Genome-wide investigation of the AP2/ERF gene family in ginger: evolution and expression profiles during rhizome and inflorescence development

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Genome-wide investigation of the AP2/ERF gene family in ginger: evolution and expression profiles during rhizome and inflorescence development

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Abstract

Background: AP2/ERF transcription factors (TFs) constitute one of the largest transcription factors families in plants, which participate crucial roles in plant metabolism, growth and development as well as biotic and abiotic stresses responses. Although the AP2/ERF family has been thoroughly identified in many plant species and several AP2/ERF transcription factors have been functionally characterized, however, little is known about this family in ginger (*Zingiber officinale*) which is an important medicinal and food homologous vegetable. As we complete the sequencing of the ginger genome, allowed us to investigate the expression profiles of *AP2/ERF* genes in ginger on a genome-wide basis.

Results: A total of 163 *AP2/ERF g*enes were obtained in the *Z.officinale* genome and renamed according to the chromosomal distribution of the *ZoAP2/ERF* genes. Phylogenetic analysis

divided them into three subfamilies, of which 35 belonged to AP2 and125 to ERF as well as 3 to RAV subfamily respectively, which was in accordance with the number of conserved domains and gene structure analysis. A total of 10 motifs were detected in ginger *AP2/ERF* genes, and some of the unique motifs were found to be important for the function of *ZoAP2/ERF* genes.

Localization of Chromosome, gene structure and conserved protein motif, as well as the analysis of events in the duplication of genes provided deep insight into the evolutionary features of these ZoAP2/ERF genes. The expression profiles derived from RNA-seq data and real-time quantitative PCR analysis of ZoAP2/ERF during rhizome and inflorescence development were investigated and and tissue-specific *ZoAP2/ERF* genes were identified in ginger.

Conclusion: A comprehensive analysis of AP2/ERF gene expression patterns in various tissues by RNA-seq and qRT-PCR showed that they played an important role in the growth and development of ginger, and genes that might regulate rhizome and flower development were preliminarily identified. This research has been done for the first time to determine the *ZoAP2/ERF* family, which contribute to study on evolutionary characteristics and better understanding the molecular basis for development as well as further functional characterization of *ZoAP2/ERF* genes with an aim of ginger crop improvement.

Keywords: Ginger, *ZoAP2/ERF*, Inflorescence /Rhizome development, Expression patterns

Background

The **AP**ETALA 2/**E**thylene-**R**esponsive element binding **F**actor (AP2/ERF) family compose one of the largest of plant-specific transcription factors and play essential roles in various biological processes[1, 2]. Members are defined by AP2/ERF domain, which comprises one or

two AP2 DNA-binding domains with 60 to 70 conserved amino acid residues [3]. According to the number of conserved domains, the AP2/ERF family can be classified into three subfamilies, subfamily ERF with one conserved AP2 domain, AP2 subfamily with two AP2 domains, and RAVsubfamily with a single AP2 domain and an additional DNA-binding domain B3 that exists in other plant-specific transcription factors [4, 5]. Furthermore, ERF family could be clarified into ERF and DREB subfamilies according to the differences in the promoter sequence. The ERF subfamily and DREB subfamily were further divided into six subgroups consisting of clades A1-A6 and clades B1-B6 in Arabidopsis [6]. Since the first *AP* gene (*AP2-1*) *was found to* determines the identity of perianth organs in flowers of Arabidopsis [7], a large number of AP2 genes have been identified in various plants.The AP2 subfamily play significant roles in the regulation of plant growth and development, such as floral organ identity and development [8-10], leaf shape [11] and seed growth [12]. Ethylene response factors (ERFs) function in downstream of the ethylene signaling pathway, and have been proved to be involved in many functions, such as metabolic regulation [13-15], responses to biotic and environmental stresses [16-18] and plant development and growth [19]. The ERFs have also been involved in different hormones signal transduction pathways including cytokinin , ethylene, abscisic acid [20],However, RAV TFs mainly participate in the regulation of leaf senescence and biotic and abiotic stress responses [21, 22].

Ginger (*Zingiber officinale* Roscoe) is an important medicinal material containing abundant gingerol exhibits many biological properties, including antioxidant, antimicrobial, and anti-inflammatory properties, which have various effects on the central nervous system [23, 24]. Jiang et al. (2005) have reported that the rhizome had the greatest concentrations of

gingerols compared with other organs [25]. Our previous research show that concent of gingerols increased along with rhizome development and the second-node deposited more gingerols than the first node and rhizome bud. But how ginger rhizome initiation, expansion and how gingerols synthesis and accumulation are still delusive mystery. In addition, ginger seldom blossom in natural cultivation and propagate by rhizome block as the "seed". As cultivation years increases, the "seed" vigor decreased and the pathogenicity increase, which could cause great losses in reproduction.To elusive the mechanism of florescence determination and emergenceis necessity for novel cultivar in breeding.

The AP2/ERF family plays a profound role in regulating plant growth and development. At present, investigation and analysis of the AP2/ERF gene family have been performed in many plants in the frame of whole-genome, including Arabidopsis [26], rice [26,27], popular[28], grape[29], peach[30], Chinese cabbage [31], apple[32], sesame [33], pear [34], pepper [35] and tartary buckwheat [36]. However, no information about the AP2/ERF family in ginger (*Zingiber officinale*) have been reported. Because of the importance of AP2/ERF genes in various physiological processes, it is important to systematically study the AP2/ERF family of ginger. The evolutionary characteristics and tissue-specific expression of the AP2/ERF gene family in ginger could be characterized taking the advantage of recently sequenced ginger genome. In this study, we performed a comprehensive analysis of the AP2/ERF family in ginger, including gene structure, motif composition, chromosomal localization, phylogenetic tree, and compared the evolutionary relationships with *Arabidopsis thaliana*, *Solanum tuberosum*, *Oryza sativa* and *Musa acuminata*, as well as investigations into the expression profiles of these genes in various tissues, particularly in the stage of rizome

expansion and floral organ formation. This study provided valuable clues for future investigations aimed at the functional characterization of AP2/EREBP genes, and can be utilized in the genetic improvement of ginger.

Results

Genome-wide **Identification of the ZoAP2/ERF family in ginger**

A total of 163 AP2/ERF family candidate genes were obtained using the Hidden Markov (HMM) method with data from a query on the A12/ERF family (PF00847). The annotation of these genes were further checked using available ginger transcriptome data. Fifteen erroneously predicted AP2/ERF gene models were manually curated. A total of 157 *ZoAP2/ERF* genes could be mapped on the chromosome and were renamed from *ZoAP2#01* to *ZoAP2#35* , *ZoERF#001* to *ZoERF#119*, *ZoRAV#1* to *ZoRAV#3* based on their order on the chromosomal location respectively (Additional file 1： Fig S1). Six *ZoERF* genes (*Maker00067748, Maker00058996, Maker00044912, Maker00021402, Maker00004369, Maker00027389*) that could not be conclusively mapped to any linkage groups were renamed *ZoERF#120-ZoERF#125* respectively. The validated *ZoAP2/ERF* gene sequences were available in Additional file 2： Table S1.

Gene characteristics including the coding sequence length(CDS), protein molecular weight (MW), isoelectric point (PI) and domain were analyzed. Among the 163 ZoAP2/ERF proteins, ZoERF#003 and ZoERF#023 were identified to be the smallest protein with 117 amino acid, whereas the largest one was *ZoAP2*#02 (671 aa). The MW of the proteins ranged from 12.92 (ZoERF#23) to 73.07 kDa (ZoERF#02), and the pI ranged from 4.58 (ZoERF#106) to 11.17 (ZoERF#005). (Additional file 2: Table S1).

Multiple sequence alignment, phylogenetic analysis, and classification of *ZoAP2/ERF* genes

Multiple sequence alignment of ZoAP2/ERF proteins was established based on the AP2 domain involving approximately 60-70 amino acids and the B3 domain consisting of 100-120 residues. The sequence alignment of all AP2/ERF proteins showed that the YRGVR (7th amino acid to 11th amino acid), LG (52thamino acid and 53th amino acid), AA (62th amino acidand 63th amino acid) and YD (65th amino acid and 66th amino acid) elements were highly conserved. The WLG element (51th aminoacid to 53th amino acid) was more conserved in the ERF family and RAV family than in the AP2 family. In the AP2 family, WLG elements (51th amino acid to 53th amino acid) were converted into YLG elements(51th amino acid to 53th amino acid)(Additional file 3: Fig. S2). These conserved amino acid profiles may contribute to the classification of AP2/ERF genes in other species.

To explore the phylogenetic relationship of AP2/ERF proteins in ginger, we constructed a phylogenetic tree using the Maximum Likelihood (ML) method based on multiple sequence alignments of 166 *A. thaliana* AP2/ERF and 157 ginger AP2/ERF amino acid. The substitution model WAG+G+I was used. The phylogenetic distribution showed that AP2/ERF genes grouped into three major categories, AP2, ERF and RAV subfamily. Among 157 candidate *ZoAP2/ERF* genes, 35 containing two AP2 domains were assigned to the AP2 subfamily; 119 containing a single AP2 domain grouped in the lineage of ERF subfamily; only 3 encoded a single AP2 domain and a B3 domain was assigned to the RAV subfamily (Fig. 1). Interestingly, *ZoERF#092*, *ZoERF#050*, *ZoERF#012* was also found to encode one

AP2 domains, but they were distinct from the ERF subfamily and clustered in the AP2 subfamily (Fig.2a).

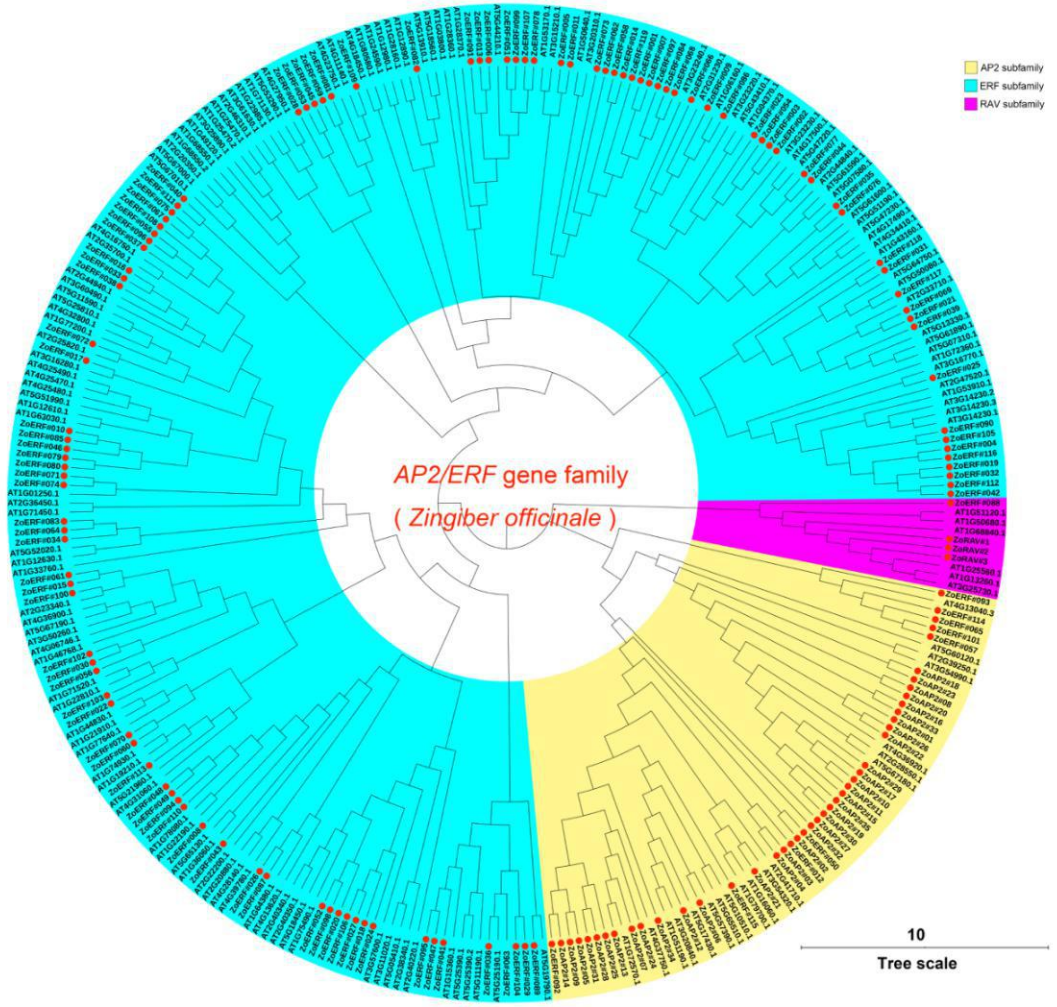


Fig.1 Unrooted phylogenetic tree representing the relationships among 163 AP2/ERF protein of ginger and Arabidopsis. The different colored arcs indicate different groups of AP2/ERF domains. The red solid circles represent AP2/ERF domain from ginger. AP2/ERF proteins from ginger with the prefix "Zo" indicate "Zingiber officinale".

Gene structure and motif composition of the ZoAP2/ERF family

By comparing the genomic DNA sequences, we obtained the intron and exon structure of *ZoAP2/ERF* genes (Fig. 2b). The coding sequences of all ginger AP2 subfamily genes were disrupted by introns, with exon numbers ranging from 5 to 13 (Fig. 2b, Additional file 2: Table S1). Excluding the *ZoAP2#32* gene with 5 exons, the other members of the AP2 subfamily contained more than 6 exons. Overall, the number of exons was conserved in the AP2 subfamily, although the exon positions varied. Most members of the ERF subfamily and RAV subfamily contained only one exon with the AP2 domain located in the exon region (Fig. 2b). In general, members with close relationship from the same subfamily share similar number and exon length. Further analysis showed that ZoAP2/ERF proteins contained, at most, two characteristic regions (Fig. 2c). All ZoAP2/ERF proteins had a highly conserved AP2 region in the N-terminal. This region is about 60-70 amino acid residues corresponding to the DNA binding region. The RAV subfamily contained the B3 region composed of 100-120 amino acids. In general, many conserved motifs were detected in transcriptional factor protein sequences, which may be involved in activating the expression of genes as potential DNA binding sites. The motifs of 157 *ZoAP2/ERF* genes were analyzed using online MEME software to further study the characteristic of ZoAP2/ERF proteins (Additional file 4: Fig. S3). A total of 10 conserved motifs were found in the ZoAP2/ERF proteins (Fig. 2d). Motif 1, Motif 6 were found in the AP2 domain regions. Motif-1, Motif-3, Motif-4, Motif-6 and Motif-9 were detected in almost all AP2/ERF proteins. All ERF subfamily genes contained Motif-1, Motif-2, Motif-4, and Motif-6. Motif-8 was detected in 29 *ERF* genes, Motf-9 was only in 2 genes (*ZoERF#088*, *ZoERF#050*), Motif-10 in 3genes (*ZoERF#095*, *ZoERF#92*, *ZoERF#115*), Motif-7 in 4ERF genes(*ZoERF#045*, *ZoERF#115*，*ZoERF#092*，

ZoERF#018）, and Motif-3 in only 3 ERF genes(*ZoERF#009*, *ZoERF#065*, *ZoERF#114*). In the AP2 subfamily, 17 genes contained six motifs including Motif-1, Motif3, Motif4, Motif5, Motif6 and Motif-9. 11 AP2 genes with Motif-10 and 15 AP2 genes with Motif-7 were detected respectively.

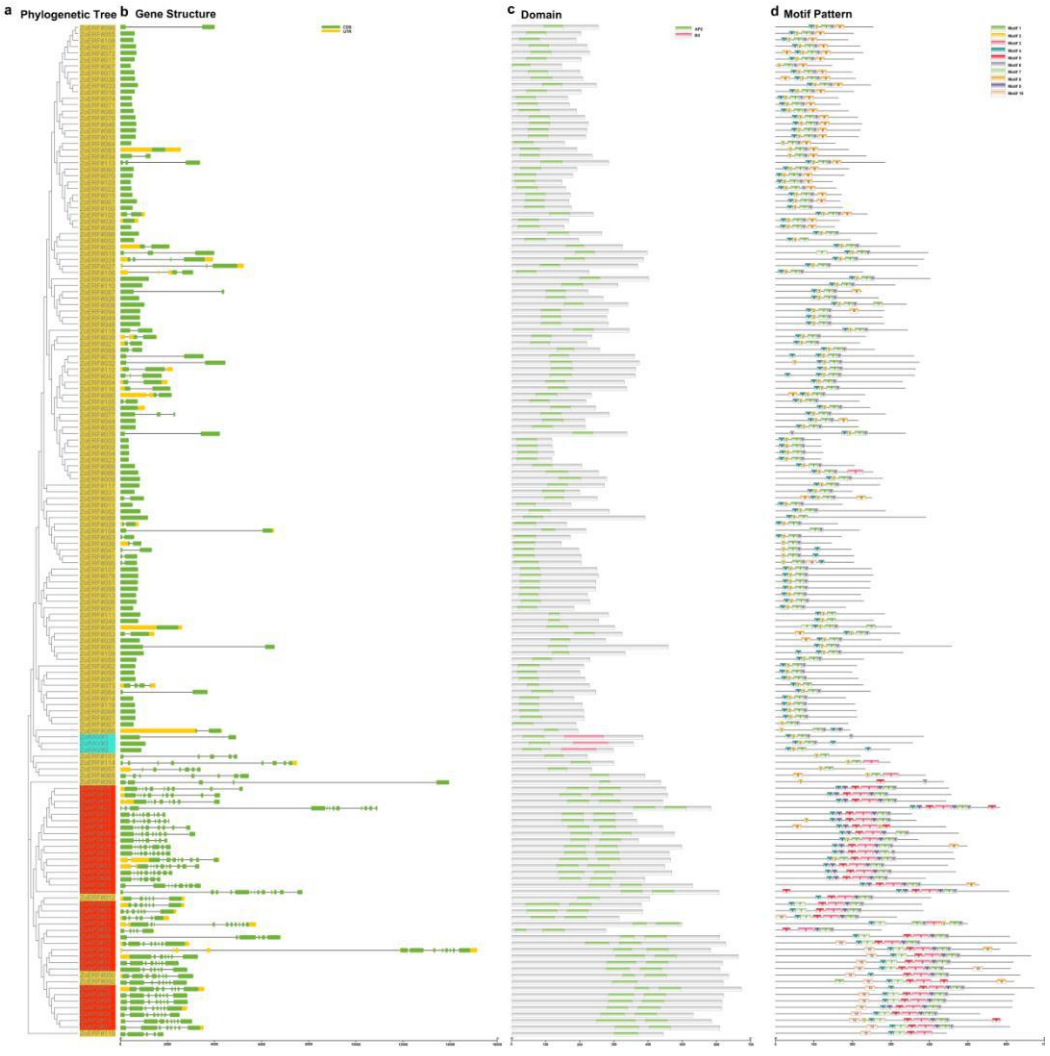


Fig. 2 Phylogenetic relationships, gene structure and architecture of conserved protein motifs in AP2/ERF genes from ginger. (a) The phylogenetic tree based on the full-length sequences of ginger AP2/ERF proteins using MEGA X software. (b) Exon-intron structure of ginger AP2/ERF genes. Yellow boxes indicate untranslated 5'- and 3'-regions; green boxes indicate exons; black lines indicate introns. (c) The motif composition of ginger AP2/ERF proteins. The motifs, numbers 1-10, are

displayed in different colored boxes. The sequence information for each motif is provided in Additional file Figure S2. The protein length can be estimated using the scale at the bottom. d The AP2 domains are highlighted by green boxes and B3 domain by pink boxes.

Chromosomal distribution and gene duplication and synteny analysis of *ZoAP2/ERF* genes

Based on chromosome mapping analysis, a total of 157 AP2/ERF TFs were found unevenly distributed on 11 ginger chromosomes (Additional file 2：Fig S1). Chromosomes 5, 9 and 11 contained the largest number of AP2/ERF TFs (21, 19 and 19, respectively), while chromosome 8 had the smallest number of AP2/ERF TFs (7 genes). Three RAV subfamily members distributed on chromosomes 2, 9 and chromosome 10. Interestingly, some transcription factors with similar conserved sequences were located on the same chromosome. Similar patterns have been found in *A. thaliana* [26], Chinese cabbage [31] and pepper genomes [35], which were thought to be caused by ancestral polyploidy events. Gene replication plays an important role in the occurrence of novel functions and gene expansion. We analyzed the duplication events of *AP2/ERF* genes in the ginger genome since Two or more genes range within a 200 kb chromosomal region were defined as tandem replication events. Eight *ZoAP2/ERF* genes clustered into six tandem repeat event regions in ginger linkage group (LG) 1,3,4,7,8 and 9 (Additional file 5：Table S2). LG7 had three clusters, indicating a hot spot of *ZoAP2/ERF* gene distribution. Four pair of tandem replication genes (*ZoAP2#15-ZoERF#30, ZoAP2#20-ZoERF#062, ZoAP2#21-ZoERF#066, ZoAP2#22-ZoERF#068*) containing different motifslocated on LG4,7,7,7 respectively, and clustered with other genes together. In addition to tandem duplications, many pairs of

segmental duplications were found in the ginger chromosomes. Analyses of homologous protein families is of great significance in establishing the kinship of species and predicting the function of new protein sequences. Many homologous genes were present on different chromosomes in ginger, indicating high conservation of the *AP2/ERF* gene family (Fig. 3). In brief, based on the above results, some *ZoAP2/ERF* genes might be produced by gene replication, and these replication events might be the main driving force of *ZoAP2/ERF* evolution.

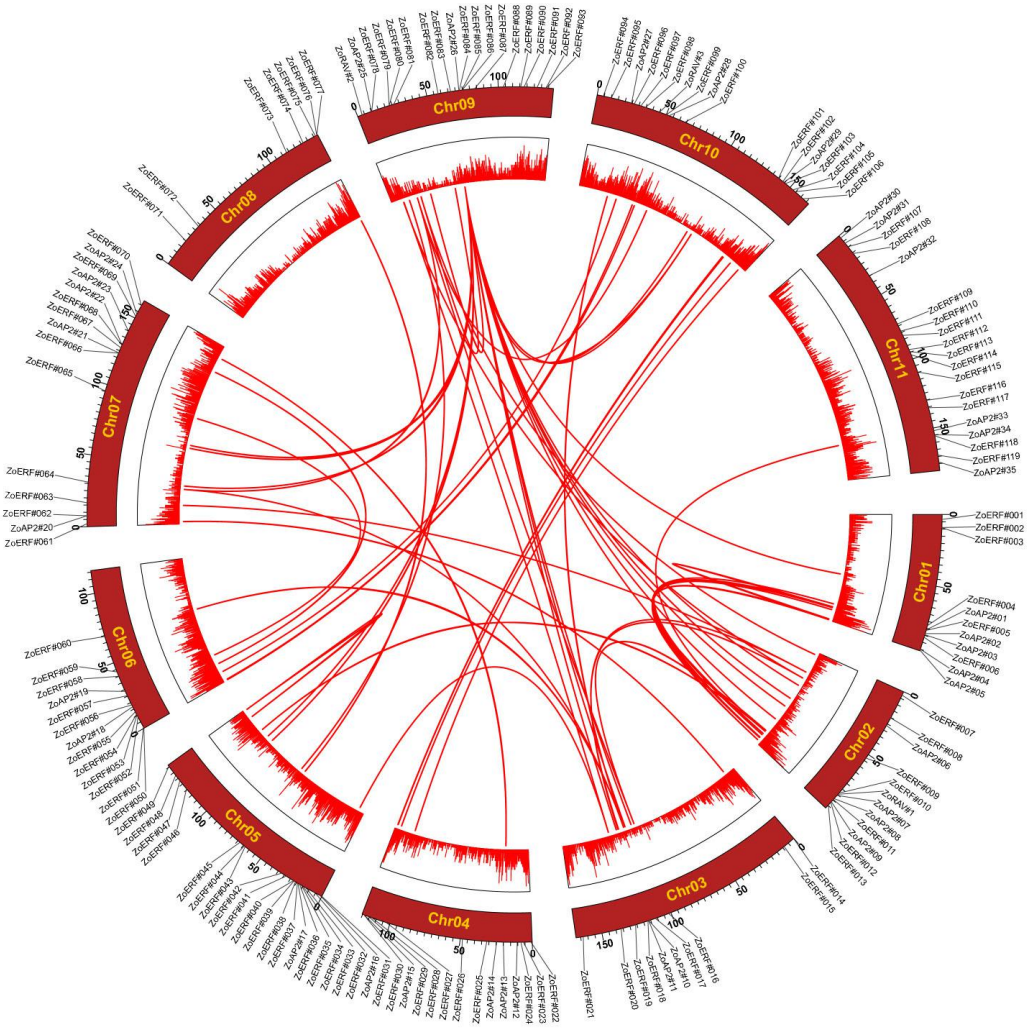


Fig.3 Schematic representations of the inter-chromosomal relationships of ginger AP2/ERF genes. The red lines indicate duplicated AP2/ERF gene pairs in ginger. The chromosome number is indicated in the middle of each chromosome.

Evolutionary analysis of *ZoAP2/ERF* genes and several different species

To deduce the evolutionary relationship of *AP2/ERF* genes, a phylogenetic tree of complete protein sequence from 5 species, including two dicotyledonous plants (*Arabidopsis thaliana* and *Solanum tuberosum*) and three monocotyledonous plants (*Oryza sativa*, *Musa acuminata* and *Zingiber officinale*), was performed. The AP2/ERF family of ginger contained three subfamilies: AP2, ERF and RAV. To explore the evolutionary relationship of each gene, a phylogenetic tree was constructed between each subfamily of ginger and other plant members of the same subfamily. Simultaneously, the motifs of the corresponding member proteins were determined. As indicated in Fig. 4a, most members of the ginger AP2 subfamily were clustered with *Musa acuminata* (33members), followed by *A. thaliana* (2 members). A total of 10 conserved motifs were detected in the protein sequences of AP2 subfamily members in all the five plants (Fig. 4b,Additional file 6： Fig. S4). Almost all members contained Motif-1, Motif-2, Motif-3, Motif-4, Motif-5 and Motif-6. In addition, most AP2 members in the same clade usually shared common motif compositions, indicating potential functional similarities among AP2 subfamily proteins.

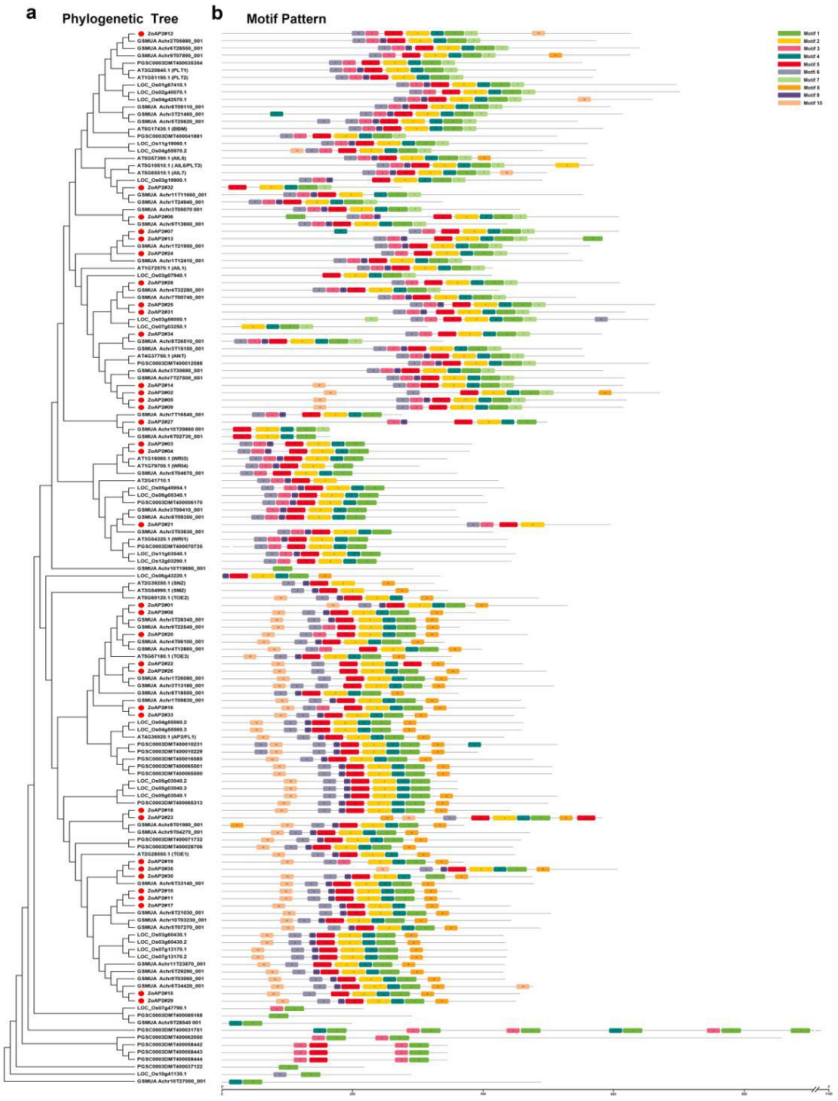


Fig.4 Phylogenetic relationships and motif compositions of AP2 proteins from five different plant species. Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method.The red solid circles represent AP2 genes from ginger. Right panel: Distribution of conserved motifs in AP2 proteins. The differently colored boxes represent different motifs and their position in each AP2 protein sequence.

The ERF subfamily of ginger contains 119 members. The phylogenetic tree constructed using the *ZoERF* genes and *ERF* members from two dicotyledonous plants, *Arabidopsis thaliana* and *Solanum tuberosum*, and three monocotyledonous plant, *Oryza sativa*, *Musa acuminata* and *Zingiber officinale*. From Fig.5 ZoERFs proteins were divided into 15 groups. A total of

10 distinct motifs were identified in the ERF subfamily of all the five plants. All the members, excluding partial of group-f, group-m and group-n, contained Motif-1, Motif-2and Motif-3 and the genes that clustered together contained common motifs. Motif-9 was unique in Group-a , group-e and group-f contained Motif-5 specifically, group-g and group-i specifically Motif-4, group-m specifically Motif-8 and group-n specifically Motif-7 (Fig. 5, Additional file 7: Fig. S5). The same phylogenetic tree method was used to analyze the clustering relationship between ZoRAV and the RAV proteins of other plants. Moreover, the members of the same clade of the phylogenetic tree had almost common motif compositions.

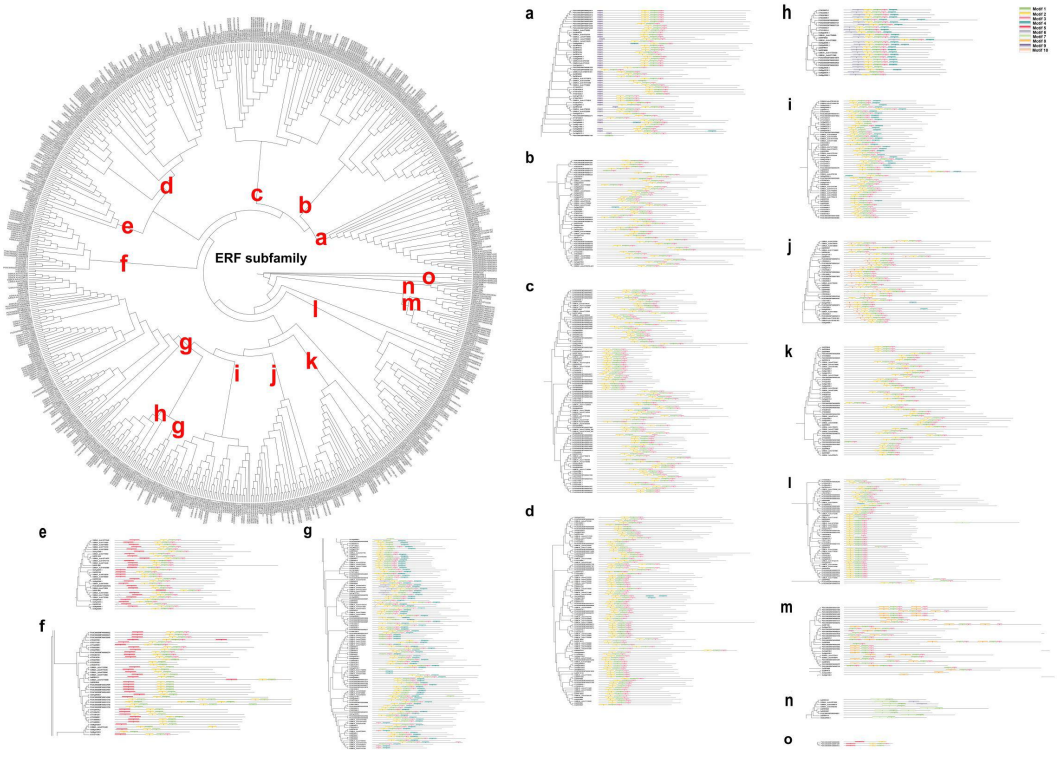


Fig.5 Phylogenetic relationships and motif compositions of ERF proteins from five different plant species. Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method. Right panel: Distribution of conserved motifs in ERF proteins. The differently colored boxes represent different motifs and their positions in each ERF protein sequence.

As illustrated in Fig. 6a, three *ZoRAV* genes were closely related to *RAV* genes in *Musa acuminata*. The protein sequences of the *RAV* genes also showed 10 distinct conserved motifs, and most of the members contained Motif-2,Motif-3 and Motif-4 (Fig.6b, Additional file 8：Fig. S6).

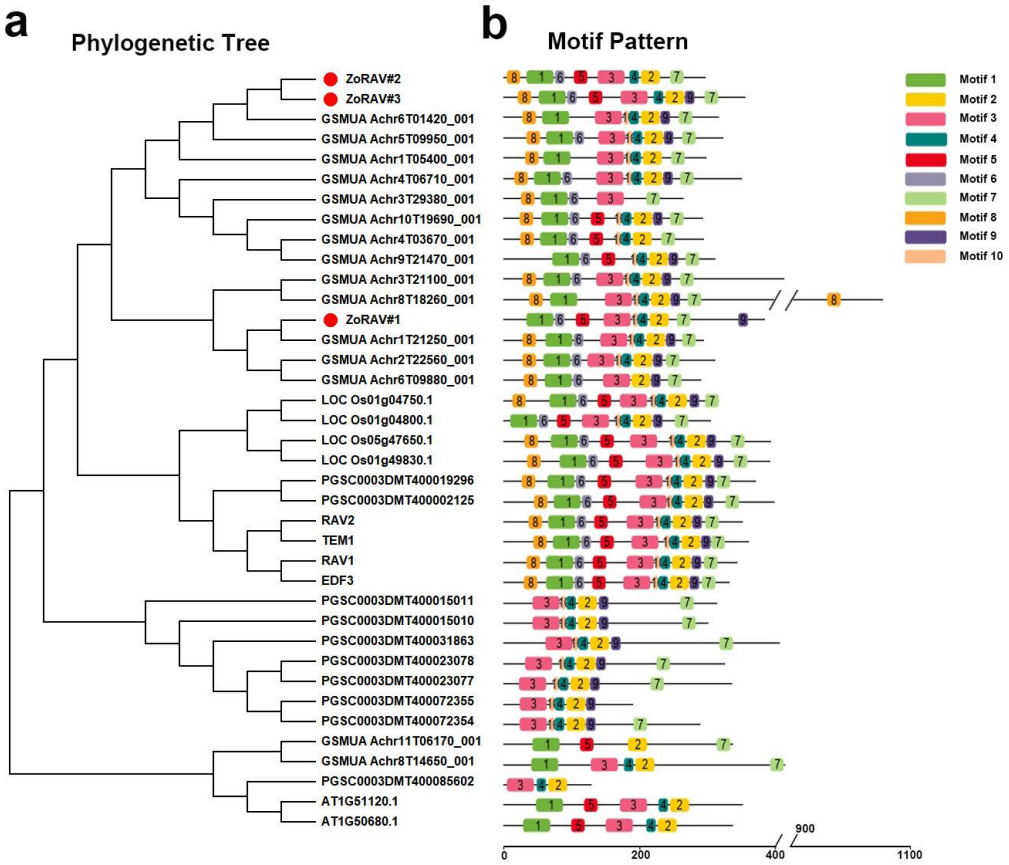


Fig.6 Phylogenetic relationships and motif compositions of RAV proteins from five different plant species.Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method.The red solid circles represent RAV genes from ginger. Right panel: Distribution of conserved motifs in RAV proteins. The differently colored boxes represent different motifs and their position in each RAV protein sequence.

In order to investigate the phylogenetic mechanisms of ginger AP2/ERF family, we constructed four comparative syntenic maps of ginger associated with other four representative plant species, including two dicots (Arabidopsis and potato) and two monocots (banana and rice) (Fig. 7). A total of 113 *ZoAP2/ERF* genes showed syntenic relationship with those in banana, followed by rice (8), potato(2) and Arabidopsis (0) (Additional file 9:Table S3). The numbers of orthologous pairs between the other four plant species (bananas, rice, potato and Arabidopsis) were 201, 9, 2 and 0. Some *ZoAP2/ERF* genes were found to be associated with at least three syntenic gene pairs (particularly between ginger and bananas *AP2/ERF* genes), such as *ZoERF#027* and *ZoERF#099*, infered that these genes may have participated an vital role of AP2/ERF gene family during evolution. The AP2 subfamily genes in ginger have homology to reference plants, and the most syntenic conservation was observed among *Musa acuminata* (201 orthologous gene pairs distributed on all LGs), *Oryza sativa* (9 orthologous gene pairs distributed on LG5, LG6, LG8, and LG10), and *Solanum tuberosum* (2 orthologous gene pairs distributed on LG6, and LG9) (Fig.7, Additional file 9: Table S3). The AP2/ERF genes were found in Arabiopsis, however, we have not found any syntenic gene paires between ginger and Arabidopsis. In the syntenic analysis of *AP2/ERF* genes of ginger and *Musa acuminata*, 49 *AP2/ERFs* were found to be associated with two syntenic gene pairs, 7 ginger *AP2/ERFs* were found to be associated with 3,4 syntenic gene pairs and *ZoERF#027* was found to be associated with five syntenic gene pairs, indicating that theses genes might play an vital role in AP2/ERF subfamily evolution . Significantly, some highly conserved syntenic blocks between ginger and banana harbored more than 80 collinear genes. In contrast, those between ginger and rice were all located in

syntenic blocks that possessed less than 50 orthologous gene pairs. However, ginger and Arabidopis have fewer gene paires, and AP2/ERF family gene was not found. We proposed that the main reason was the ginger genome having higher heterozygosity and also had more repeat sequence.

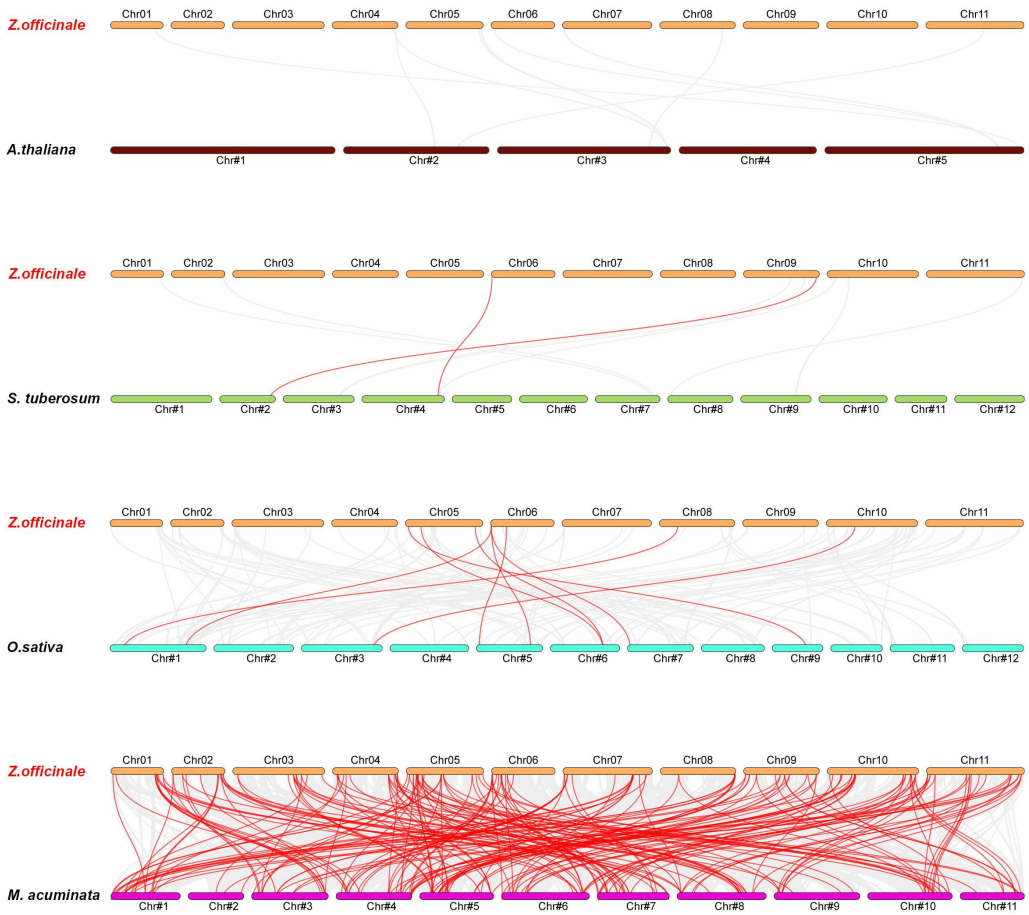


Fig.7 Synteny analysis of ERF genes between ginger and four representative plant species. The gray lines in the background indicate the collinear blocks within ginger and other plant genomes, while the red lines highlight the syntenic ERF gene pairs.

To further investigate the evolutionary constraints acting on *AP2/ERF* gene family, the Ka/Ks ratios of the *AP2/ERF* gene pairs were analysized (Additional file 10: Table S4). All segmental and tandem duplicated *ZoAP2/ERF* gene pairs, and the majorityof orthologous

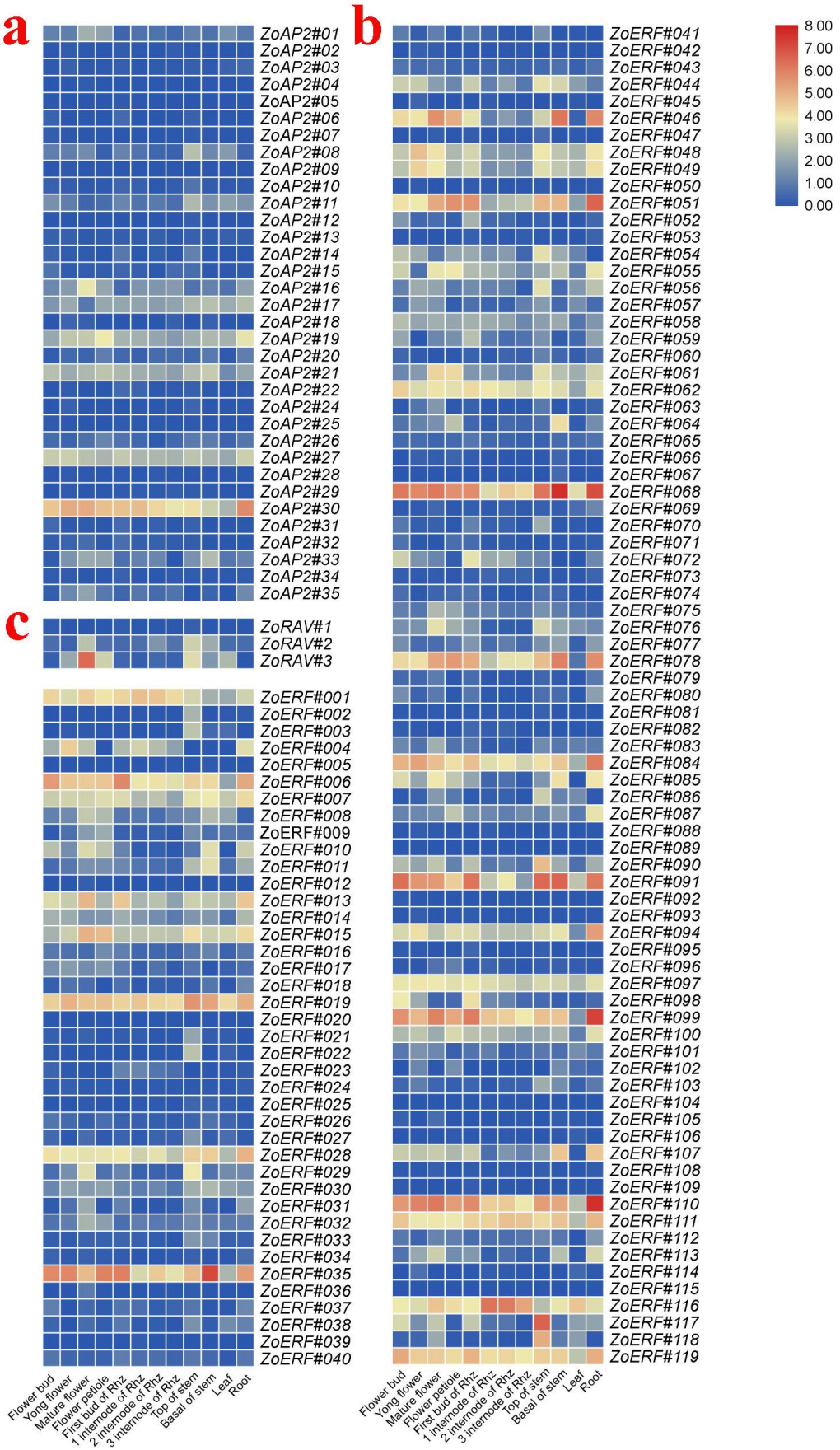
AP2/ERF gene pairs had Ka/Ks < 1, suggesting that the ginger *AP2/ERF* gene family might have experienced strong purifying selective pressure during evolution.

The syntenic analysis provided reliable evidence to sustain and validate the previous phylogenetic groupings and motif distribution. In summary, these data shown that the ginger AP2/ERF gene family was highly conserved and the ginger *AP2/ERF* genes were closer to the *Musa acuminata* genes than to the *Oryza sativa* genes. The *AP2/ERF* genes might have evolved from the common ancestor in different plants.

Expression profiling of ginger *AP2/ERF* genes with RNA-seq

To investigate the potential functions of the ZoAP2/ERF genes in the different developmental stage of ginger organs/tissues, we used RNA-seq data to detect their expression paterns. (Fig. 8).The reliability of the transcriptome data was further validated by quantitative real-time PCR (qRT-PCR) experiments which were carried out on eight representative samples for 12 selected *ZoAP2/ERF* genes (Fig. 9). Among the 35 *ZoAP2* subfamily , *ZoAP2#23* was not expressed in all detected samples, which may be pseudogenes or had special temporal and spatial expression patterns not examined in our libraries. Seventeen *ZoAP2* genes were expressed in all 12 samples tested (FPKM > 0) and four *ZoAP2* genes showed constitutive expression (FPKM > 1 in all samples). Some genes exhibited preferential expression across the detected tissues. Two genes in root(*ZoAP2#17,ZoAP2#27,ZoAP2#30*), Three genes in mature florescence (*ZoAP2#16,ZoAP2#33,*),one gene in anthocaulus (*ZoAP2#19*), two genes in leaf *ZoAP2#21*/33) and one genes in top of stem meristem (*ZoAP2#08*) showed the highest transcript expression levels. The expression of some genes exhibited significant trends in

307 different development stages. For example, the expression levels of *ZoAP2#16/19/30/33* and
308 *ZoAP2#35* were gradually increased whereas *ZoAP2#27* was gradually reduced along with
309 the flower development (Fig. 8a).
310 Among the 119 *ZoERF* subfamily, 69 genes were expressed in all 12 samples tested
311 (FPKM > 0) and twenty-two *ZoERF* genes showed constitutive expression (FPKM > 1 in all
312 samples). Some genes displayed preferential expression across the detected tissues. Three
313 genes in young flower bud (*ZoERF#004/048/049*), Severn genes in mature flower
314 (*ZoERF#001/*008/013/015/029/117/118,),Three genes in top of stem (*ZoERF#002/003/056*),
315 Fifteen genes in root (*ZoERF#007*/014/028/051/061/080/084/094/099/100/107/110/111/112/
316 113),one gene in second internode of rhizome ,eight genes in basal of stem(*ZoERF#010/011/*
317 *019/044/078/084/091*) and four genes in first bud of rhizome (*ZoERF#006/035/052/062*)
318 showed the highest transcript abundances. The expression of some genes exhibited significant
319 trends in different development stages. For example, the expression levels of
320 *ZoERF#15/30/31/61/087*and *ZoERF#110* were gradually increased whereas *ZoERF#014/054/*
321 *091/098* were gradually reduced during differnent development stage of the flower . These
322 gene , as *ZoERF#001/006/013/019/046/051/055/062/068/085/099* were reduced in flower
323 emerged stage then increased in flower elongation stage. These gene , as *ZoERF#004/035/048*
324 */049/084* and *ZoERF#100* were increased in emerging inflorescence then reduced in
325 inflorescence elongation stage (Fig. **8**b).Among the 3 ZoRAV subfamily, *ZoRAV#1*and
326 *ZoRAV#2* have no significant fluctuation in development stage,while *ZoRAV#3* was highly
327 expressed in mature inflorescence (Fig. **8**c).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ZoERF#036
ZoERF#037
ZoERF#038
ZoERF#039
ZoERF#040
Flower bud
Yong flower
Mature flower
Flower petiole
First bud of Rhz
1 internode of Rhz
2 internode of Rhz
3 internode of Rhz
Top of stem
Basal of stem
Leaf
Root

Fig.8 Expression profiles of the ginger AP2/ERF genes. a Hierachical clustering of expression profiles of ginger AP2/ERF genes in 12 samples including different tissues and developmental stages.

The transcriptional levels of all157 *ZoAP2/ERF* genes in different whole-rhizome developmental (rhizome bud,first inter-node,second inter-node,third inter-node)stages were also investigated and the results showed that the expression of several *ZoAP2/ERF* genes were associated with the rhizome development in ginger. The transcripts of *ZoAP2#17*/19/27/30 were gradually reduced during different developmental stages of rhizome. *ZoERF#001/006/013/ 084/099/107/119* were increased between the rhizome bud and first rhizome inter-node, and reduced in the following development stage.

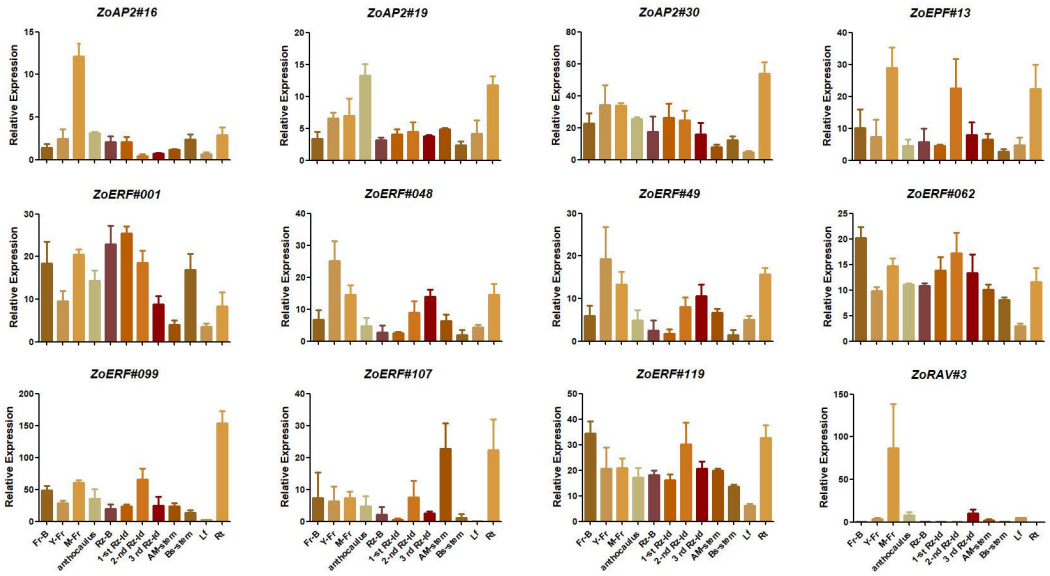

Fig.9 Expression analysis of 12 AP2/ERF genes in 12 samples by qRT-PCR. Data were normalized to TUB-2 gene and vertical bars indicate standard deviation.

Discussion

AP2/ERF genes comprise a large family of transcription factors that are ubiquitous to all plant species which play vital roles in various physiological processes, and it has been intensively studied in many plants, such as A. thaliana [26], populus [28]. However, no studies have investigated the *AP2/ERF* genes in ginger. The genome-wide analysis of *AP2/ERF* gene families have been widely carried out in many species whose genomes have been sequenced [26-36]. In this study, a search for *AP2/ERF* genes in the ginger genome resulted in the identification of 163 members, including 125 ERF subfamily members, 35 AP2 subfamily members and 3 RAV subfamily members, which were designated *ZoAP2#01 -ZoAP2#35*, *ZoERF#001-ZoERF#125*,*ZoRAV#1-ZoRAV#3* on the basis of their chromosomal location respectively. The similar numbers of AP2/ERF genes in other plants were found, such as rice with 157 *AP2/ERF* genes, including 139 *ERF* members, and arabidopsis with 145 AP2/ERF members, including 121 *ERF* genes. Concurrently, the genome size of the three plants was different, *Oryza sativa japonica* (466 Mb), Arabidopsis(125 Mb) and 3.4Gb in ginger, thus indicating that the number of AP2/ERF superfamily members was relatively stable and that there was no absolute correlation with genome size.

The gene structure of *ZoAP2/ERF* genes were assessed in this study. As Fig. 2b shown, 63.03% (75/119) of *ZoERF* genes had no introns, while the number of introns in the AP2 subfamily genes ranged from 1 to 8. The gene structure of *AP2/ERF* genes in ginger was similar to that of *AP2/ERF* genes in tartary buckwheat [36]. The difference between the structure of AP2 subfamily and ERF subfamily genes supported a vast differentiation in the genome evolution.

The domains and motifs of transcription factors are often related to DNA binding ,protein interaction and transcriptional activity[37]. Motif analysis showed that AP2 domain of the AP2/ERF genes in ginger contained Motif-1, Motif-2, Motif-4, and Motif-6 (Fig. 2c). Motif-3, Motif-7 and Motif-8 were specifically detected in different groups of the ERF subfamily, suggesting that they play an important role in this subfamily. The conserved structural domains of the ginger AP2/ERF proteins were assessed. Multiple sequence alignments revealed that sixteen AP2/ERF proteins (ZoAP2#27/32 and ZoERF #033/068/075/080/082/088/089/ 079/093/108/113/104/117/120) had sequence variation in their AP2 domain. Most characterized AP2/ERF proteins exhibited binding preference to their cognate cis-acting GCC-box or DRE motifs with the assistance of AP2 domain. In previous , variations in the YRGVR motif in AP2 domain might influence normal interactions of *AP2/ERF* genes with downstream target genes, and therefore these sixteen AP2/ERF proteins might be worthy to further characterize their functions and binding specificities [34, 38].These results propose that although some motifs/domains of the AP2/ERF family genes were highly conserved, the new evolutionary motifs/domains might perform new functions in some specific plants, and the functions of these new evolutionary motifs/domains require further verification.

Chromosomal fragments, duplications of individual genes or entire genomes have long been considered a major source of evolution, including novel functions and expression patterns [39]. In this study, most duplicated *ZoERF* genes were expressed in different tissues / organs, indicating that these genes had specific or redundant cellular functions in development. Evidence for differences between duplicate genes can be deduced from the

386 expression patterns of the *ZoERF#076* and *ZoERF#077* genes. *ZoERF#076* highly expressed
387 in mature inflorescence and basal of stem , but *ZoERF#077* was not expressed .The
388 composition and location of their motifs(Motif 4,2,1,6) are identical (Fig. 8), and thus we
389 conjecture that the reason for their different expression pattern might be due to the mutation
390 of genes involved in the process of replication, which leads to a loss of function of
391 *ZoERF#077*.
392 Additionly, functional differences may biring about differences in gene pair expression
393 patterns. For instance, the mRNA abundance of *ZoERF#062* peaked in bud of inflorescence ,
394 but *ZoAP2#20* was highly expressed in root (Additional file 10: Figure S6). *ZoAP2#20* (Motif
395 4,9,5,3,9,1,6) contains motif-3,5,9, but *ZoERF#062*(Motif 4,2,1,6) does not (Fig. 8); therefore,
396 we speculate that the alteration of the motifs of the two genes during replication may be one
397 of the reasons of the functional differences.
398 Gene functions can be preliminarily predicted by analysis of the gene expression profiles [40].
399 Tissue-specific expression patterns indicated that most of the AP2 subfamily genes (17/35,
400 49.6%) , ERF subfamily genes(75/119, 63.0%) (Fig. 8) and RAV subfamily genes (2/3,
401 66.7%)(Fig. 12) were expressed in all tested tissues. However, 22 *ZoAP2/ERFs* were
402 expressed at higher abundance in roots, and similar results have been found in other plant
403 species [28, 30]. Ginger is usually not easy to blossom, sometimes once in 10 years in
404 cultivation. However,the flowering echanism of ginger is still unclea. In model plants such
405 as *Arabidopsis thaliana*, transformation of vegetative to reproductive growth have
406 been intensive studied, AP2/ERF family is involved in controlling flowering. In our
407 study,Interesting 49 were abundantly expression in inflorescence development . According to

408 the phylogenetic tree, we found that AP2 genes (*ZoAP2#01, ZoAP2#16 and ZoAP2#33*) that
409 were highly expressed in mature inflorescence were clustered together (Fig. 1). Exploration of
410 the evolutionary relationship between these AP2 genes and other AP2 genes in other plants
411 revealed a similar evolutionary relationship of *ZoAP2#16/33* and AT4G36920.1 (*FL1*) and
412 identical motif compositions (Fig. 4). Moreover, *FL1* was identified as a gene participated in
413 the specification of floral organ identity, establishment in *A. thaliana* and, thus, this provides
414 a direction for us to further characterize the function of ZoAP2#16/33 [41]. In ginger ,
415 *ZoAP2#16/33* have relative higher expression ininflorescence compared to leaf and other
416 vegetative organs,indicating that *ZoAP2#16/33* may be also associated with the regulation of
417 the flower development. Indeed, it is essential to further validate whether *ZoAP2#16/33* can
418 also promote inflorescence initiation and growth in ginger. In Arobidopsis,
419 ANT(AT4G37750) and AIL6(At5g10510) are key regulon in floral organ positioning, identity
420 and growth [42,43,44]. Howere , the homolog of Arabidopsis *ANT* and *AIL6* in ginger
421 (*ZoAP2#02/05/09/14, ZoAP2#32*) were not expression flower initiation and growth phase ,
422 suggesting the functional divergence of AP2 genes during evolution. Motif compositions
423 analysis indicate that *ZoAP2#02/05/09/14* gain another motif element (Motif 10) in N
424 -terminal compared with there homolog ANT whlie *ZoAP2#32 have* lost Motif 6/3/9
425 compaired with it homolog *AIL6 in* Arabidopsis (Fig. 4) . It supposed that gain and loss of
426 motif may result in the function divergence.Therefore, we need to further screening the *AP2*
427 */ERF* genes possesses a function similar to *ANT* or *ANT-like* genes which plays an important
428 role in regulating the initiation and development of flowers in ginger.

ZoERF#013 exhibited that the higher expression in all floral development stage and the highest expression in muature inflorescence , while its othologs in arabidopsis , *ERF12*, regulates the floral meristem identity [45], indicating that *ZoERF#006* may share similar functions in ginger.

Rhizome is the economic organ of ginger, the process of rhizome enlargement is the focus of attention in cultivation. Zhou et al.,(2016) reported that *AtERF11* (At1g28370) promotes internode elongation by promoting both GA biosynthesis and signaling pathways[46]. Based on phylogentic analysis, ZoERF#066 is the otholog of *AtERF11*.*ZoERF#006* is also highly expressed in the internodes of rhizome, which is probably related to the elongation of rhizome. ERF139 is a key factor within a negative regulatory cascade that controls vessel expansion in poplar [47]. In ginger , *ZoERF #72* is the otholog of *ERF139*, according the expression pattern, the highest expression in leaf buds, and gradually reduced from tender to old internodes during the rhizome growth and development. Therefore, we need to further verify whether *ZoERF#006* possesses a function similar to *ERF139* and plays an vital role in regulating the growth and development of rhizome.

Overall, these above findings provide insight into the potential functional roles of ginger *AP2/ERF* genes. The comprehensive analyses were beneficial to screening candidate *AP2/ERF* genes for further functional characterization, and for the genetic improvement in the agronomic characters of ginger.

Conclusions

A comprehensive analysis of *AP2/ERF* gene family in ginger was carried out in this study. 163 full-length *AP2/ERF* genes were identified and further classified into three subfamily,

with high similar gene structures and motif compositions with in the same subfamily or subgroups. Phylogenetic comparison and synteny analysis of *AP2/ERF* genes from several different plants provided valuable clues about the evolutionary characteristics of *AP2/ERF* in ginger. *ZoAP2/ERF* genes played vital roles in ginger growth and development as indicated by their expression patterns . The phylogenetic and gene expression analysis will shed light on the functional analysis of *ZoAP2/ERF* genes. In short, these results provide a valuable resource for better understanding the biological roles of individual *AP2/ERF* genes in ginger.

Methods

Genes identification and classification

The largest number of AP2/ERF genes were found in ginger genome (Data from our ginger genome research project) by two BLASTP methods. The candidate genes were searched by BLASTP using a score value of ≥100 and e-value ≤ e- 10. Then the hidden Markov model (HMM) file from the Pfam protein family database (http://pfam.xfam.org/) corresponding to the AP2 domain (PF00847) and the B3 domain (PF02362) was downloaded. *AP2/ERF* genes were retrieved from the ginger genomic database by HMMER3.0. The default parameter was determined and the cutoff set to 0.01. Using the PFAM and SMART programs to determine the existence of AP2 core sequence and genes contains AP2 domain were further verified by HMMER. Finally, 163 AP2/ERF gene models were identified in the ginger genome for further analysis. The basic information of the identified AP2/ERF proteins were obtained using the tools at the ExPasy website (http:// web.expasy.org/protparam/).

Sequence analysis

The structural differences between *Zingiber officinale AP2/ERF* (*ZoAP2/ERF*) genes were

investaged by studying the conserved motifs of the encoded AP2/ERF proteins. Alignment of FtAP2/ERF protein sequences with ClustalW default parameters. The exon-intron structure of the *ZoAP2/ERF* genes were determined by Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/) and the conserved motifs of AP2/ERF proteins were evaluated by MEME online program (http:/meme.nbcr.- net/meme/intro.html) [48].

Chromosomal distribution and gene duplication of *ZoAP2/ERF* genes

The method of mapping *ZoAP2/ERF* genes on the chromosome of ginger according by Xing et al.,2018 [49]. Analysis of gene replication events using Multiple collinear scanning toolkits (MCScanX). The syntenic analysis maps of the Dual Systeny Plotter software was constructed to determine the syntenic relationship between *ZoAP2/ERF* genes and *AP2/ERF* genes in other selected plants[50].

Phylogenetic analysis and classification of the *ZoAP2/ERF* gene family

According to the number of AP2 conserved domain and the existence of B3 conserved domain, *ZoAP2/ERF* genes were divided into different groups. The AP2/ERF protein sequences of *Arabidopsis thaliana*, *Solanum tuberosum*, *Oryza sativa* and *Musa acuminata* were downloaded from the UniProt database (https://www.uniprot.org/). The phylogenetic trees were constructed by the neighbor-joining (NJ) method, the parameters refer to Xing et al. [49].

Plant materials

Zingiber officinale accessions (LAIWU No.2) used in this study. The materials including ginger flowers, flower bud , anthocaulus , stem, rhizome bud , fist and second rhizome

494 inter-node, functional leaves,leaf bud ,root were collected in Oct.2019. The collected samples
495 were quickly placed in liquid nitrogen and stored at- 80 °C for further extract RNA.

496 **Expression analysis of *ZoAP2/ERF* genes by real-time PCR**

497 Using the *Zingiber officinale* (“southwest” cultivar) genome sequence database to download
498 the corresponding sequences of *ZoAP2/ERF* genes. Meanwhile, the qRT-PCR primers were
499 designed using Primer5 software (http://frodo.wi.mit.edu/) (Additional file 1: Table S4).
500 Analysis of spatial and temporal expression of some *ZoAP2/ERF* gene by qRT-PCR. *TUB2*
501 gene are expressed in almost all tissues with little difference in expression levels and are often
502 used as internal reference genes. The *ZoTUB2* gene was used as an internal control, and each
503 qRT-PCR experiment with SYBR Premix Ex Taq II (TaKaRa) was performed at least three
504 times using a CFX96 Real Time System (Bio-Rad). The experimental data were processed by
505 the $2^{-\triangle\triangle CT}$ method[51] .Statistical analysis All the data were analyzed by analysis of variance
506 using the Sigma Plot 10.0 (SYSTAT software, USA) statistics program, and the means were
507 compared by the least significant difference test (LSD) at significance levels of 0.05 and 0.01.

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643 quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods-A Companion To Methods in
644 Enzymology. 2001,25:402-8.

645 **Declarations**

646 **Ethics approval and consent to participate**

647 Not applicable

648 **Consent for publication**

649 Not applicable

650 **Availability of data and materials**

651 The datasets used and/or analysed during the current study are available from the
652 corresponding author on reasonable request.

653 **Competing interests**

654 The authors declare that they have no competing interests.

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662 **Authors' contributions**

663 H.T.X.,Y.L. and H.L.L. coordinated the project, conceived and designed experiments, and
664 edited the manuscript.H.T.X., X.L.L., X.L.W., Y.S.J., Y.R., L.W. and Y.Z. performed
665 experiments. H.T.X., Y.L.,and H.L.L.analyzed data and wrote the draf of the manuscript. All
666 authors read and approved the final manuscript.

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683 **Additional information**

684 Additional file1: Table S1: List of the 163 ZoAP2ERF genes identified in this study.

685 Additional file2: Fig S1: Schematic representations for the chromosomal distribution of ginger

686 AP2ERF genes.

687 Additional file3: Fig S2: Alignment of multiple ZoAP2ERF and selected AP2 domain amino

688 acid sequences.

689 Additional file4: Fig S3: Analysis and distribution of conserved motifs in ginger AP2ERF

690 proteins.

691 Additional file5: Table S2: List of the tandem repeat gene pairs of AP2ERF in ginger.

692 Additional file6: Fig S4: Analysis and distributio of conserved motif in AP2 subgroup of

693 ginger and other species.

694 Additional file7: Fig S5: Analysis and distributio of conserved motif in ERF subgroup of

695 ginger and other species.

696 Additional file8: Fig S6: Analysis and distributio of conserved motif in RAV subgroup of

697 ginger and other species.

698 Additional file9: Table S3: Ginger and other species AP2ERF synteny gene pairs list.

699 Additional file10: Table S4: Ka/Ks ratio.

700 Additional file11: Table S5: The primer sequences of qRT-PCR.

Figures

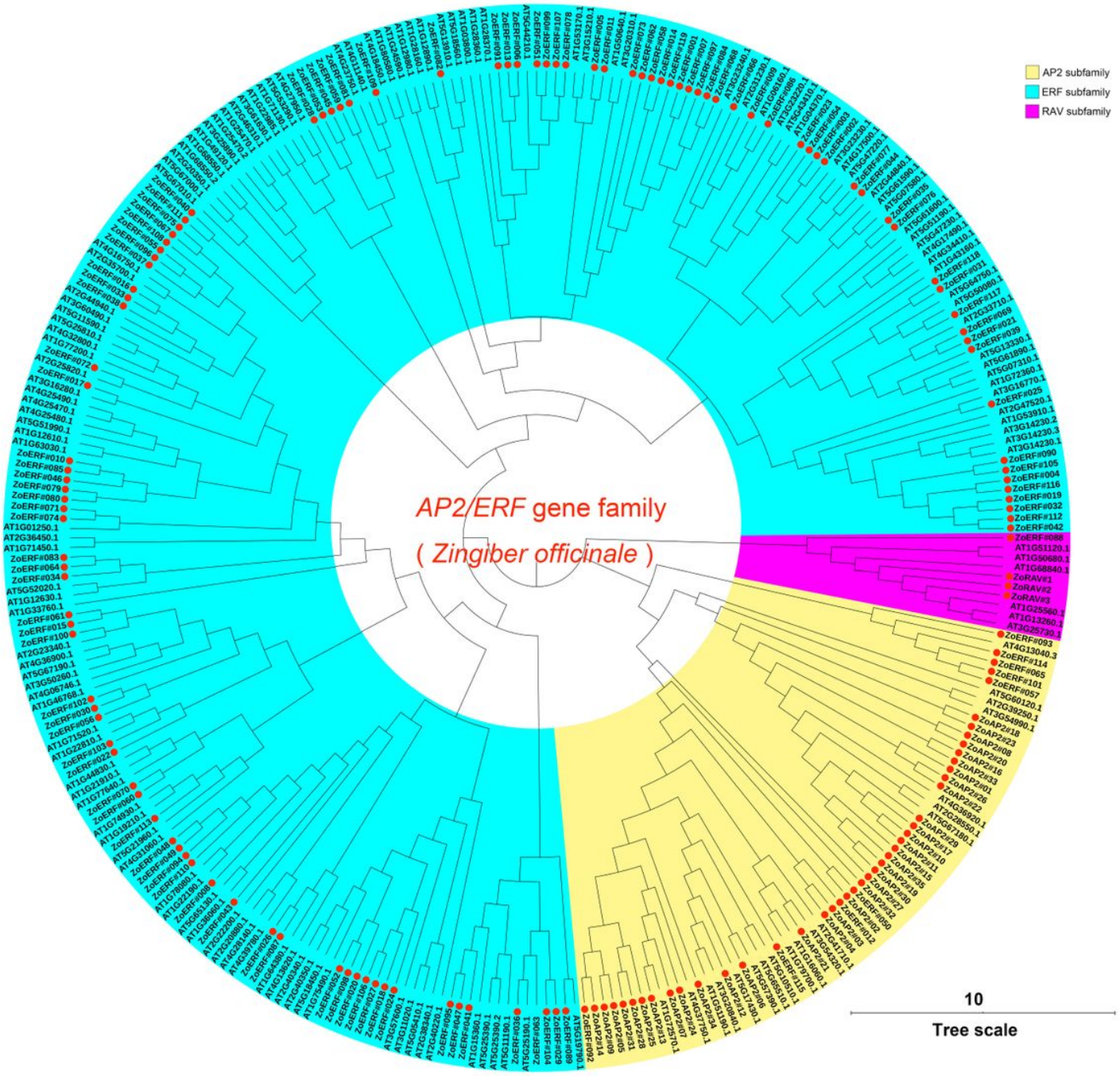

Figure 1

Unrooted phylogenetic tree representing the relationships among 163 AP2/ERF protein of ginger and Arabidopsis. The different colored arcs indicate different groups of AP2/ERF domains. The red solid circles represent AP2/ERF domain from ginger. AP2/ERF proteins from ginger with the prefix “Zo” indicate “Zingiber officinale”

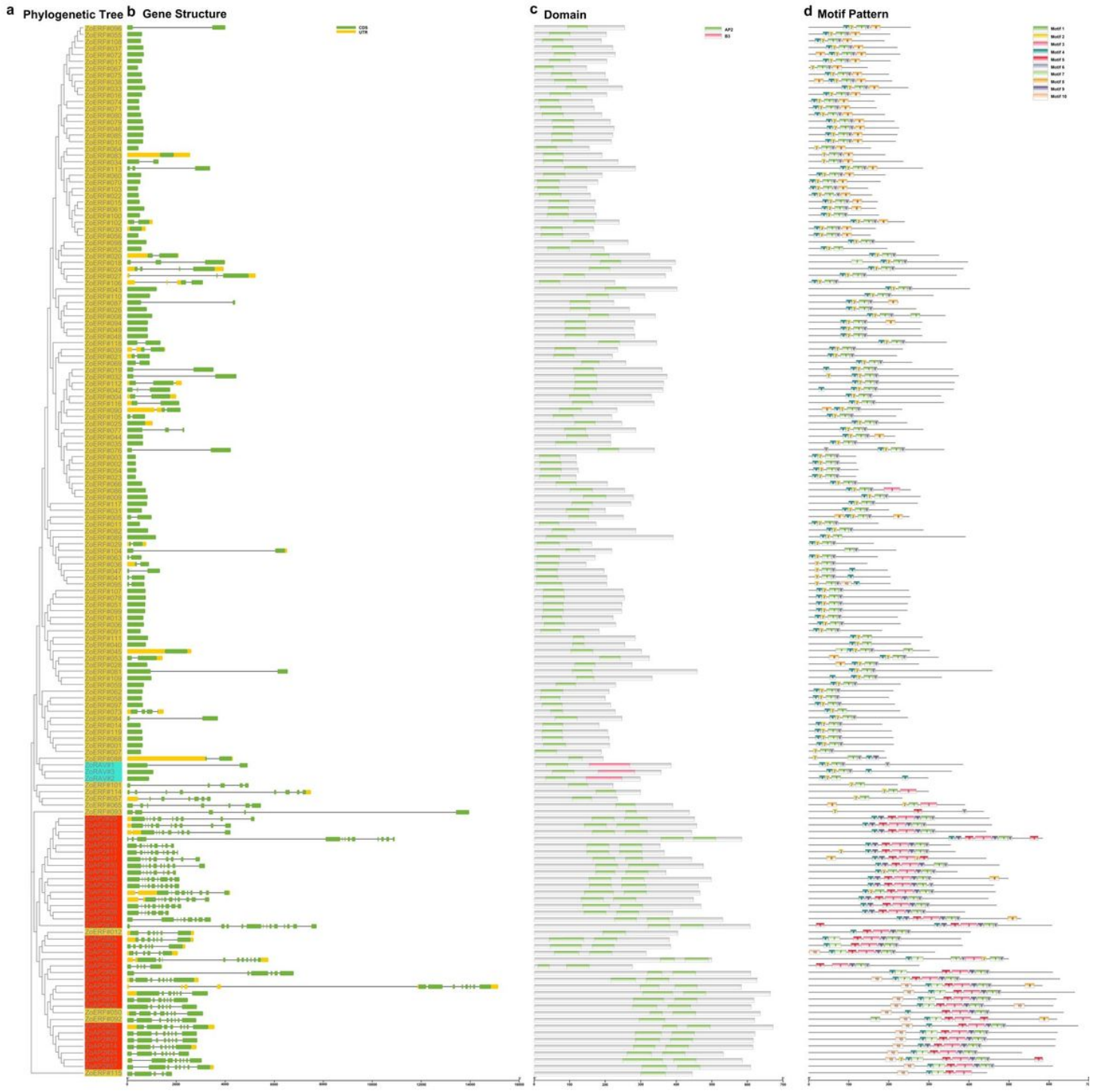

Figure 2

Phylogenetic relationships, gene structure and architecture of conserved protein motifs in AP2/ERF genes from ginger. (a) The phylogenetic tree based on the full-length sequences of ginger AP2/ERF proteins using MEGA X software. (b) Exon-intron structure of ginger AP2/ERF genes. Yellow boxes indicate untranslated 5'- and 3'-regions; green boxes indicate exons; black lines indicate introns. (c) The motif composition of ginger AP2/ERF proteins. The motifs, numbers 1-10, are displayed in different colored boxes. The sequence information for each motif is provided in Additional file Figure S2. The protein length can be estimated using the scale at the bottom. d The AP2 domains are highlighted by green boxes and B3 domain by pink boxes.

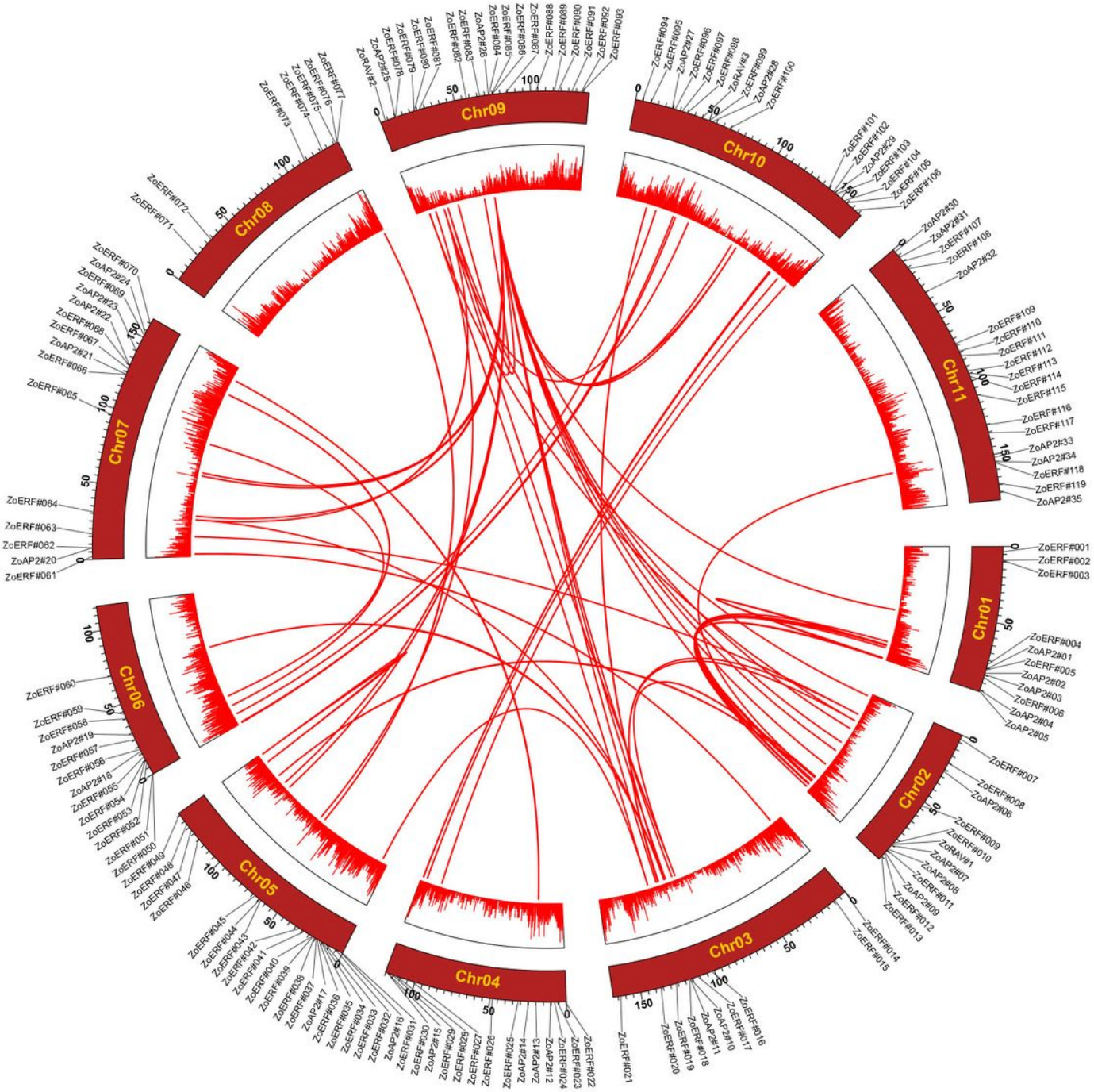

Figure 3

Schematic representations of the inter-chromosomal relationships of ginger AP2/ERF genes. The red lines indicate duplicated AP2/ERF gene pairs in ginger. The chromosome number is indicated in the middle of each chromosome

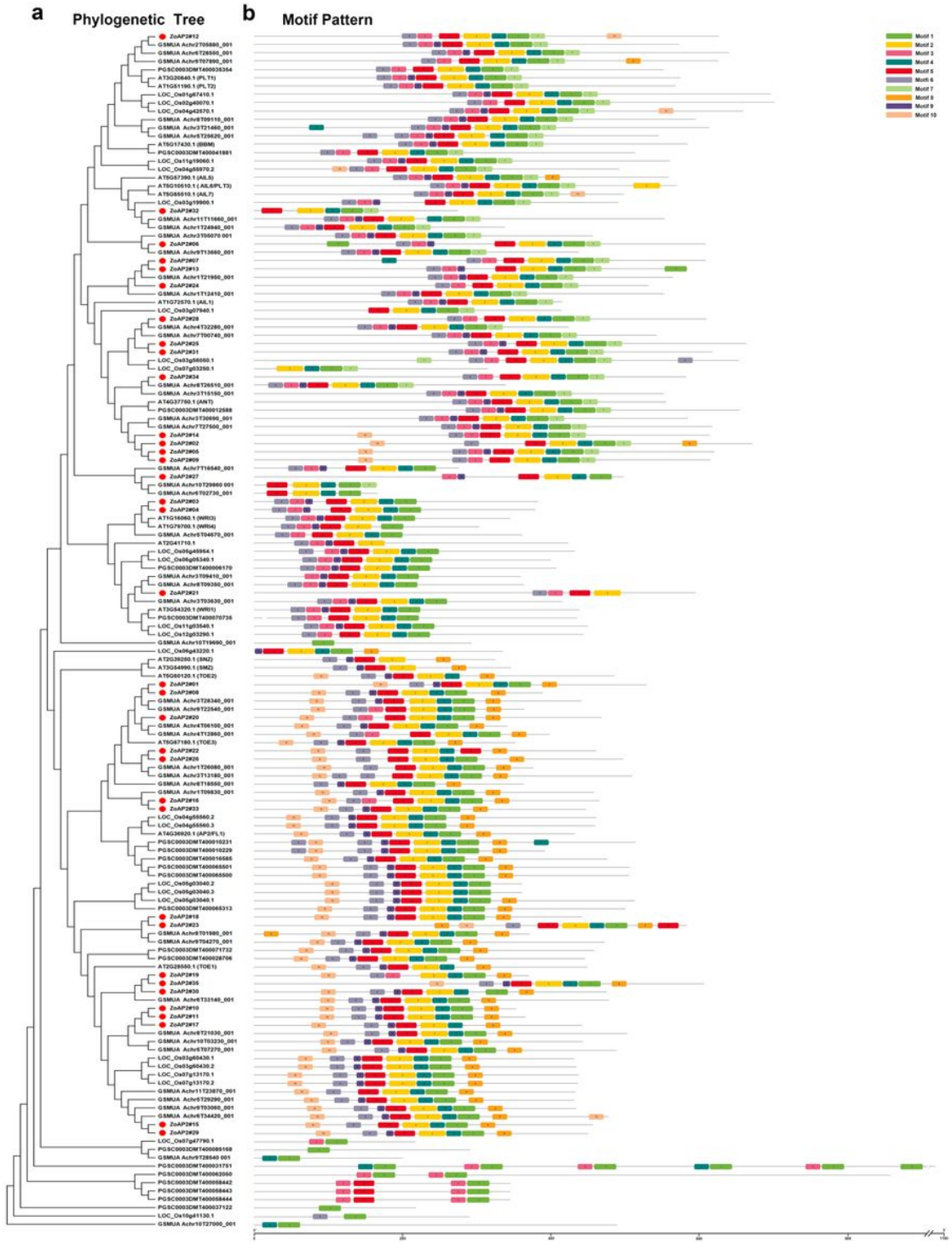

Figure 4

Phylogenetic relationships and motif compositions of AP2 proteins from five different plant species. Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method. The red solid circles represent AP2 genes from ginger. Right panel: Distribution of conserved motifs in AP2 proteins. The differently colored boxes represent different motifs and their position in each AP2 protein sequence

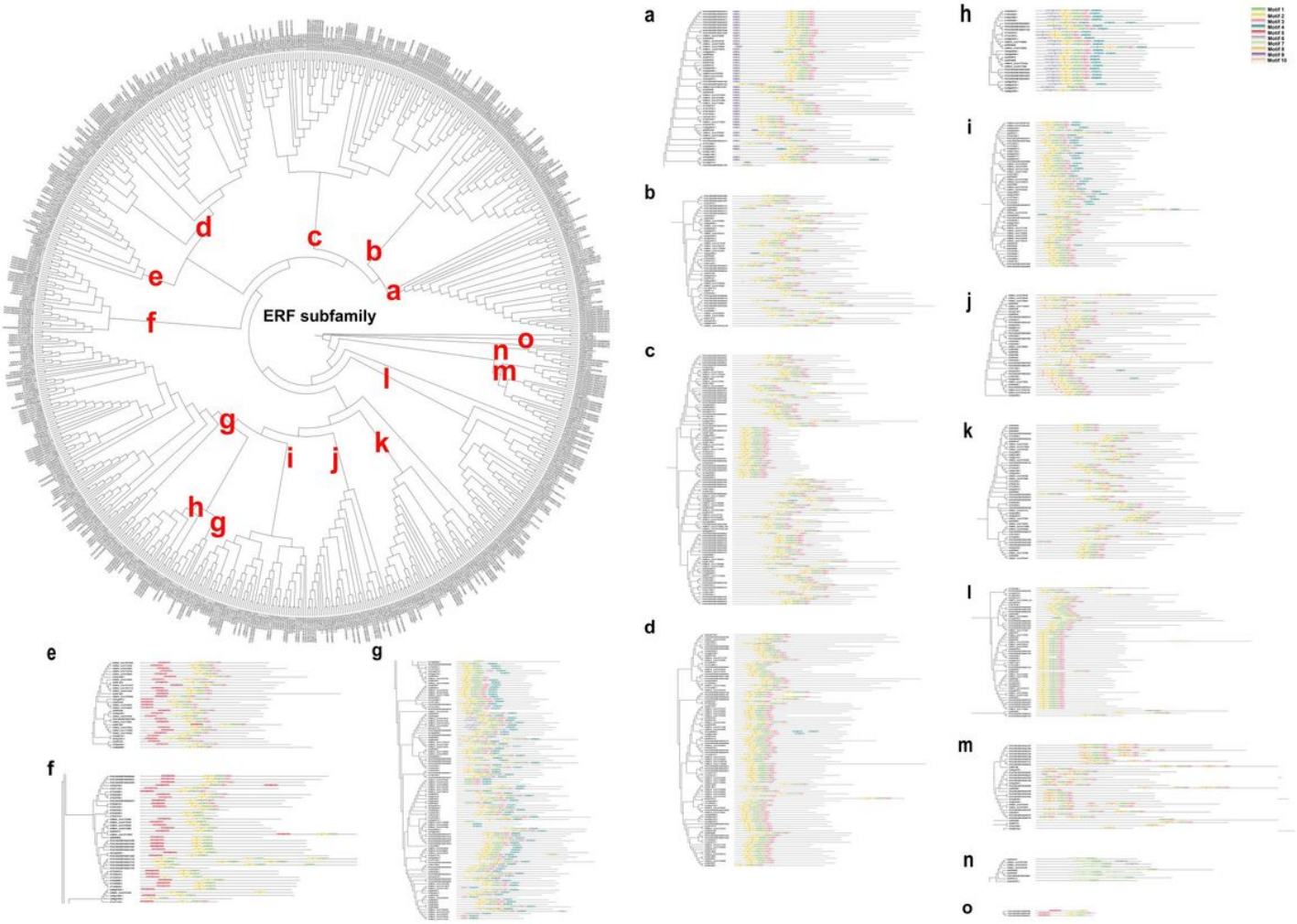

Figure 5

Phylogenetic relationships and motif compositions of ERF proteins from five different plant species. Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method. Right panel: Distribution of conserved motifs in ERF proteins. The differently colored boxes represent different motifs and their positions in each ERF protein sequence

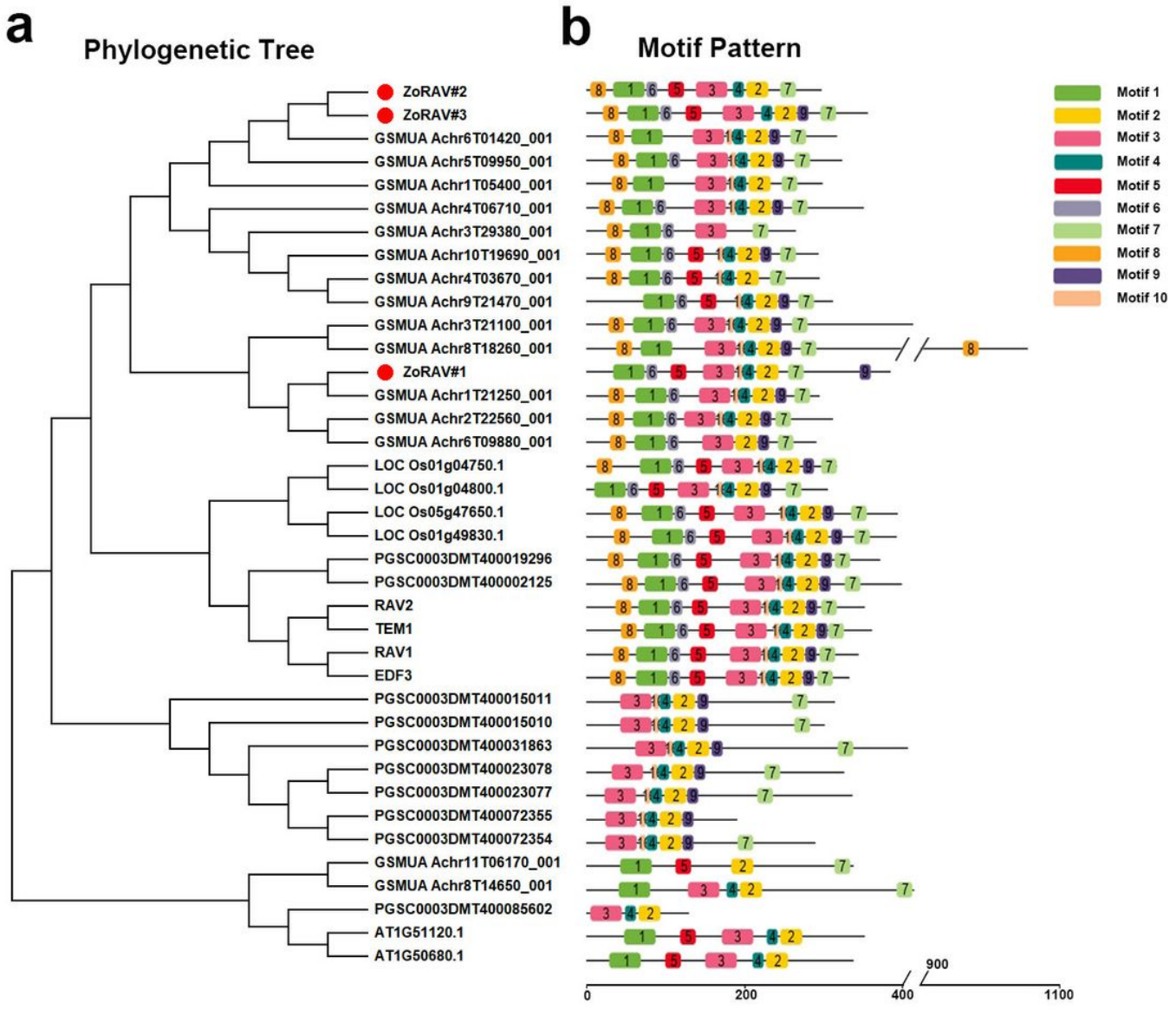

Figure 6

Phylogenetic relationships and motif compositions of RAV proteins from five different plant species. Left panel: An unrooted phylogenetic tree constructed using MEGA X with the neighbor-joining method. The red solid circles represent RAV genes from ginger. Right panel: Distribution of conserved motifs in RAV proteins. The differently colored boxes represent different motifs and their position in each RAV protein sequence.

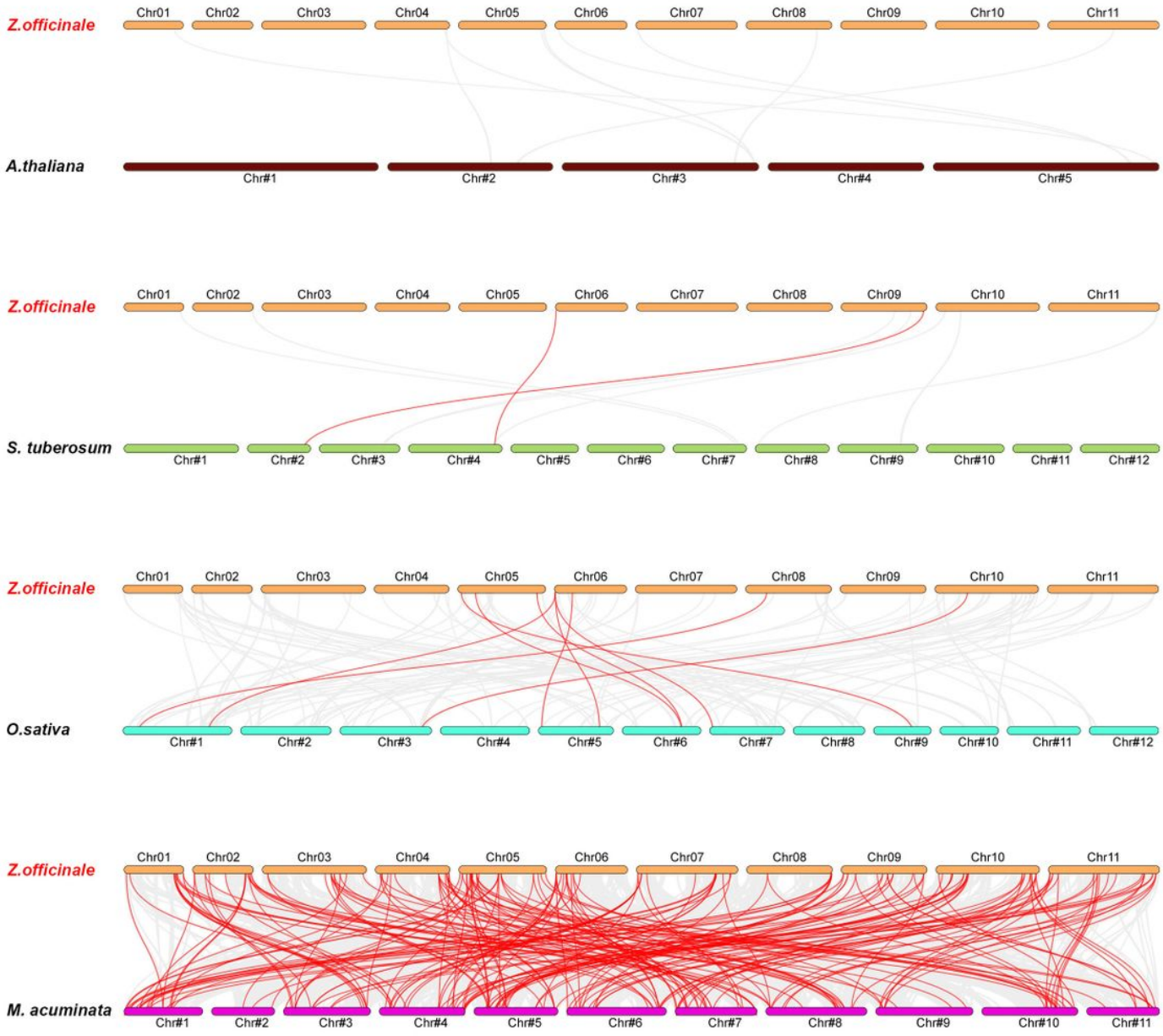

Figure 7

Synteny analysis of ERF genes between ginger and four representative plant species. The gray lines in the background indicate the collinear blocks within ginger and other plant genomes, while the red lines highlight the syntenic ERF gene pairs.

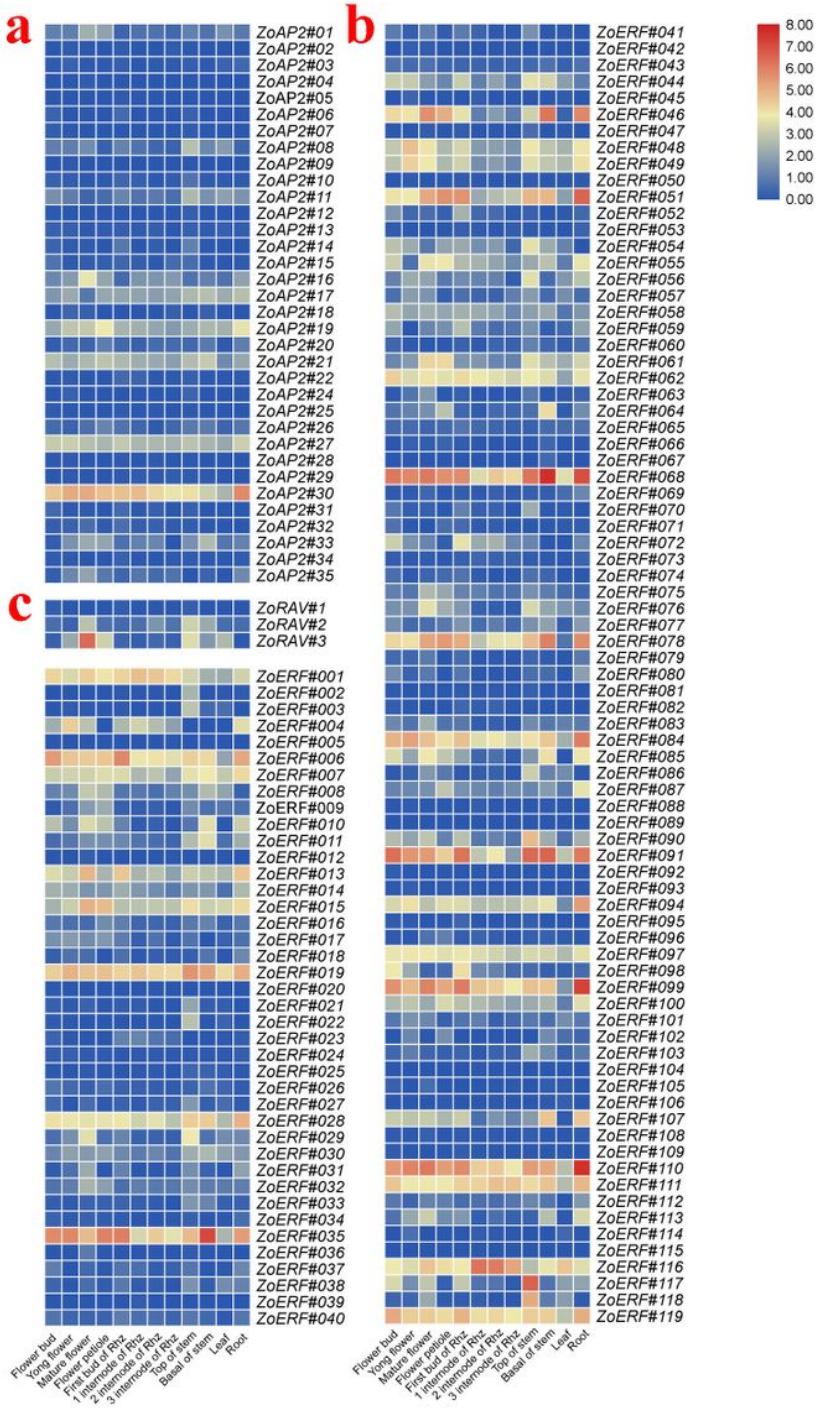

Figure 8

Expression profiles of the ginger AP2/ERF genes. a Hierachical clustering of expression profiles of ginger AP2/ERF genes in 12 samples including different tissues and developmental stages.

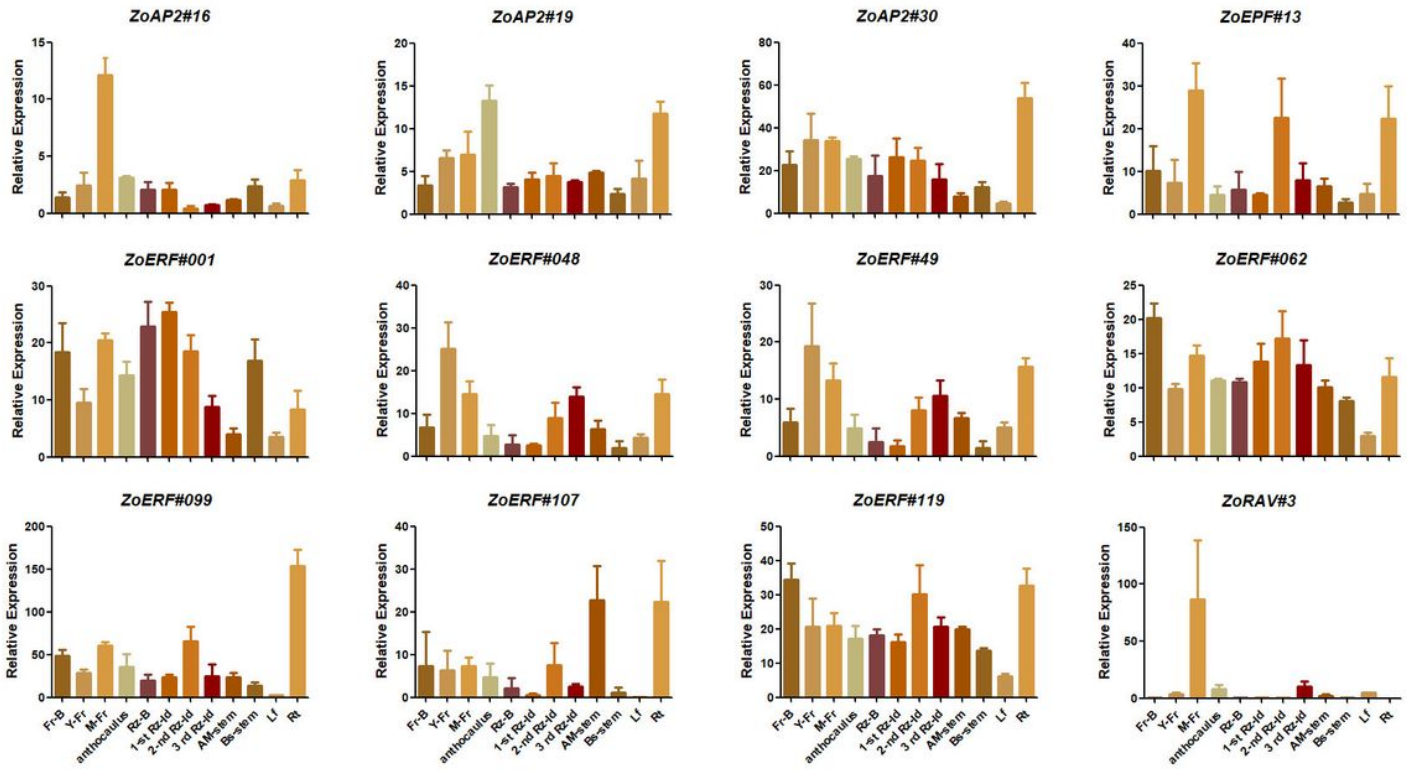

Figure 9

Expression analysis of 12 AP2/ERF genes in 12 samples by qRT-PCR. Data were normalized to TUB-2 gene and vertical bars indicate standard deviation.

Supplementary Files

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

- Additionalfile1FigS1SchematicrepresentationsforthechromosomaldistributionofgingerAP2ERFgenes.jpg
- Additionalfile2TableS1Listofthe163ZoAP2ERFgenesidentifiedinthisstudy.xls
- Additionalfile3FigS2AlignmentofmultipleZoAP2ERFandselectedAP2domainaminoacidsequences.jpg
- Additionalfile4FigS3AnalysisanddistributionofconservedmotifsingingerAP2ERFproteins..png
- Additionalfile5TableS2ListofthetandemrepeatgenepairsofAP2ERFinginger.xls
- Additionalfile6FigS4.AnalysisanddistributioofconservedmotifinAP2subgroupofgingerandotherspecies.png
- Additionalfile7FigS5AnalysisanddistributioofconservedmotifinERFsubgroupofgingerandotherspecies.png
- Additionalfile8FigS6AnalysisanddistributioofconservedmotifinRAVsubgroupofgingerandotherspecies.png
- Additionalfile9TableS3GingerandotherspeciesAP2ERFsyntenygenepairslist.xls
- Additionalfile10TableS4KaKsratio.xls
- Additionalfile11TableS5TheprimersequencesofqRTPCR.xls