conserved domains (α1 and α2), while NF-YB and NF-YC have conserved domains similar to the Histone Fold Motif (HFM) of H2B and H2A histones, respectively [10]. It is worth noting that these subunits can only regulate transcription when they form heterotrimeric complexes and cannot function independently [11]. In the initial stages, NF-YB and NF-YC form a dimer, including conserved domains, within the cytoplasm. Following their initial dimerization, NF-YB and NF-YC migrate to the nucleus, where they interact with NF-YA to form a transcriptionally-active heterotrimeric complex, which can bind to the CCAAT sequence in promoters and function as a transcription factor [10, 12, 13]. Moreover, NF-Y subunits can form complexes with transcription factors from other families, adding to their ability to regulate the transcription of downstream genes [14, 15]. As a type of combinatorial transcription factor, NF-Y complexes are known to function as regulators of plant growth, development, and stress responses [15–17].

The NF-Y gene family has gained significant attention in recent years for its role in fruit and seed development. One well-known plant NF-Y gene, Arabidopsis LEAFY COTYLEDON 1 (LEC1, AtNF-YB9), has been identified as a critical regulator of embryogenesis and seed development [18–22]. In tomato fruit, a specific subgroup of NF-YB (SlNF-YB8a, SlNF-YB8b, and SlNF-YB8c) can interact with a particular subgroup of NF-YC, forming NF-Y heterotrimers with two distinct NF-YAs, which facilitate flavonoid biosynthesis [17]. Rice LEC1-like transcription factor, OsNF-YB9, has also been identified to interact with SPK and play a role in seed development [23]. Several studies have conducted genome-level analysis to investigate the NF-Y transcription factor family in different fruit development stages across various species. For example, 34 VvNF-Ys have been characterized in the grape genome, and their expression patterns in grape berries at different developmental stages have been explored [24]. In castor bean, 25 *RcNF-Y* genes have been identified, and six candidate genes (including two LEC1-type members, *RcNF-YB2*, and *RcNF-YB12*) have been found to be highly or specifically expressed in endosperms, suggesting their significant role in seed development and storage reservoir accumulation [25]. In peach, 24 PpNF-Y genes have been characterized, and several candidate genes (such as *PpNF-YA4* and *PpNF-YB12*) were found to be highly expressed in fruit, indicating their potential involvement in fruit development regulation [26].

However, there is a lack of basic information regarding the NF-Y transcription factors in *Zingiber officinale*.

The present study aimed to conduct a comprehensive analysis of the NF-Y family in ginger. Specifically, we examined various aspects of the family, such as gene structure, motif composition, chromosomal localization, and the phylogenetic tree. We compared these findings with those of other species, including *Arabidopsis thaliana*, *Solanum tuberosum*, *Oryza sativa*, and *Musa acuminata*, to gain insights into the evolutionary relationships of ginger. Furthermore, we investigated the expression profiles of NF-Y genes in different ginger tissues, with a particular focus on the stage of rhizome expansion and floral organ formation. Our study provides valuable information that can be leveraged to identify candidate NF-Y family genes involved in regulating growth and abiotic stress responses and for the genetic improvement of ginger.

Materials and methods

Plant materials

The seedlings of *Z. officinale* 'LAIWU No.2' utilized in this research were cultivated in the greenhouse located at the Institute of Special Plants, Chongqing University of Arts and Sciences. Prior permission was obtained for collecting the materials, and a specimen (voucher number Li_Z026) was stored at the Herbarium of Chongqing University of Arts and Sciences (www.cqwu.net). Inquiries can be directed to Huamin Liu at liuhuanin@126.com. To investigate the expression patterns of *ZoNF-Y* genes, we collected samples from six-month-old seedlings, including ginger flowers, flower buds, anthocaulus, stems, rhizome buds, 1^{st,} 2nd and 3rd rhizome internodes, functional leaves, rhizome buds, and roots. To explore the involvement of *ZoNF-Y* genes in response to abiotic stresses, we used two-month-old seedlings with five leaves. Our sampling strategy aimed to capture the maximum variation across different developmental stages and under different abiotic stresses. To impose drought and salinity stresses, we watered the ginger plants with a 15% PEG6000 solution and a 200 mM NaCl solution, respectively. Heat and cold stresses were induced by subjecting the plantlets to 40℃ and 4℃, respectively. We collected leaf samples at various time points after stress treatment, including 0, 1, 3, 6, 12, 24, and 48 h for cold, drought, and salt treatments, and 0, 1, 3, 6, 12, and 24 h for heat treatment. The collected samples were immediately frozen in liquid nitrogen and stored at -80℃ for downstream analyses. Our experimental design ensured that we captured the dynamic changes in gene expression in response to different abiotic stresses.

Gene identification and classification

We identified the largest number of NF-Y genes in the ginger genome (as part of our ginger genome research project) using two BLASTP methods. We searched for candidate genes with a score value of ≥100 and e-value of ≤e-10. To verify the presence of conserved domains, we used HMMER v3.0 software (available at https://www.ebi.ac.uk/Tools/Hmmer/) with default parameters, setting the cutoff to 0.01. Additionally, we used the Pfam and SMART databases to confirm the existence of *NF-Y* core sequences. In total, we identified 36 *NF-Y* gene models in the ginger genome for further analysis. To obtain basic information on the identified NF-Y proteins, we employed tools available at the ExPasy website (http://web.expasy.org/protparam/). Our analysis pipeline ensured a thorough identification and confirmation of NF-Y genes in the ginger genome.

Sequence analysis

To investigate the structural differences among ZoNF-Y genes in *Z. officinale*, we analyzed the conserved motifs of NF-Y proteins. We aligned the Zo*NF-Y* protein sequences using ClustalW with default parameters. The exon-intron structure of ZoNF-Y genes was determined using the Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/). To evaluated the conserved motifs of *NF-Y* proteins, we utilized the MEME online program (http://meme.nbcr.net/meme/intro.html) and the Tbtools software, as described by Bailey and Chen in 2015 and 2020 [27, 28], respectively. By employing this analysis pipeline, we gained insights into the structural differences among ZoNF-Y genes in *Z. officinale* through the examination of conserved motifs and gene structures.

Chromosomal distribution and duplication of ZoNF-Y genes

We followed the method outlined by Xing et al. (2021) [5] to map ZoNF-Y genes to ginger chromosomes. To analyze gene duplication events, we utilized the multiple collinear scanning toolkits (MCScanX). Syntenic analysis maps of *NF-Y* synteny relationships were constructed using the Dual Synteny Plotter software, as described by Chen in 2020 [28]. The parameters associated with gene duplication events, such as Ks (synonymous substitution rate) and Ka (nonsynonymous substitution rate), were computed using the TBtools software, as described in reference 31. To determine the date (T) of the duplication events, we used the formula $T=Ks/2\lambda$, where λ represents the estimated clock-like rate of synonymous substitution, which is $1.5\times10-8$ substitutions/synonymous site/year in dicots [29]. Our comprehensive analysis approach enabled us to elucidate the synteny relationships and evolutionary history of ZoNF-Y genes in ginger.

Phylogenetic analysis and classification of the ZoNF-Y gene family

We obtained the NF-Y protein sequences of *A. thaliana* and *O. sativa* from the EnsemblPlants database (http://plants.ensembl.org/index.html). To compare the NF-Y protein sequences from ginger, rice, and Arabidopsis, we performed multiple sequence alignments using MUSCLE with default parameters. The resulting alignments were used to generate a ML (maximum likelihood) phylogenetic tree with partial deletion, 80% cutoff, the JTT+G amino acid substitution model, and 1000 bootstrap replicates, using the MEGA X program. We visualized the resulting ML tree using iTOL (https://itol.embl.de/), following the method described by Xing in 2021 [5]. Our analysis allowed us to compare the evolutionary relationships among NF-Y proteins from different plant species.

Expression analysis of ZoNF-Y genes by RNA-seq and qRT-PCR

We performed RNA-seq analysis using samples collected from plants at 12 h after exposure to cold, heat, drought, and salt treatments. Total RNA was extracted using the TRIzol kit (Invitrogen, USA) following the manufacturers' instructions, and further purified using an mRNA purification kit from Promega (China). We enriched approximately 20 μg of total RNA from each sample using oligo (dT) magnetic beads, digested them into short fragments, and synthesized the first- and second-strand cDNA at BGI (Shenzhen, China). After purification, we ligated the fragments to sequencing adaptors and analyzed them using an Illumina Hiseq 2000 sequencing system. Our RNA-seq approach enabled us to obtain comprehensive gene expression profiles in response to different stress conditions. We employed a rigorous algorithm to identify differentially expressed genes involved in the ginger defense response. To control for false positives, we set the false discovery rate (FDR) at 5% to determine the P value threshold for multiple comparison tests and analyses. We used a P value threshold of <0.001 and an absolute value of log2 ratio >1 to assess the significance of differences in gene expression. The transcriptome data were deposited in the NCBI Short Read Archive (Project Accession Number: SRP064226). To identify the corresponding sequences of ZoNF-Y genes based on the the genome sequence of "Zhugen" cultivar of *Z. officinale*, we designed qRT-PCR primers using Primer Primier 5 software (http://frodo.wi.mit.edu/) (Table S8) and analyzed the spatial and temporal expression patterns and abiotic stress responses of selected ZoNF-Y genes. Our combination of RNA-seq and qRT-PCR allowed us to perform comprehensive analyses of gene expression and validate our findings. The TUB2 gene is constitutively expressed in almost all tissues with little variation in expression levels and is often used as a reliable reference gene. In our study, we used the ZoTUB2 gene as an internal control for qRT-PCR experiments using SYBR Premix Ex Taq II (TaKaRa), which were performed at least three times using a CFX96 Real Time System (Bio-Rad). The qRT-PCR reaction conditions were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Each reaction was conducted in triplicate as three biological replicates. We analyzed the data using the $2^{-\triangle\triangle CT}$ method [30], which allowed us to accurately quantify relative changes in gene expression levels. Our use of a reliable internal reference gene and rigorous qPCR methodology ensured the reliability and reproducibility of our results.

Statistical analysis

We conducted statistical analyses using Sigma Plot v14.0 (SYSTAT software, USA) to analyze all of the collected data. Differences between means were evaluated using the Fisher's least significant difference (LSD) test at a significance level of 0.05 and 0.01, respectively. Our approach to statistical analysis allowed us to accurately interpret and present the experimental results with reliable statistical support.

Results

Genome-wide identification of the NF-Y family in ginger

After eliminating redundant sequences, we identified a total of 36 ZoNF-Y genes in the ginger genome. These genes were categorized into three subfamilies: 10 *ZoNF-YAs*, 16 *ZoNF-YBs*, and 10 *ZoNF-YCs* (Table S1). For clarity, we named the ZoNF-Y genes based on their subfamily branch and chromosome locations, using the format *ZoNF-YA1 to ZoNF-YA10*, *ZoNF-YB1 to ZoNF-YB16*, and *ZoNF-YC1* to *ZoNF-YC10* (Fig. 1). The distribution of the identified 36 ZoNF-Y genes was uneven across 11 chromosomes (Fig. 1 and Table S1). Chromosomes 06 and 11 contained the largest number of ZoNF-Y genes, with a total of seven genes located on these two chromosomes. Conversely, chromosome 01 contained no ZoNF-Y genes, representing the lowest number of ZoNF-Y genes (Figure S1). Furthermore, we observed high densities of ZoNF-Y genes located at the proximal end of chromosome 06 and the distal end of chromosome 11. Our clear nomenclature and detailed chromosomal distribution analysis provided insight into the genomic organization and diversity of the ZoNF-Y gene family in ginger.

The analysis conducted indicated that ZoNF-YC3, consisting of a mere 141 amino acids (aa), was the smallest protein among the 36 ZoNF-Y proteins, whereas ZoNF-YB3, with 713 aa, was identified as the largest protein. The protein molecular weight (MW) ranged from 15.40066 kDa (ZoNF-YC3) to 78.15038 kDa (ZoNF-YB3), and the isoelectric points (pIs) ranged from 4.67 (ZoNF-YB4) to 10.13 (ZoNF-YA9) (Additional file Table S1). Our detailed analysis of gene characteristics provides

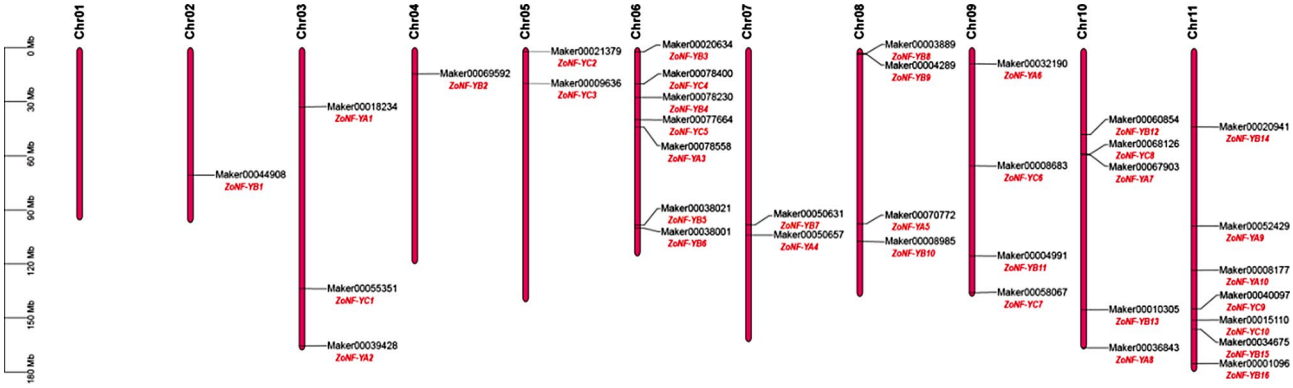

Fig. 1 Genomic distributions of 36 ZoNF-Y genes on 11 ginger chromosomes

important insights into the diversity of the ZoNF-Y protein family in ginger.

Multiple sequence alignment, phylogenetic analysis, and classification of ZoNF-Y genes

The ZoNF-Y proteins contain conserved domains and relatively less conserved N- or C-terminal transcriptional regulation regions (Fig. 2). Specifically, ZoNF-YA proteins contained a conserved region comprising two subdomains for the NF-YB/C interaction and DNA binding (Fig. 2A). In the core domains of ZoNF-YB and ZoNF-YC members, we found conserved regions for DNA binding and protein-protein interactions, which included the HFM of the H2B and H2A, respectively (Fig. 2B and C). Our analysis provides new insights into the conserved domains and structural characteristics of the ZoNF-Y protein family in ginger.

As shown in Fig. 3, the NF-Ys were divided into three distinct subclades: NF-YA, NF-YB, and NF-YC. Specifically, 10 ZoNF-Ys, 11 OsNF-Ys, and 10 AtNF-Ys belonged to the NF-YA subunit, while 14 ZoNF-Ys, 11 OsNF-Ys, and 12 AtNF-Ys were assigned to the NF-YB subunit. Furthermore, 11 ZoNF-Ys, 12 OsNF-Ys, and 13 AtNF-Ys belonged to the NF-YC subunit. Interestingly, we found that ZoNF-YB14, ZoNF-YB15, and AtNF-YB11 were distinct from the NF-YB subfamily and clustered with the NF-YC subfamily (Fig. 3). Our results reveal that NF-Y genes are grouped into three subfamilies, NF-YA, NF-YB, and NF-YC, in ginger.

Gene structure and motif composition of the ZoNF-Y family

The intron and exon structure of ZoNF-Y genes was obtained by comparing their genomic DNA sequences (Table S1). Our findings revealed that all ZoNF-YA genes contained introns, with five *ZoNF-YAs* containing four introns, four *ZoNF-YAs* containing four introns, and one *ZoNF-YA* containing nine introns. While six *ZoNF-YBs* and three *ZoNF-YCs* lack introns entirely, the remaining *ZoNF-YBs* and *ZoNF-YCs* have 1–16 introns. Overall, the gene structure of *ZoNF-YA* members is more conserved when compared to *ZoNF-YBs* and *ZoNF-YCs*.

Conserved motifs found in TF protein sequences have been shown to potentially act as DNA binding sites and activate gene expression. To gain further insight into the characteristics of ZoNF-Y proteins, we analyzed the motifs of the 36 ZoNF-Y genes using the online MEME software (Fig. 4 and Figure S2). Our analysis identified a total of 10 conserved motifs in the ZoNF-Y proteins (Fig. 4b). The distribution of motifs in the three ZoNF-Y subunits was found to be unique (Fig. 4c). Motifs 3, 5, and 7 were present in nearly all ZoNF-Y proteins, with Motif 5 exclusively found in ZoNF-YA members. Motifs 1, 2, 6, and 10 were conserved in ZoNF-YB proteins, with Motif 6 exclusively present in ZoNF-YB members. ZoNF-YC proteins showed a different distribution, with Motifs 3 and 1 present in one subclade (*ZoNF-YC5*, *6*, *8*, *9*) and Motifs 8, 4, and 9 present in another subclade (*ZoNF-YC1*, *2*, *3*, *4*, *7*, *10*). Motif 2 was common to both ZoNF-YA and ZoNF-YB subunits, while Motif 3 was present in both ZoNF-YB and ZoNF-YC subunits. Taken together, each subunit had a distinct set of conserved motifs.

Analysis of cis-acting elements of ZoNF-Y promoters

The detected elements include various hormone response elements (Fig. 5), such as ABRE (which responds to abscisic acid), GARE-motif, P-box, and TATC-box (which respond to gibberellin), TCA-element and SARE (which respond to salicylic acid), CGTCA-motif and TGACG-motif (which respond to MeJA), and TGA-element, TGA-box, AuxRR-core, AuxRE, and TGA-element (which respond to auxin). Additionally, to investigate abiotic stress response elements, we identified the presence of MBS (which responds to drought), LTR (which responds to low temperatures), WUN-motif (which responds to wound), TC-rich repeats (which respond to defense and stress), DRE (which responds to dehydration, low temperature, and salt stress), and GC-motif (which is specific to anoxic inducibility). We also observed various light-responsive elements, such as TCCC-motif,

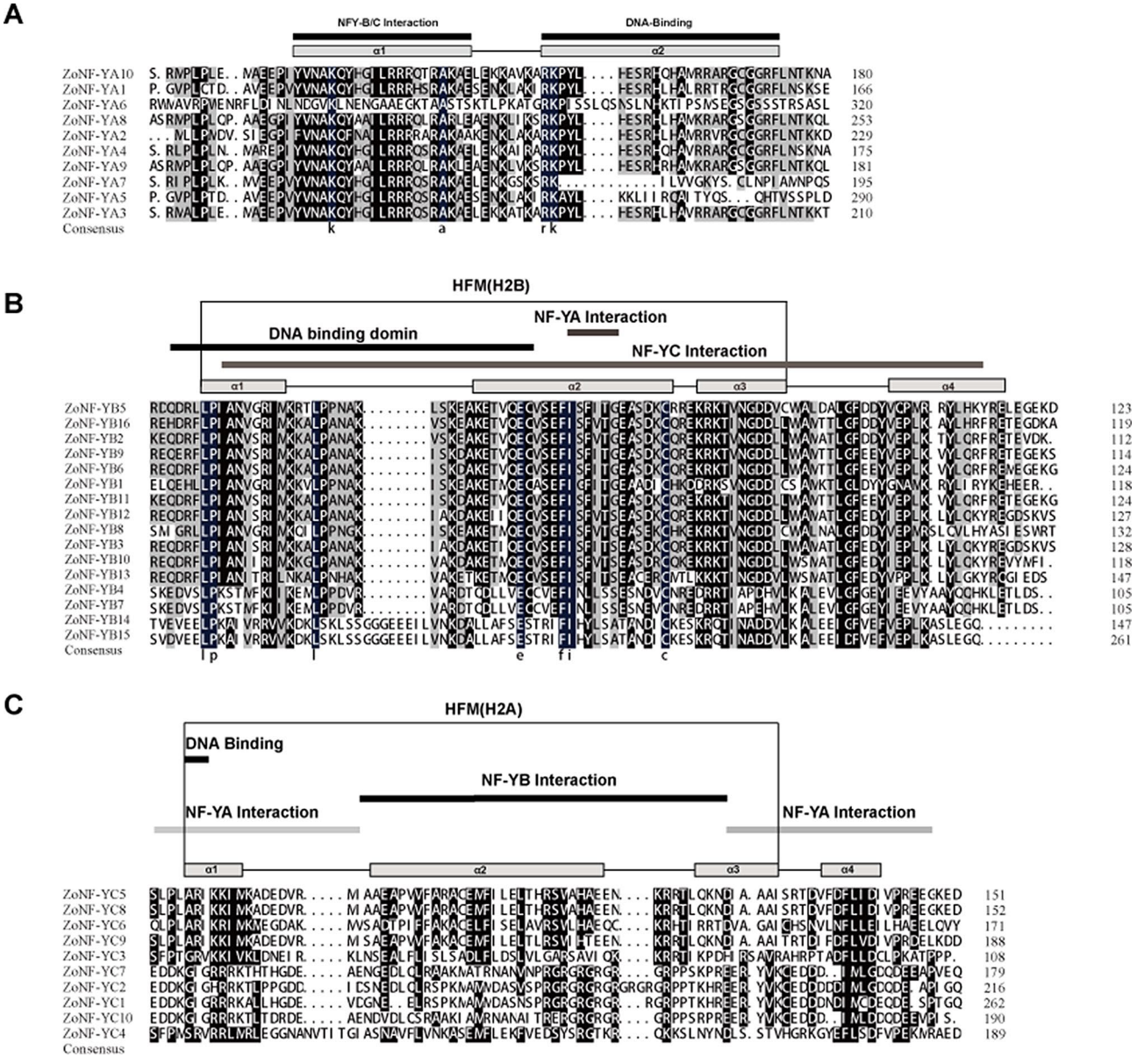

Fig. 2 Alignments of ginger NF-Y domains. (**A**) ZoNF-YA subfamily; (**B**) ZoNF-YB subfamily; (**C**) ZoNF-YC subfamily

GATA-motif, chs-CMA1a, G-box, MRE, Box 4, Gap-box, TCT-motif, GA-motif, AE-box, LAMP-element, Box II, ATCT-motif, chs-CMA2a, I-box, AT1-motif, ATC-motif, ACE, chs-Unit 1 m1, ACA-motif, chs-CMA2b, L-box, CAG-motif, GTGGC-motif, GT1-motif, 3-AF1 binding site, Sp1, AAAC-motif, GATT-motif, TGGCA, sbp-CMA1c, 4 cl-CMA2b, Pc-CMA2a, and LS7. Additionally, we observed the presence of certain response elements in ZoNF-Y family genes, including GCN4-motif (related to endosperm expression), ARE (linked to anaerobic induction), circadian (associated with circadian control), MBSI (regulating flavonoid biosynthetic genes), CAT-box and NON-box (regulating meristem expression), motif I (regulating root-specific expression), and RY-element (regulating seed-specific expression). Additionally, we observed a higher frequency of TC-rich repeats, TGA-element, ARE, ABRE, G-box, Box 4, LTR, CGTCA-motif, and CGTCA-motif in the cis-regulatory elements of most ZoNF-Y genes. The cis-element patterns within the *ZoNF-Y* gene family differ, and we observed a unique pattern in *ZoNF-YA9* and *ZoNF-YB4* consisting of the WUN-motif associated with wound responsiveness. The RY-element, a seed-specific regulation cis-element, was present solely in *ZoNF-YA8* and *ZoNF-YB4*.

Additionally, the CAT-box was found in the cis-regulatory regions of several ZoNF-Y genes involved in meristem regulation, including *ZoNF-YA4*, *ZoNF-YA6*, *ZoNF-YB15*, *ZoNF-YB2*, *ZoNF-YB3*, *ZoNF-YB4*,

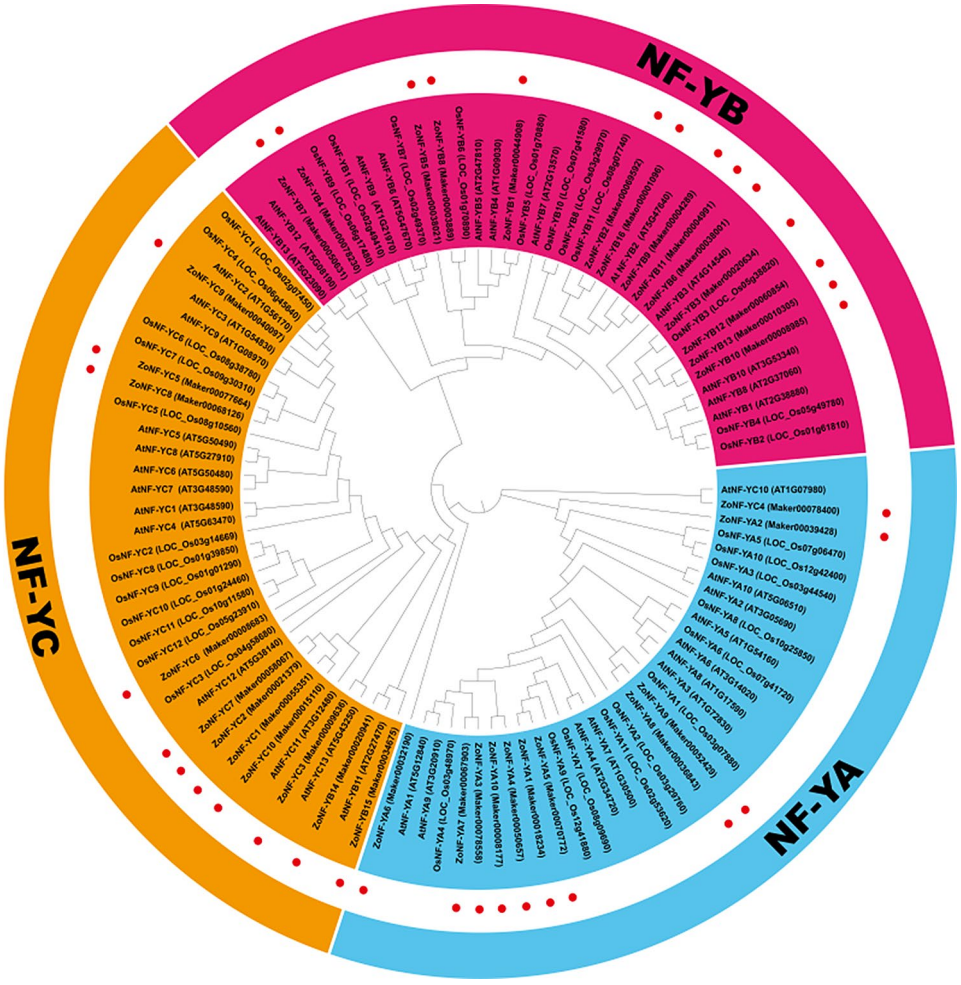

Fig. 3 Unrooted phylogenetic tree representing the relationships among NF-Y proteins of ginger, rice and Arabidopsis. The different colored arcs indicate different groups of the NF-Ysubfamily. NF-Y proteins from ginger with the prefix "Zo" indicate "*Zingiber officinale*"

ZoNF-YB6, *ZoNF-YB9*, *ZoNF-YC4*, and *ZoNF-YC6*. Furthermore, the MBSI flavonoid biosynthetic gene regulation cis-element was detected in *ZoNF-YB7* and *ZoNF-YC7*. The MYB binding site MBS element, which is involved in drought-inducibility, was detected in the cis-regulatory regions of various ZoNF-Y genes, including *ZoNF-YA1*, *ZoNF-YA3*, *ZoNF-YA4*, *ZoNF-YA5*, *ZoNF-YA6*, *ZoNF-YA7*, *ZoNF-YB1*, *ZoNF-YB11*, *ZoNF-YB14*, *ZoNF-YB2*, *ZoNF-YB4*, *ZoNF-YB6*, *ZoNF-YB8*, *ZoNF-YB9*, *ZoNF-YC1*, *ZoNF-YC3*, *ZoNF-YC4*, *ZoNF-YC6*, *ZoNF-YC7*, *ZoNF-YC8*, and *ZoNF-YC9*.

Gene duplication analyses of ZoNF-Y genes

According to Cannon et al. (2004) [31], duplications, both tandem and segmental, are essential for expanding gene families and creating new functions. In this study, we did not identify any tandemly duplicated pairs of ZoNF-Y genes, however, we found 16 segmental duplication events (Fig. 6 and Table S3). Genes replication plays a critical role in the expansion of gene family and the discovery of novel functions. In the ginger genome, we examined the tandem duplication events of NF-Y genes using the criterion of two or more genes located in a 200-kb chromosomal region. Our analysis revealed no tandem duplication events of NF-Y genes in the ginger genome. Instead, we identified 16 pairs of segmentally duplicated genes, indicating a high level of conservation within the NF-Y gene family. Analyzing homologous protein families is crucial for establishing species kinship and predicting functions of new protein sequences. Several homologous genes were found on different chromosomes in ginger, suggesting the high conservation of NF-Y gene family (Fig. 7). Based on these results, it is possible that some NF-Y genes originated from the 16 segmental duplication events, indicating that duplication events are the key driving force shaping ZoNF-Y gene evolution.

Evolutionary analysis of ZoNF-Y genes

In order to explore the phylogeny of the ginger NF-Y family, we created four comparative syntenic maps. These

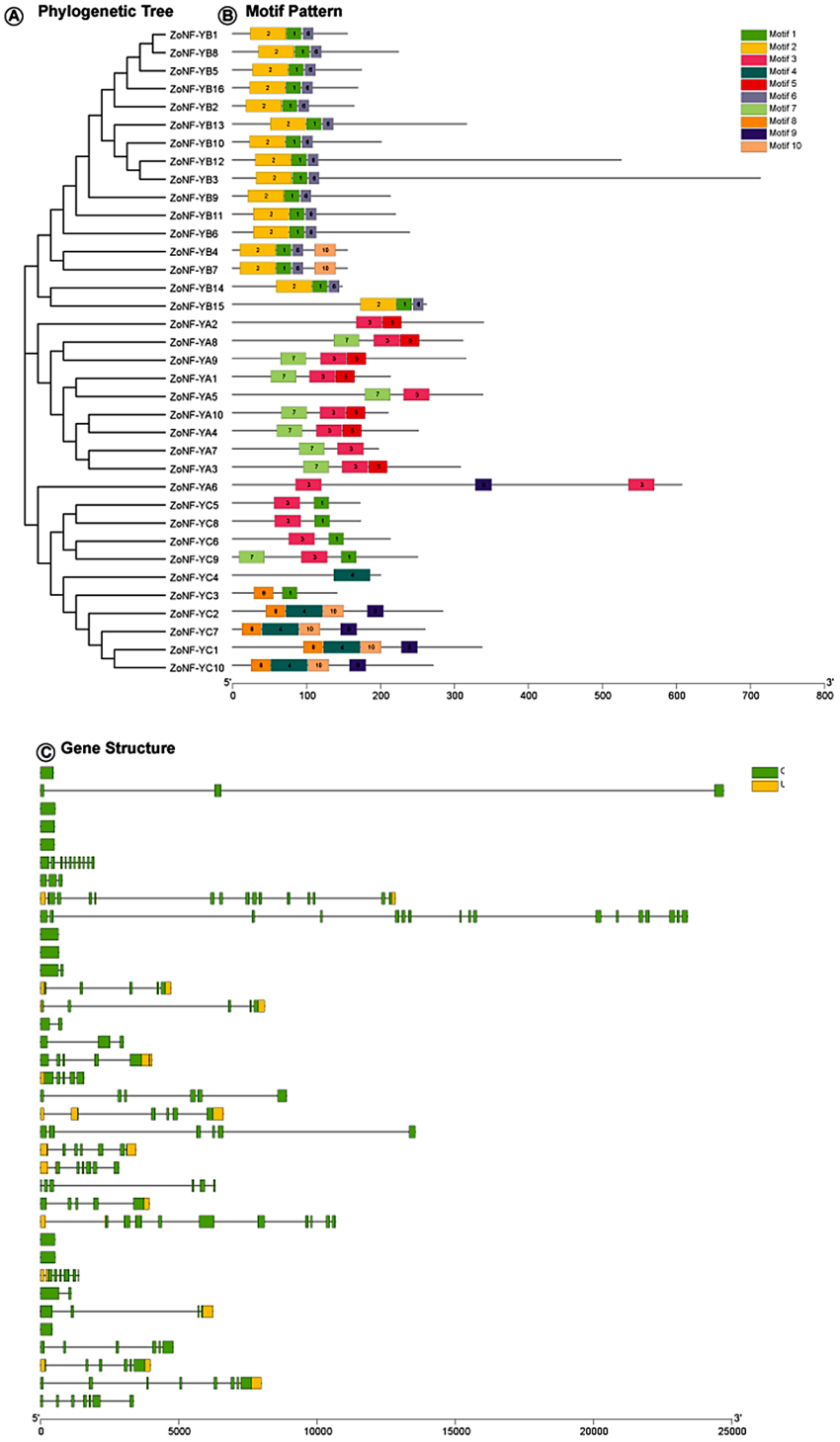

Fig. 4 Phylogenetic relationships, gene structure, and conserved protein motifs in NF-Y genes from ginger. (**a**) The phylogenetic tree displays the full-length sequences of ginger NF-Y proteins and is generated using the MEGA X software. (**b**) The motif composition of ginger NF-Y proteins is indicated with different colored boxes labeled as numbers 1–10, and sequence information for each motif is provided in Additional file: Fig. S2. (**c**) The exon-intron structure is depicted with 5′- and 3′- untranslated regions highlighted in yellow boxes, exons in green boxes, and introns represented by black lines. The protein length scale is provided at the bottom for estimation

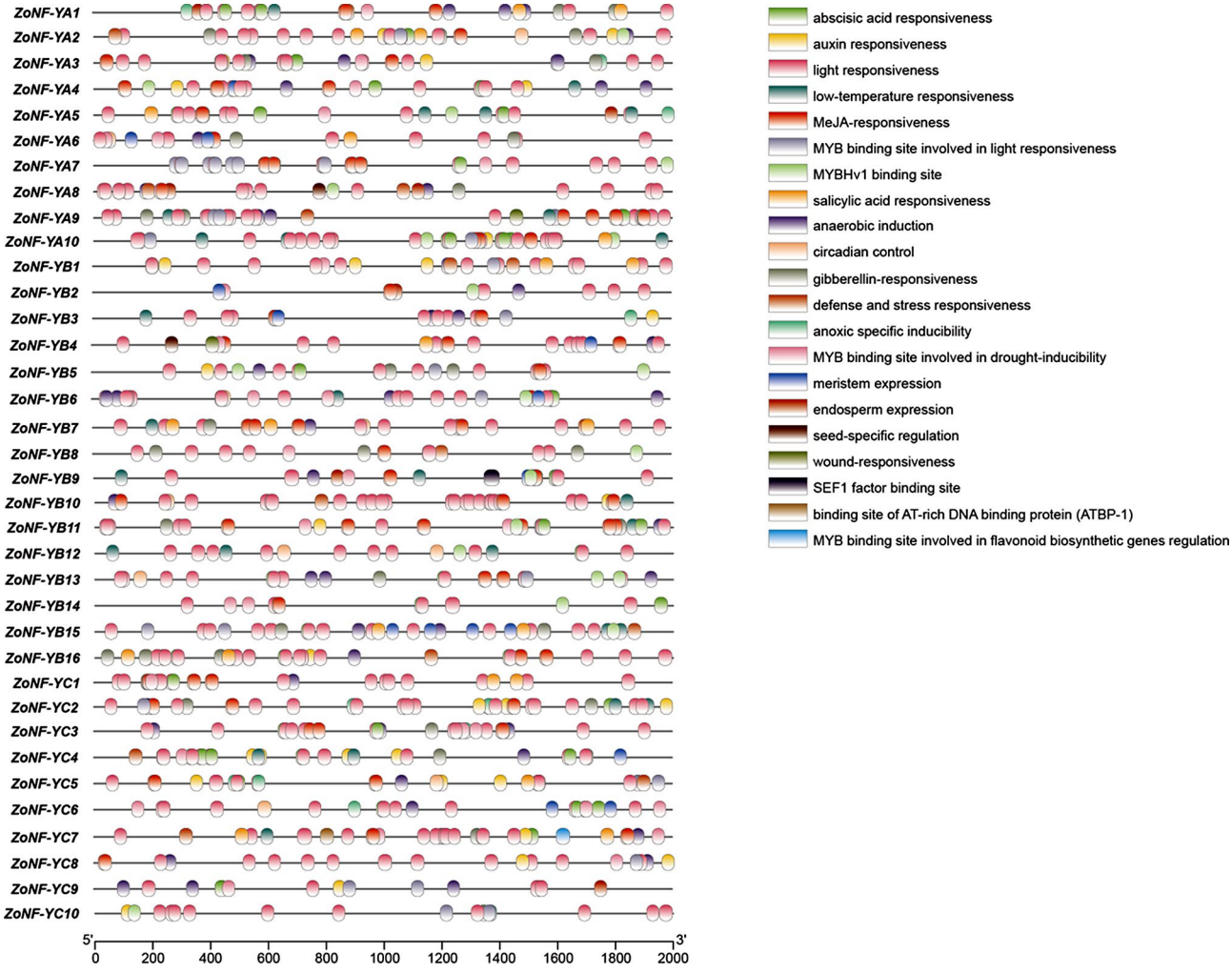

Fig. 5 *Cis*-acting elements of *ZoNF-Y* promoters. Different colors represent different types of *cis*-acting elements

maps allowed us to examine the genetic relationships between ginger and four other important plant species: two dicots (Arabidopsis and potato) and two monocots (banana and rice). The results indicated that 23 ZoNF-Y genes showed significant syntenic relationships with banana, followed by rice (1), potato (0), and Arabidopsis (0) (Fig. 7 and Table S4). We analyzed the orthologous pairs between ginger and the four other plant species (banana, rice, potato, and Arabidopsis) and found that there were 32, 1, 0, and 0 pairs, respectively. Some ZoNF-Y genes, including *ZoNF-YA6*, *ZoNF-YC1*, *ZoNF-YC2*, and *ZoNF-YC10*, were associated with at least three syntenic gene pairs between ginger and banana NF-Y genes. This suggests that these genes may have played an important role in the evolution of the NF-Y gene family. The presence of NF-Y genes was confirmed in Arabidopsis, but no syntenic gene pairs were identified between ginger and Arabidopsis.

The presence of more than 80 collinear genes in highly conserved syntenic blocks between ginger and banana was observed to be significant. On the other hand, in the case of ginger and rice, all collinear genes were present in syntenic blocks containing fewer than 50 orthologous gene pairs. Although fewer gene pairs were identified between ginger and Arabidopsis, they did not include any NF-Y family genes. This observation can be attributed to the recent whole genome duplication events in the ginger genome, which we propose as the primary reason behind these findings.

To explore the evolutionary constraints affecting the NF-Y gene family, we analyzed the Ka/Ks ratios of the NF-Y gene pairs (Table S5). Our results indicated that the ginger NF-Y gene family may have undergone significant purifying selective pressure during evolution since almost all orthologous NF-Y gene pairs and segmentally duplicated ZoNF-Y gene pairs had Ka/Ks values of <1.

To support and substantiate the previous phylogenetic grouping and motif distribution, we conducted a syntenic analysis, which yielded reliable evidence. Our analysis showed that the ginger NF-Y gene family is remarkably

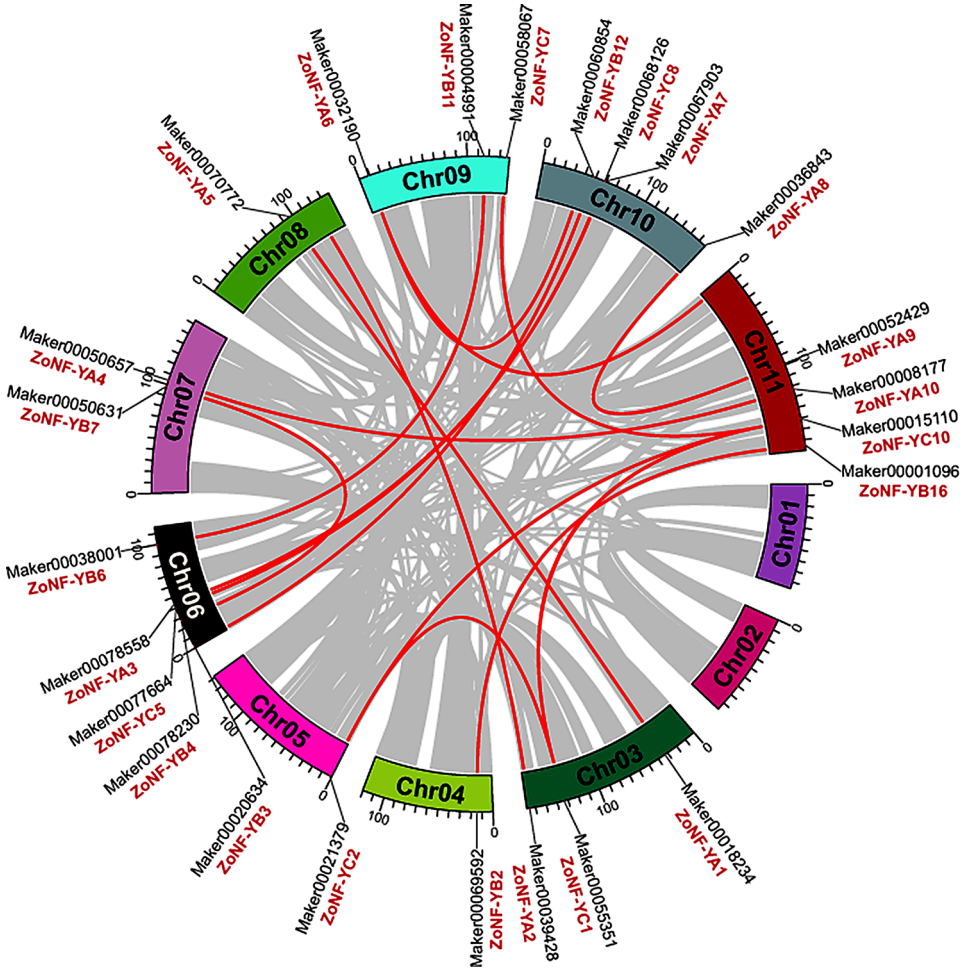

Fig. 6 Schematic representation of the inter-chromosomal relationships among ginger's NF-Y genes. The red lines indicate the duplicated pairs of NF-Y genes in ginger. The chromosome number of each chromosome is shown in the middle

conserved, and the ginger NF-Y genes are more closely related to the *M. acuminata* genes than to the *O. sativa* genes. Based on these findings, it is possible that the NF-Y genes evolved from a common ancestor that existed in various plant species.

Expression profiling of ginger NF-Y genes in different tissues

In order to explore the possible roles of NF-Y genes in varying developmental stages of ginger tissues/organs, we assessed their expression patterns using RNA-seq data (Fig. 8 and Table S6). Our findings showed that four ZoNF-Y genes (*ZoNF-YB7*, *ZoNF-YB4*, *ZoNF-YC5* and *ZoNF-YC8*) exhibited high expression levels in all tissues, with FPKM values > 5. Three ZoNF-Y genes (*ZoNF-YA1*, *ZoNF-YB1* and *ZoNF-YC10*) showed no expression in any of the tissues (FPKM = 0). Moreover, some ZoNF-Y genes exhibited tissue-specific expression patterns. For instance, *ZoNF-YA9* and *ZoNF-YB16* were highly transcribed in the 3rd rhizome internode, while *ZoNF-YA7*, *ZoNF-YB10* and *ZoNF-YC1* were markedly expressed in the mature flower.

To ensure the accuracy and consistency of our transcriptome data, we carried out qRT-PCR experiments on eight representative samples, comprising nine NF-Y genes (Fig. 9). Our results demonstrated that out of the 36 ZoNF-Y genes, four genes (*ZoNF-YA1*, *ZoNF-YB1*, *ZoNF-YB3*, and *ZoNF-YC10*) exhibited no expression in any of the tested samples. This suggests that these genes may be pseudogenes or have unique temporal or spatial expression patterns that were not captured in our data. Furthermore, 20 ZoNF-Y genes were expressed in all 12 tested samples (FPKM > 0), and 12 ZoNF-Y genes displayed constitutive expression with FPKM values greater than one across all samples. Additionally, some genes demonstrated preferential expression patterns in the examined tissues. The transcript expression levels of *ZoNF-YA8* and *ZoNF-YB5* were highest in roots, *ZoNF-YA7*, *ZoNF-YB10*, *ZoNF-YB13*, *ZoNF-YC1*, and *ZoNF-YC7* in mature inflorescence, *ZoNF-YB6*, *ZoNF-YB9*, and *ZoNF-YB15* in flower petiole, and *ZoNF-YA4* in the

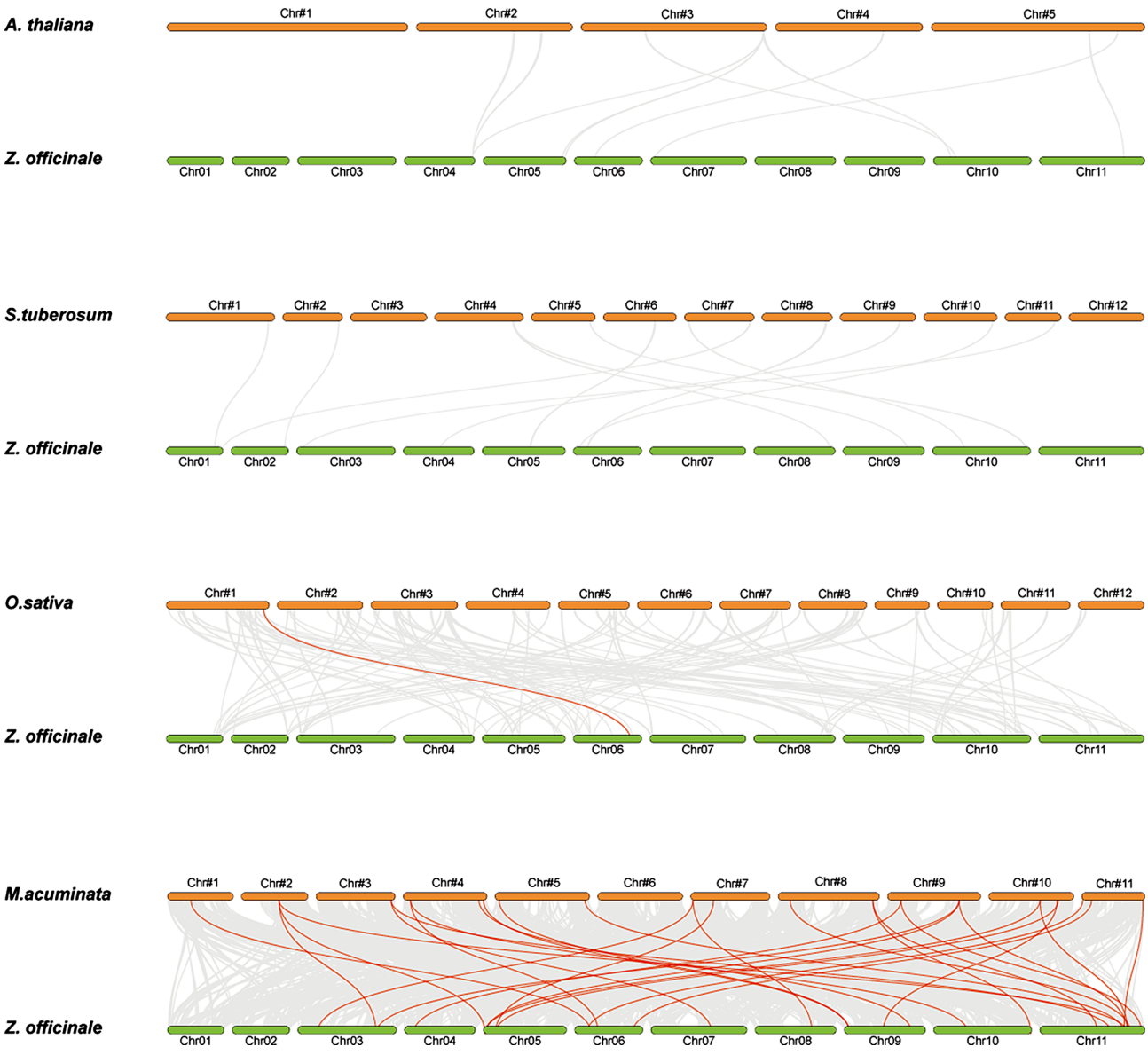

Fig. 7 Synteny analysis of NF-Y genes between ginger and four representative plant species. The gray lines in the background represent the collinear blocks between the ginger genome and those of the other plants. The red lines highlight the syntenic NF-Y gene pairs

basal stem. Interestingly, some genes displayed significant expression trends across different developmental stages. Specifically, during the ginger rhizome development (rhizome bud, first inter-node, second inter-node, third inter-node), the expression levels of *ZoNF-YB12* and *ZoNF-YC6* gradually increased, while the transcripts of *ZoNF-YA8/C3/C5* decreased. Moreover, the expression levels of *ZoNF-YA4/B4/B7/B10/C1/C7* gradually increased during flower development (Fig. 8).

Using qRT-PCR, we investigated gene expression in 12 different tissues by randomly selecting 9 ZoNF-Y genes. Our results revealed that *ZoNF-YB15* was more abundant in the pedicel compared to other tissues. On the other hand, *ZoNF-YB2* and *ZoNF-YC1* showed higher expression in mature inflorescence than in other tissues. *ZoNF-YB12* and *ZoNF-YC6*, however, exhibited higher abundance in the first internode, second internode, and third internode than in other tissues (Fig. 9). Our findings are consistent with the RNA-seq data.

Expression patterns of NF-Y genes in response to abiotic stress treatments

To explore the potential functions of NF-Y genes under various abiotic stresses, we analyzed their expression levels under cold, heat, drought, and salt treatments using RNA-seq data. Out of the 36 ZoNF-Y genes, only one showed no expression in any of the four treatments. In contrast, the other 35 ZoNF-Y genes were induced by

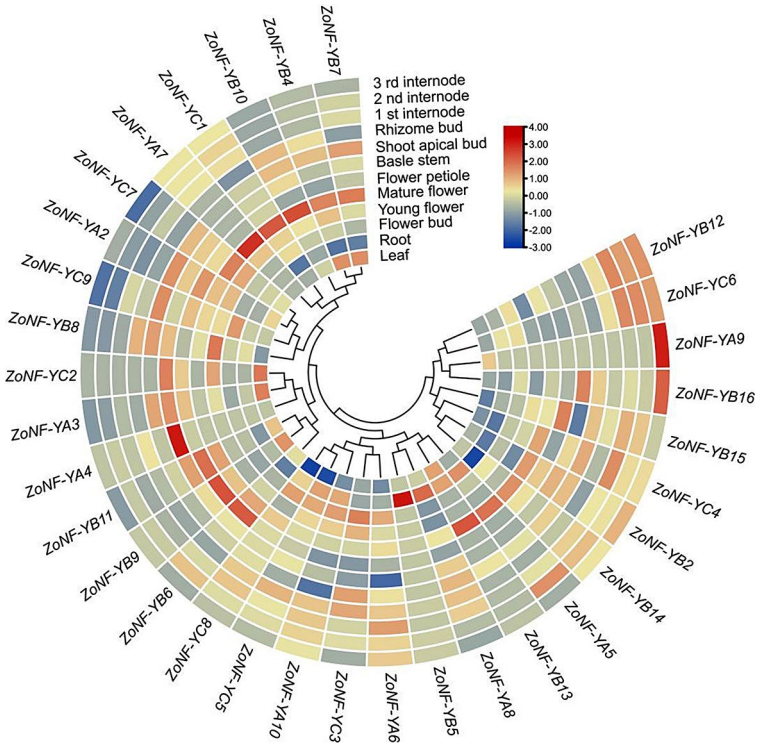

Fig. 8 Expression profiles of the ginger NF-Y genes. Hierachical clustering of expression profiles of ginger NF-Y genes in 12 samples including different tissues and developmental stages

at least one of the stress treatments (Fig. 10). Additionally, we observed that 18, 24, 12, and 11 Zo*NF-Y* members were down-regulated by cold, drought, heat, and salt treatments, respectively (Table S7). Notably, ZoNF-YB5 was reduced under all four treatments, suggesting that ZoNF-Y genes may be involved in signaling pathway crosstalk in response to different abiotic stresses, or that the functions of different genes are complementary.

To investigate further, we randomly selected 13 genes and analyzed their expression levels under cold, heat, salt, and drought stress treatments using qRT-PCR. Our results showed that all 13 genes were significantly induced by stress at one or more time point/s (Fig. 11), which was consistent with the RNA-seq data at 12 h post-treatment. Notably, the gene response was delayed in salt conditions compared to drought conditions, with gene expression levels gradually increasing over time and peaking at 12–24 h under salt stress. In contrast, genes responded immediately to drought, with expression levels peaking at 3–1 h after treatment (Fig. 11).

Discussion

The NF-Y genes comprise a large family of TFs that are ubiquitous in all plant species and play vital roles in various physiological processes in eukaryotes. Each NF-Y subunit is encoded by multiple genes in plants [9]. Genome-wide analyses of *NF-Y* family genes have been widely conducted in many species with sequenced genomes, such as Arabidopsis, grape, tomato, banana, rice and peach [24, 26, 29, 32–34]. However, no study has investigated the NF-Y genes in ginger. In this study, a search for NF-Y genes in the ginger genome resulted in the identification of 36 members, including 10 ZoNF-YA subfamily members, 16 ZoNF-YB subfamily members, and 10 ZoNF-YC subfamily members. Similar numbers of NF-Y genes were found in other plants. For instance, in Arabidopsis, 28 NF-Y genes were identified, while in *Brassica rapa* and *Oryza sativa*, 39 and 52 NF-Y genes were identified, respectively. The significant differences in gene numbers could be due to variations in genome size and intrinsic genetic diversity of each species. Furthermore, an increase in the number of NF-Y genes might indicate their involvement in functional diversification and adaptive evolution, particularly in response to biotic and abiotic stresses.

The gene structure of ZoNF-Y genes was assessed in this study. As depicted in Figs. 2b and 25.00% (9/36) of ZoNF-Y genes had no introns, while the number of introns in the NF-Y subfamily genes ranged from 1 to 16. The gene structure of NF-Y genes in ginger was found to be similar to that in Arabidopsis, Tartary buckwheat and tomato [32, 35–37]. The disparity in the structure between the ZoNF-YA subfamily and ZoNF-YB subfamily genes supports significant divergence in genome evolution.

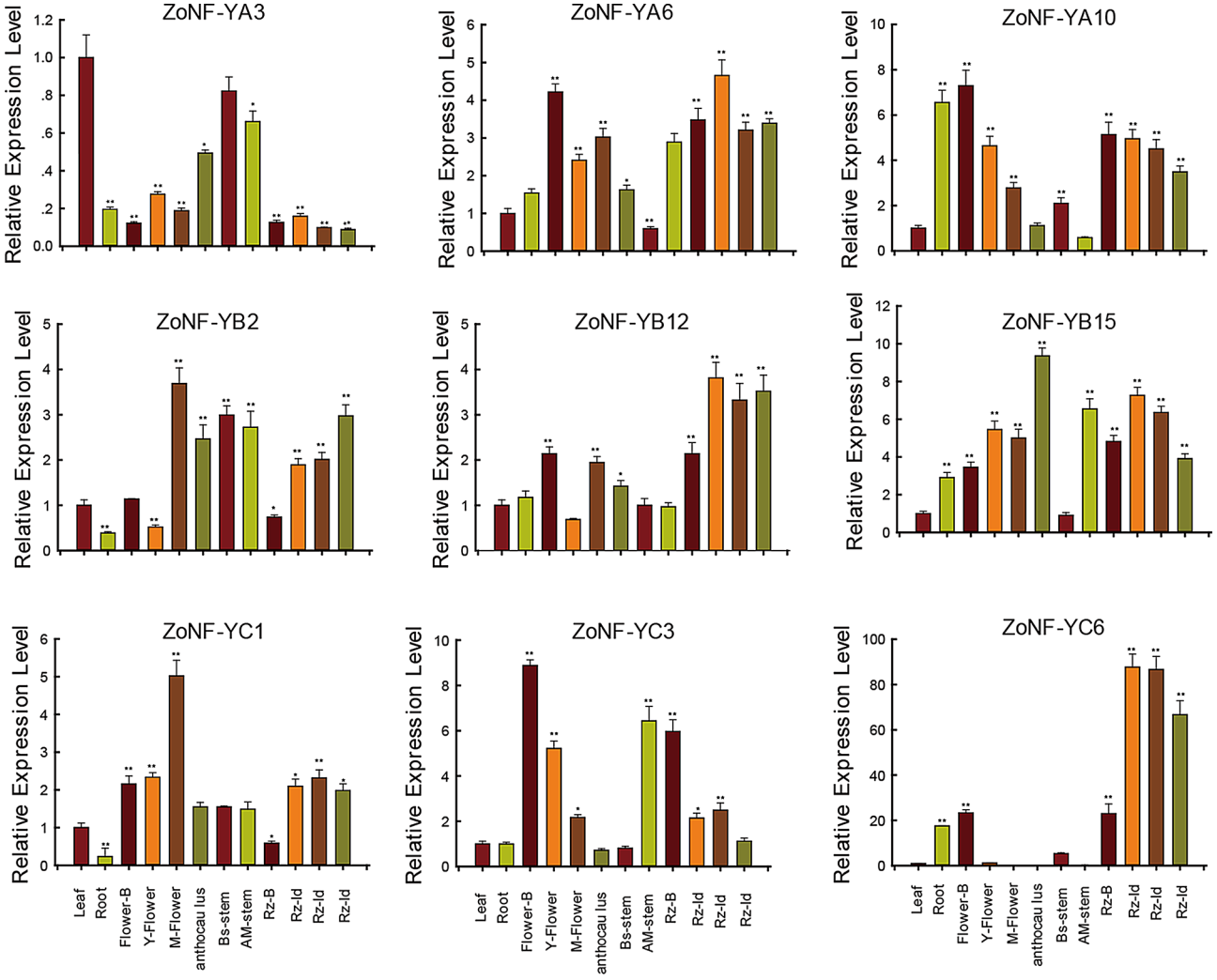

Fig. 9 Expression analysis of 9 NF-Y genes in 12 samples by qRT-PCR. Data were normalized to *TUB-2* gene, and vertical bars indicate standard deviation. Values are presented as means ± SD ($n = 3$). (* $p < 0.05$, ** $p < 0.01$, Student's *t*-test)

The number and type of motifs also represent the structural basis of gene functional diversity. The domains and motifs of TFs are often associated with DNA binding, protein interaction and transcriptional activities [9]. Motif analysis revealed that motif 5 was exclusively present in ZoNF-YA members, while Motifs 6 was only found in ZoNF-YB members. In one subclade of ZoNF-YC proteins (*ZoNF-YC5*, *6*, *8*, *9*), motifs3 and 1 were detected, whereas another subclade of ZoNF-YC proteins (*ZoNF-YC1*, *2*, *3*, *4*,*7*,*10*) contained motif-s 4,8, and 9 (Fig. 4). These results suggest that different motifs play important roles within specific subfamily. Multiple alignments demonstrated that ZoNF-Y proteins prosses conserved regions that are highly similar across animals and plants [32]. Analysis of ZoNF-YB and ZoNF-YC protein sequence alignments indicated the presence of a histone fold motif (HFM) in these domains (Fig. 2B, C). HFM shares low sequence identity but exhibits high structural similarity to all histone proteins, consisting of a short α-helix (α1) followed by a β-strand segment, a long helix (α2), another β-strand, and a short a-helix [35, 38].

The amplification of chromosomal fragments, individual genes or whole genomes has been widely recognized as one of the primary drivers of evolution, contributing to the generation of new functions and expression patterns [39, 40]. In our study of ginger, we observed a distinctive expansion of the ZoNF-Y family, primarily resulting from fragmentary duplications (Figs. 1 and 6, Table S3). Most of the duplicated ZoNF-Y genes were expressed in various tissues or organs, indicating their specific or redundant cellular roles during ginger development. Moreover, the diversity in gene function could result in differences in the expression patterns of gene pairs, as exemplified by the distinct expression of *ZoNF-YA8* in roots compared to *ZoNF-YA9* in the 3rd internode of ginger rhizome. Interestingly, despite the dissimilar expression patterns,

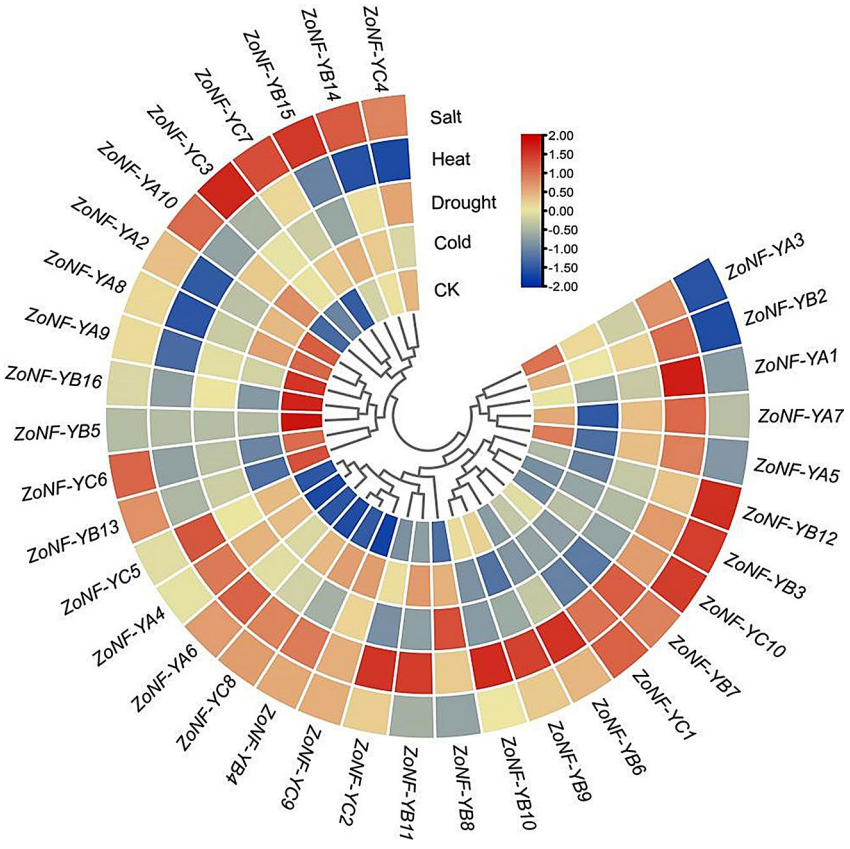

Fig. 10 Expression profiles of *ZoNF-Y* genes under abiotic stress treatments

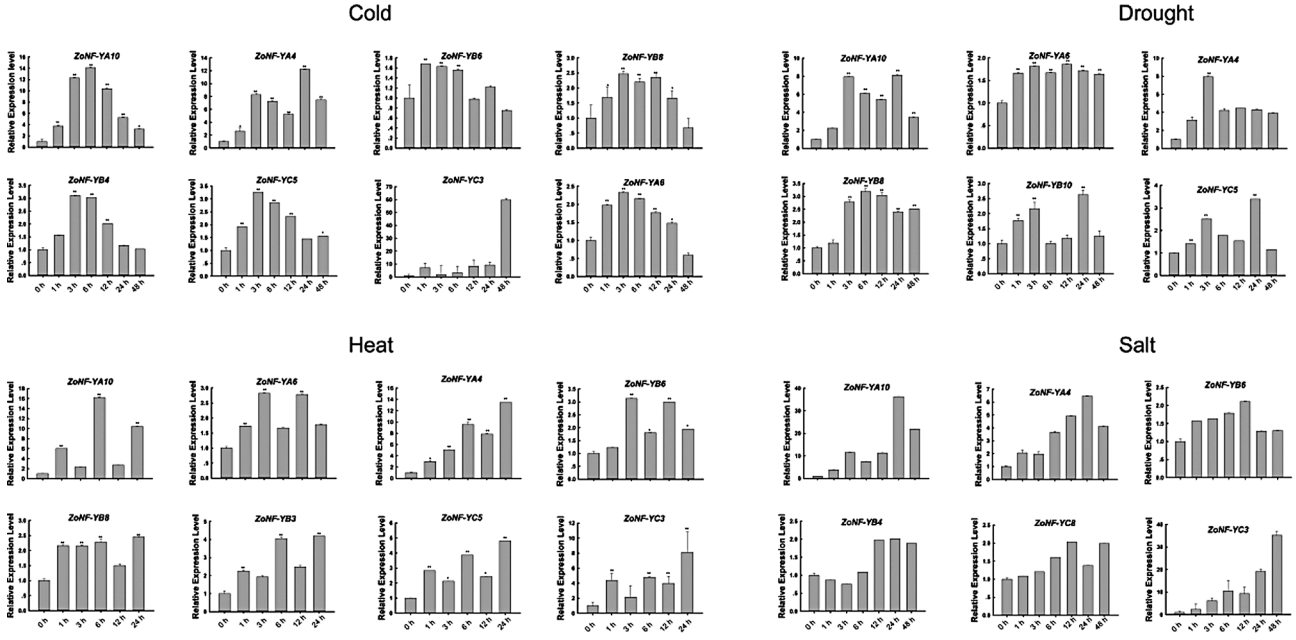

Fig. 11 Expression analysis of NF-Y genes under abiotic stresses by qRT-PCR. Data were normalized to *TUB-2* gene, and vertical bars indicate standard deviation

their motifs (Motif-7, Motif-3, Motif-5) exhibited identical arrangements and content (Fig. 4), suggesting that that the functional variations between *ZoNF-YA8* and *ZoNF-YA9* could be attributed to gene mutations during duplication. Notably, *ZoNF-YA7* exhibited the highest RNA concentration in fully matured flowers, whereas *ZoNF-YA3* displayed high expression levels in both the basal stem and shoot apical buds (Table S6). *ZoNF-YA7* shared Motif-7 and Motif-3 with *ZoNF-YA3*, while ZoNF-YA3 possessed Motif-7, Motif-3, and Motif-5 (Fig. 4). Therefore, it is reasonable to speculate that modifications in the motifs of these two genes during duplication may have led to their functional discrepancies.

The prediction of gene functions often relies on interpreting phylogenetic analyses and examining gene expression profiles. These methods frequently utilize the known functionalities of homologs from different species [36, 41]. Based on tissue-specific expression patterns, the majority of NF-Y genes (Fig. 8) were detected in all examined tissues. *ZoNF-YB8* exhibited higher expression levels in young flowers, shoot apical buds, and rhizome buds. Its rice homolog, *Os-NF-YB7*, plays significant roles during the development of both floral and vegetative meristems [9, 42]. Overexpression of *At-NF-YB2* in *A. thaliana* leads to accelerated primary root elongation due to faster cell elongation and/or division [43]. In this investigation, root tissue demonstrated heightened *ZoNF-YB5* expression, a homolog of *At-NF-YB2*. The transition from vegetative to reproductive growth has been extensively studied in model plants like *A. thaliana*, where NF-Y genes have been identified as regulators of flowering [44, 45]. In our study, 21 ZoNF-Y genes showed significant expression during inflorescence development. Phylogenetic analysis revealed that highly expressed NF-Y genes in the flower petiole (*ZoNF-YB6*, *ZoNF-YB9*, and *ZoNF-YB11*), were co-clustered (Fig. 1). Their *A. thaliana* homologs, *At-NF-YB2* and *At-NF-YB3*, are essential for flowering promotion under prolonged light exposure [46, 47]. NF-Y's role in flowering time has also been reported in monocots as well. Rice *NF-YB11*, known as *DTH8/Ghd8/LHD1*, acts as a suppressor in the photoperiodic flowering signal network by reducing the expression of multiple floral regulators during unfavorable long-day situations [48–50]. Therefore, *ZoNF-YB6/9/11* might be involved in the regulation of flower development. It is essential to verify whether *ZoNF-YB6/9/11* can stimulate inflorescence onset and growth in ginger.

The rhizome serves as the primary economic organ of ginger, as it contains the majority of starch and proteins that accumulate during ginger rhizome development [51]. Studies have suggested that OsNF-Ys (*OsNF-Y8/9/10/11*) may be involved in starch biosynthesis for seed reserves and seed storage protein accumulation. Similarly, *AtNF-YB6* regulates oil production during seed development in *A. thaliana* [52]. Based on phylogenetic analysis, *ZoNF-YC6* has been identified as an ortholog of *OsNF-Y8/9/10/11* and *AtNF-YB6*. Additionally, *ZoNF-YC6* exhibits high expression levels in the internodes of the rhizome, indicating its potential role in rhizome formation.

Various environmental stress responses, including cold, heat, drought, and salinity, involve the participation of the NF-Y family genes [9, 11]. NF-Ys have been found to act as regulators of drought tolerance in several plant species. Overexpression of *At-NF-YB1* in transgenic Arabidopsis and *Zm-NF-YB2*, the maize ortholog, in transgenic maize (*Zea mays*) plants has been observed to result in improved plant performance and survival under drought stress [53]. *StNF-YB3.1* in potato (*Solanum tuberosum*) has been demonstrated to enhance drought resistance by inducing ABA-mediated stomatal closure [54]. In ginger, drought stress was shown to significantly increase the expression of *ZoNF-YB8*, *ZoNF-YB5*, *ZoNF-YB7*, and *ZoNF-YB16*. However, their expression patterns differed in response to drought treatment. *ZoNF-YB8* showed a quick response to drought stress within 1 h, peaked at 6 h, and then gradually decreased from 12 to 48 h (Fig. 11). The response to drought stress of *ZoNF-YB6* and *ZoNF-YB8* was noticeable within 1 h and increased gradually until 3 h, after which it began to decrease from 6 to 12 h and then peaked at 24 h. Moreover, *ZoNF-YB8* exhibited responsiveness not only to drought but also to heat and salinity stress treatments. The cis-elements present in *ZoNF-YB8*, such as TC-rich repeats and MBS elements, are known to be associated with stress responsiveness and defense mechanisms. Although *ZoNF-YB2*, *ZoNF-YB3*, *ZoNF-YB6*, *ZoNF-YB11*, and *ZoNF-YB12* were induced by drought, their expression levels were lower than those of *ZoNF-YB5*, *ZoNF-YB7*, *ZoNF-YB8*, and *ZoNF-YB16*, indicating that the latter group of genes is more important in cold response. Interestingly, the majority of the ZoNF-Y gene family members were significantly induced by both heat and salt stress, suggesting that these ZoNF-Y genes might serve as key components in signaling pathways that respond to environmental stresses.

Conclusions

In this study, we conducted a comprehensive analysis of the NF-Y family genes in ginger. A total of 36 full-length NF-Y genes were identified and analyzed for their structural features, phylogenetic relationships, and expression patterns. Based on similarities in gene structure and motif compositions, we classified the ZoNF-Y genes into three subfamilies. To infer the evolutionary characteristics of NF-Y genes in ginger, we compared them with NF-Y genes from various other plants through phylogenetic and synteny analyses. The expression patterns of

ZoNF-Y genes in ginger revealed their significance in growth and development. By performing phylogeny and gene expression analyses on ginger rhizome development and its response to abiotic stress treatments, we gained insights into the functional analysis of ZoNF-Y genes. These findings provide valuable information on the potential functional roles of ginger NF-Y genes. These comprehensive analyses can facilitate the screening of candidate NF-Y genes and further functional characterization and genetic improvement of agronomic traits in ginger.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10588-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

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Author contributions
H.T.X. and H-L. L. coordinated the project, conceived and designed experiments, and edited the manuscript. H-L. L., M. G., M. X., W. Z. performed experiments. H-L. L., H.T.X. and Z. C. analyzed data and wrote the draft of the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets generated and/or analysed during the current study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/). The transcriptome data were deposited in the NCBI Short Read Archive (Project Accession Number : SRP064226). These datasets are also available from the corresponding author (Hong-Lei Li) on reasonable request by email lihonglei215@163.com.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
The authors declare that there are no conflict of interest.

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