Genome-Wide identification and Analysis of the AP2/ERF Gene Family in *Fragaria vesca*

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Abstract

Background: The AP2/ERF superfamily consists of primary transcription factors in plants that play a critical role in numerous aspects of various physiological stages and responses to stress stimuli. Nevertheless, there is little information related to AP2/ERF in strawberry, an important perennial fruit and model plant for horticulture. **Results:** In this study, 117 AP2/ERF genes were identified in strawberry and were grouped into four types of genes, AP2 (17), ERF (94), RAV (5) as well as soloist (1), according to the gene structure, phylogenetic tree and conserved domains. The duplication events and synteny analysis combination of genes offered a good opportunity to understand the evolutionary process of the FvAP2/ERF family. Moreover, identified orthologous genes and expression profiles of genes across various tissue, developmental stages and different treatments predicted potential functions of some AP2/ERF genes in strawberry.

Conclusions: In this study, 117 genes were identified in the AP2/ERF family of strawberry, and their structure, chromosomes location, evolutionary relationship, promoter sequence and expression profile were investigated. Our findings provide valuable clues to gain better insights into each FvAP2/ERF gene under different types of biological developments and in response to stressors.

Background

Plants face numerous environmental stressors, such as high salinity, drought, pathogen attack and wounding. For reproduction and survival under various environmental conditions, plants have to evolve a series of transcription factors to regulate their defense mechanism. For instance, the AP2/ERF transcription factors, a large gene family in green lineage, have essential roles in regulating plant growth, breeding and environmental pressures. AP2/ERF transcription factors belong to the AP2/ERF superfamily and include four types, AP2, ERF, RAV and soloist. In the AP2/ERF superfamily, the most prominent feature of gene sequences is the AP2 domain, which consists of about 6070 amino acids involved in DNA binding of down-stream genes [1, 2]. Based on the number of domains, the AP2/ERF proteins are classified into three families, those with double AP2 domains belong to the AP2 family protein, while those with a single AP2 domain belong to the ERF family protein, and the RAV family protein has a single AP2 domain and a single B3 domain that is a plant-specific DNA-binding domain. In light of differences in the promoter sequences of ERF genes, the ERF family is further divided into two major subfamilies, the ERF subfamily (ethylene-responsive transcription factor) and DREB subfamily (dehydration/CBF response element binding) [1].

Since the first AP2 gene involved in the development of flowers was cloned from *Arabidopsis thaliana*, numerous AP2/ERF genes have been identified in different plants [3]. Considerable evidence demonstrated that the AP2/ERF family has important functions in the regulation of a variety of biological processes relevant to growth, development and various responses to environmental pressures. TFs (transcription factors) from the AP2 family have been shown to participate in the regulation of plant growth and development, for example, flower development [4, 5], leaf shape and seed growth [6]. ERF

genes are predicted to be involved in the response to hormonal signal transduction [7], abiotic/biotic stress [8, 9] and plant developmental processes [10, 11]. Recently, many proteins of the RAV family in pathogen infections and abiotic stresses were identified [12, 13].

Cultivated strawberries (*Fragaria ananassa*) originated ~300 years ago; it is recognized as an important berry fruit in the horticultural industry and is among the youngest crop species [14]. After grape, its cultivation area ranks only second in the world. In contrast to cultivated strawberry and woody fruit trees, wild strawberry (*Fragaria vesca*) is a versatile experimental perennial plant system and an emerging model, owing to its small-sized genome [15]. Information obtained from studies of all aspects of plant growth, physiology, and biochemistry of *Fragaria vesca* should be applicable to or may inform studies on other horticulture plants.

With the increasing demand for the qualities of strawberry fruits, comprehending the potential regulation mechanism of plant growth and development has been a heated matter. The TFs from the AP2/ERF family play a crucial role in controlling plant growth and development. To date, the AP2/ERF genes have been extensively studied in various plants, including *Arabidopsis thaliana* [1], rice [1], pear [16], Musa [17] and apple [18]. However, there is poor knowledge about the AP2/ERF proteins in strawberry (*Fragaria vesca*). By reason of the significance of the AP2/ERF genes in various physiological mechanisms, it would be meaningful to conduct a systematic investigation of the AP2/ERF genes in strawberries. The recent ending of the wild strawberry genome sequencing offered an opportunity to identify and dissect the gene structure, evolutionary relationship and expression profile of AP2/ERF proteins at the genome-wide level [15]. In this study, 117 AP2/ERF genes of strawberry were identified and grouped into AP2, ERF, RAV and the soloist family. We completely investigated gene characters, including protein sequence length, isoelectric point, molecular weight, gene structure and chromosome distribution and conducted analysis of the evolutionary relationship of the FvAP2/ERF family through phylogenetic trees, gene duplication events and synteny analysis. Comprehensive expression investigations were carried out to determine the association of specific AP2/ERF family representatives in various biological developments of strawberry. These results offered significant indications for useful investigations of AP2/ERF family representatives in strawberry.

Methods

FvAP2/ERF family identification in strawberry

To obtain all AP2/ERF proteins in *Fragaria vesca*, we used two different methods, HMMSCAN and BLASTP. First, the hidden Markov model (HMM) file (PF00847) was downloaded from the Pfam database (<http://pfam.xfam.org/family/PF00847>). HMMER tool was used to search the potential AP2/ERF protein from the *Fragaria vesca* 4.0 genome (<https://www.rosaceae.org/organism/Fragaria/vesca>). The default parameters were adopted, and the cutoff value was set to 0.01. Second, the AP2/ERF protein sequences of *Arabidopsis thaliana* were adopted as queries to search for candidate AP2/ERF proteins by a BLASTP alignment against all wild strawberry protein sequences [1]. For all candidate protein sequences searched

by these two approaches, we first removed the incomplete and redundant protein sequences. Then, all candidate protein sequences with the AP2 domain were checked by the NCBI CDD tool. All candidate proteins had to contain at least one AP2 domain. Finally, 117 AP2/ERF protein sequences were identified in wild strawberry, and the sequence length, isoelectric point and molecular weight of FvAP2/ERF proteins were predicted by the ExPasy website (<http://web.expasy.org/protparam/>).

Phylogenetic analysis and classification of strawberry AP2/ERF genes

The full sequence of the identified AP2/ERF proteins in strawberry was used to set up multiple sequence alignments by MEGA X with default parameters. Based on the alignment full-length sequences of FvAP2/ERF proteins and the AP2/ERF classification scheme, all FvAP2/ERF proteins were classified into four families. The un-rooted phylogenetic tree was built by the Neighbor-Joining (NJ) method of MEGA X, with the following parameters: Poisson model, pairwise deletion and 1000 bootstrap replications.

Gene structures and motif composition analysis

The exon/intron structures of FvAP2/ERF genes were analyzed by the Gene Structure Display Server (GSDS: <http://gsds.cbi.pku.edu.cn>) [19]. Conserved motifs of FvAP2/ERF proteins were identified using the MEME online tool (<http://meme.nbcr.net/meme/intro.html>) [20], with the maximum number of motifs, 25.

Chromosomal distribution, gene duplication and syteny analysis of FvAP2/ERF genes

According to physical position information from the *Fragaria vesca* 4.0 genome database, all FvAP2/ERF genes were mapped to strawberry chromosomes. The gene duplication events of AP2/ERF in strawberry were obtained using the Multiple Collinearity Scan toolkit (MCScanX) with the default parameters [21]. The result of collinear analyses of the orthologous AP2/ERF genes from strawberry, *Arabidopsis thaliana*, tomato, grape, peach and apple was exhibited by Dual Syteny Plotter software.

Promoter sequences

The promoter sequences of FvAP2/ERF, which were 2000 bp upstream of the transcription start site, and the cis-elements in the promoter were researched using the PlantCARE online tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Plant growth conditions, RNA isolation and Illumina sequencing

Wild strawberry Hawaii4 (National Clonal Germplasm Repository accession # PI551572) [15] were grown in a growth chamber with 14 h light at 22 °C and 10 h dark at 20 °C. The fruits were harvested at the white, turning initial, turning middle and red stages, respectively and the stages were determined based on days post anthesis (DPA). The white stage is at 20 DPA, turning initial is at 25 DPA, turning middle is at 27 DPA and red stages is at 30 DPA. At least eight fruits were combined to form one biological replicate and three biological replicates were collected. Total RNA from samples (white stage, turning initial stage, turning middle stage and red stage) was isolated using RNAPrep Pure Plant Plus Kit (TIANGEN BIOTECH) and the RNA-seq data were generated from Illumina HiSeq™.

Data research and gene expression analysis

The expression profile data for each FvAP2/ERF gene in different tissues, organs and treatments were obtained from the NCBI SRA database (<https://www.ncbi.nlm.nih.gov/sra/>) or from less effective RT-PCR experiments because the number of AP2/ERF genes in strawberry was abundant and there were comprehensive verified RNA-seq data about strawberries in NCBI. And the ggplot2 tool of R Programming Language was used to produce heatmaps.

Results

Identification of FvAP2/ERF proteins in strawberry

A total of 152 sequences were matched as candidate FvAP2/ERF family genes based on HMMsearch and BLASTP search. Based on the result of the CDD tool, among these 152 genes, we removed 35 candidate genes without the AP2 domain. Finally, 117 complete genes were identified and annotated as being the AP2/ERF family of strawberry with complete AP2 domains. According to domain structures, 117 AP2/ERF genes were assigned into four families (see Figure 1). Ninety-two of 117 genes were grouped into the ERF family and encode one AP2/ERF domain. Fifteen genes were placed in the AP2 family and encode a protein that contains two AP2/ERF domains. Five genes were classified as the RAV family and encode a protein containing a single AP2/ERF and one B3 domain. Additionally, gene FvH4_2g18520 was assigned as a soloist and contains a single AP2 domain, the sequence of which has high homology to the soloist of *Arabidopsis* (AT4G13040.3). Interestingly, the two remaining genes, ERF-85 and ERF-48, encoded two AP2 domains but were distinct from the AP2 family and were more closely related to the ERF family. Thus, these genes were grouped into the ERF type. The AP2 family had similar situations in that AP2-13 and AP2-17 encoded a single AP2 domain, but their amino acid arrangements were more related to the AP2 family and were grouped into the AP2 family. In order to differentiate each family representative, these genes were assigned a new name on the basis of categorization of the family

as well as the arrangement of the chromosome locus. The results demonstrate that all 117 FvAP2/ERF genes were mapped on chromosomes 1 to 7 (see Figure 2).

Gene characteristics, including the protein molecular weight, the length of the protein sequence and isoelectric point, were predicted (Additional file 1). Within the AP2/ERF family, ERF-15 was found to be the smallest protein containing 136 amino acids, while the largest one was AP2-10 with 827 amino acids. The molecular weight of the proteins ranged from 15,147.75 to 89,808.32 kDa, and the isoelectric point ranged from 4.59 to 10.4.

Phylogenetic relationship, conserved motif composition and gene structure of the strawberry AP2/ERF family

The phylogenetic relationships of FvAP2/ERF were determined through multiple sequence alignment of full sequences of proteins. The phylogenetic relationship (Figure 3) demonstrated that the FvERF family could be grouped into ten groups corresponding to groups I to X according to the classification of the ERF group in *Arabidopsis*[1]. Among the 94 AP2/ERF proteins of strawberry, 3 belong to group I, 7 to group II, 14 to group III, 14 to group IV, 7 to group V, 10 to group VI, 10 to group VII, 8 to group VIII, 18 to group IX and 3 to group X. For reference, the current knowledge regarding the functions of the genes, the functions of which have already been verified by silence/overexpression of genes or mutant plant, in the AP2/ERF family is summarized in Table I. As shown in table I, AP2/ERF is mainly involved in the stress responses, phytohormone responses and plant growth, and similar sequences of proteins usually indicate the same function of genes. Table I reveals that most members of the FvERF family group III may regulate fruit development, and the ERF family groups IV and VI may be involved in stress responses, especially abiotic stress.

In order to acquire extra information about the AP2/ERF family in strawberry, the gene structures of all 117 FvAP2/ERF proteins were examined. As shown in Figure 3b, all RAV genes possess three exons, AP2 genes have three to eight exons (2 genes with three exons, 3 genes with four exons, 5 genes with five exons, 2 genes with six exons, 4 genes with 7 exons and 1 gene with eight exons), and most ERF genes have two exons (80 genes with two exons, 12 genes with three exons, 2 genes with six exons, 1 gene with seven exons and 1 gene with twelve exons). Additional investigation revealed that AP2/ERF genes hold a single intron in their AP2 and B3 domains. The location of introns was coincident with the alignment clusters of AP2/ERF genes, in general. There were no introns in the domains of the RAV family and ERF family (excluding group VII and ERF-45), while three types of intron phases were found in different AP2 genes (excluding AP2-13).

An illustration of the structure of all AP2/ERF proteins in strawberry was set up through the MEME motif study consequences. As shown in Figure 3c, motif 1 was the AP2 domain widely distributed in the AP2/ERF family, and motif 9 and 10 were the B3 domain; other than that, representatives of FvAP2/ERF in the uniform clusters were generally discovered to have similar motif compositions. For instance, the

array of motifs 4, 2, 1 and 3 was common for the ERF family (excluding group X), whereas motif 5 was unique to the AP2 family. The FvAP2/ERF from the uniform cluster, such as ERF-32, 31, 12 and 15; ERF-72, 56 and 01; ERF-92, 43 and 67, exhibited a seriously conserved motif composition, revealing that the protein structures were similar, and their functions were possibly conserved. In general, homologous gene structures and the similar motif distribution of the FvAP2/ERF representatives in the uniform cluster, as well as the consequences of the phylogenetic tree, could enhance the dependable results of the family and group classifications.

Chromosomal distribution, gene duplication and collinear analysis of the FvAP2/ERF gene family

To further investigate the relationship between the differentiation of genetic functions and gene duplication events in strawberry, the chromosomal location of each AP2/ERF gene was identified from the genomic data of strawberry. All 117 FvAP2/ERF genes were mapped on seven chromosomes (see Figure 2). The smallest number of FvAP2/ERF genes was found on chromosomes 1 and 3 (both 10 genes), whereas chromosome 6 contained the highest number of FvAP2/ERF genes (23 genes). The most ERF genes appeared on chromosomes 2, 5, 6 and 7 (16, 19, 16 and 17, respectively), there was an absence of AP2 genes on chromosome 5, and five members of the RAV family were mapped on chromosome 36.

Gene duplication usually plays a vital role in gene expansion and the occurrence of new gene functions; on this account, we analyzed the gene duplication events of the FvAP2/ERF family. Fourteen FvAP2/ERF genes were classified into seven tandem repeat event regions on strawberry chromosomes 2, 4 and 7. In addition to the tandem duplication events, seven segmental duplications with 14 AP2/ERF proteins were also found using BLASTP and MCScanX tools. Only two pairs of genes in the AP2 family were identified, and others were identified in the ERF family. These results suggested that certain FvAP2/ERF genes may be generated through gene tandem and segmental duplication events, which are keys that drive AP2/ERF gene evolution in strawberry.

In order to further infer the phylogenetic relationship of the strawberry AP2/ERF genes, we analyzed the collinear relationships of strawberry associated with six representative plants, including rice, *Arabidopsis*, tomato, grape, apple and peach (Figure 4). The results showed that FvAP2/ERF genes were collinear with apple (173), followed by peach (102), grape (75), tomato (60) and *Arabidopsis* (31) (Additional file 3–7). Among the rest, some syntenic pairs (with 11 FvAP2/ERF proteins) were found in both strawberry and the other plants, revealing these orthologous genes probably occurred earlier than the ancestral divergence. Moreover, some syntenic pairs (with 26 FvAP2/ERF genes) were identified in four berry plants, indicating that these genes possibly played vital roles during fruit development. Interesting, some AP2/ERF genes in strawberry were identified to be correlated with no less than two syntenic gene pairs (especially with peach and apple), such as ERF-05, ERF-06, ERF-11 and ERF-31, indicating these genes were possibly previously critical characters of the AP2/ERF gene family. It is worth noting that certain AP2/ERF syntenic gene pairs identified between strawberry and berry plants were anchored to the extremely conserved collinear blocks, the extents of which exceeded 110 genes, indicating these genes had similar functions.

Analysis of cis-acting elements in the promoters of AP2/ERF family genes

The different cis-acting elements in the promoters of genes might indicate that the functions of these genes are different. To better understand the FvAP2/ERF gene expression regulatory mechanisms, a 2000-bp genomic sequence upstream of the transcription start site in each AP2/ERF gene was used to search the PlantCARE databases. As shown in Figure 6, four types of cis-elements were identified in the promoters of FvAP2/ERF genes. The first was the responses of plant hormones, such as MeJA (methyl jasmonate), abscisic acid, gibberellin and salicylic acid and in particular, MeJA and abscisic acid responsive element, which are more ubiquitous in the promoter of the ERF family genes than in the AP2 and RAV family genes. For example, MeJA responsiveness accounted for fifteen percent of the total numbers of cis-elements in the ERF family, but in other families, eight percent of cis-elements were involved in MeJA responsiveness. Among these genes, ERF-87 took part in both MeJA and abscisic acid response, indicating that ERF-87 played a key role in phytohormone regulation mechanisms. Abundant stress-related responses elements, such as low temperature, drought, anoxic conditions and wounding, were found in the promoters of FvAP2/ERF genes. Interesting, most members of the FvAP2/ERF family were mainly involved in the anoxic response.

The third component was responses of plant development and growth. Some genes in the FvAP2/ERF family are involved in the expression of specific plant tissues, circadian control, flavonoid biosynthesis and cell cycle regulation. In addition, the response to light was the most important response element in the promoter of FvAP2/ERF genes. Sixty of 117 FvAP2/ERF genes contained at least 10 light response elements, which indicated AP2/ERF genes play key roles in the light regulations in strawberry.

Expression profile analysis of AP2/ERF of strawberry in different development stages and tissue

Despite the high numbers of members of the AP2/ERF family, we obtained abundant reliable data of FvAP2/ERF gene expression, including in the root, leaf, seed and flower (microspore, pollen, perianth, receptacle, carpel and anther) tissues from the NCBI short read archive (SRA) database (SRP035308), which was previously verified [65]. As shown in Figures 7 and 8, among 117 FvAP2/ERF genes, six genes (AP2-17, ERF-06/21/25/61/74) in the root, eight (ERF-06/20/25/27/61/74/75, RAV-02) genes in the seed, one (ERF-27) genes in the flower, three (AP2-01, ERF-/61/74) genes in the leaf and six (ERF-20/23/27/47/74/75) genes in fruit showed high transcript abundance (RPKM>100). Interesting, ERF-61 was expressed in most tissue, indicating that ERF-61 plays an important role in the vegetative growth of strawberry. ERF-74, ERF-75 and ERF-76 from chromosome six were expressed in most tissues and organs of strawberry, which suggested these genes are probably involved in plant development and breeding.

During the development of the flower, certain AP2/ERF genes are expressed in the flower and not in the pollen, such as ERF-25, ERF-27, ERF-39 and ERF-61, whereas in pollen, only two FvAP2/ERF genes, ERF-75 and ERF-93, were expressed. Significantly, ERF-75 plays a part in the various tissues and

stages of flower, which implies it perhaps is the primary regulatory gene of flower morphogenesis. To understand the comprehensive functions of FvAP2/ERF genes in different stages of the achene and receptacle, we obtained the RNA-seq data from SRA databases (SRA065786) [66] and sequenced different stage of fruits. As shown in Figure 8, 57 FvAP2/ERF genes were expressed in early-stage fruit development, and 16 FvAP2/ERF genes were expressed in fruit development and ripening. Among these genes, ERF-73, ERF-74 and ERF-75 were expressed across all stages of fruit development, and decreased transcript levels of ERF-25, ERF-27 and ERF-61 were similar during different stages of fruit development. Interesting, ERF-3, ERF-08, ERF-09, ERF-90 and ERF-91 were expressed only in different tissues of the achene (not the embryo), which revealed these genes may be responsive to the development of the achene. There were the remarkable expressions of ERF-20 and ERF-23 in fruit development and ripening but not in early-stage fruit development, which indicated they determined the quality of strawberry fruit. In general, these results showed that few AP2/ERF genes engage in physiological processes of most tissues, and transcription of most genes occurred in specific tissues.

Expression profile analysis of AP2/ERF in strawberry under abiotic and biotic stress

To investigate whether the expression levels of AP2/ERF family genes are regulated by exogenous abiotic and biotic stress, representative data of RNA-seq (*Phytophthora* stress, salt stress, salicylic acid treatment and methyl jasmonate treatment) from SRA databases (SRR3743193–3743198, PRJNA508389, SRR7157737 and SRR7157738) were obtained that were previously verified [14, 67, 68]. Expression profiles of individual genes in each treatment are shown in a heat map (Figure 9). After *Phytophthora* infection, 11 of 117 FvAP2/ERF genes showed significant changes. For example, transcription levels of AP2-17, ERF-06, ERF-21 and ERF-91 were repressed, whereas expression of ERF-11, ERF-17, ERF-25, ERF-29, ERF-55, ERF-89 and ERF-90 was induced by *Phytophthora*. Interesting, under the salt stress, sixteen FvAP2/ERF genes in the root were up-regulated, such as ERF-04, ERF-05 ERF-13, ERF-15, ERF-49, and transcription of only two genes was decreased obviously. Among the rest, RAV-02 was the most remarkable—the transcription level was 28 times higher than that in the control. ERF-04, ERF-50 and RAV-03 decreased significantly after MeJA treatment for 0.5 h (26-fold, 3-fold and 4-fold, respectively). Moreover, transcription levels of the AP2/ERF gene 4 h after treatment were similar to the previous levels except for RAV-03. After MeJA treatment for 24 h, four genes, AP2-09, AP2-13, ERF-08 and ERF-32, reached peak values and were more than 7-, 2-d, 40- and 2-fold higher than those, respectively, after MeJA treatment for 4 h. However, SA treatment hardly induced peculiar expression profiles of FvAP2/ERF genes, different from all other treatments considered. In general, the AP2/ERF family is involved in the regulation of stress response in strawberry, and diverse members played different roles under different stresses.

Discussion

Plant AP2/ERF genes are among the important transcription factors involved in the responses to environmental stressors and plant growth and reproduction. Previous studies on the AP2/ERF gene family have been reported for various plant species, such as *Arabidopsis thaliana* [1], rice [1], pear [16], Musa [17] and apple [18]. However, strawberry is a model plant for horticulture, and this family has never been identified in depth in strawberry. Therefore, in this study, we performed a genome-wide analysis of the AP2/ERF gene family in strawberry and identified 117 genes in the FvAP2/ERF family, 17 in the AP2 family, 94 in the ERF family, 5 in the RAV family and only 1 in the soloist family.

Evolution of the AP2/ERF gene family of strawberry

Gene duplication events in advanced plants are principal sources of evolutionary innovation and play pivotal roles in speciation [69]. Previous studies demonstrated at least 75 AP2/ERF genes in the common ancestor of extant angiosperms, while we only found 117 AP2/ERF genes in strawberry, the number of which was lower than that in *Arabidopsis* (147) [70], pear (191) [16], peach (131) [71], grape (149) [72] and apple (259) [18]. Furthermore, the number of both tandem duplication (11.9%) and segmental duplication (11.9%) events was seven pairs of genes in strawberry, far lower than that in *Arabidopsis*, pear, peach, grape and apple, which may be one of the reasons that the number of AP2/ERF genes in strawberry is low. These results also indicated the evolution rate of the AP2/ERF family in strawberry was slow and aligned with earlier studies. Davis et al. thought that the evolutionary track of the AP2/ERF gene family possibly experienced two phases: post-duplication functional divergence as well as a dull evolutionary pace of the process in general, which is on account of the stronger functional restriction after functional divergence [73, 74]. Furthermore, Semon et al. found that widely expressed genes were far more inclined to be restricted compared with those with narrower expression, which were aligned to the prior subfunctionalization of slow-evolving genes [75], and the other studies predict prior conservation of genes expressed in various tissues [76]. In strawberry, certain genes had similar situations, such as FvERF-74, FvERF-75 and FvERF-76, i.e., they were highly expressed in various tissues and were collinear with grape, apple and peach. Although they may be retained after duplication events and were from the same chromosomes, their functions were divergent under various environmental pressures (see Figure 9). During evolution processes, plants need to both develop and guard themselves to survive and breed by keeping balances between growth and defense [77]. Thus, some genes usually show different functions in different situations, for example, the gene of group III in Table I. We found abundant cis-elements of light response, anoxic specific inducibility and auxin response at the same time in promoter sequences of the FvAP2/ERF family. Although we currently predicted unknown genes through identified homologous genes, preferences of AP2/ERF genes in strawberry between growth and defense will depend on the environment to a great degree in the future.

Potential roles of FvAP2/ERF genes in plant growth and development

Genes from the uniform clusters have similar gene structures, which determine their behaviors. Thereby, comparison with identified functions of ERF genes from other species contributes to predict functions of candidate orthologous genes of strawberry. In *Arabidopsis*, TINY, AP2/ERF homologous genes repressed plant growth, and CBF2 can delay leaf senescence and extended plant longevity. Overexpression of SIAP2a, SIERF.B3 and SIERF6 in tomato resulted in a dramatic delay of fruit ripening, and ectopic expression of ERF36 reduced plant growth and early flower by controlling stomatal density. Similarly, some AP2/ERF genes of pear and peach activated bud break. In addition, most members of the AP2/ERF family contributed to or repressed ethylene synthesis, such as MdDREB1, LeERF1 and AdERF9. Although strawberries are considered to be a non-climatic plant and are insensitive to ethylene, FvERF is involved in the ripening of fruit. For instance, FvERF-06 was identified in regulating furaneol synthesis, a key aroma of fruit, by activating FaQR expression [78]. In this study, the phylogenetic tree of full-length amino acid sequences suggested the FvERF family is grouped into ten groups, consistent with the number of ERF groups in *Arabidopsis*. FvERF-06 and FvERF-61 are members of the same cluster in the phylogenetic tree; they showed similar profiles and were highly expressed in the root, seed and early stage of fruit development, indicating they not only control the development of fruit but also play a critical role in the root and seed. Furthermore, FvERF-06 was most closely related to *Arabidopsis* genes AtERF-60, AtERF-59 (WIN1) and AtERF-58 (WIN2). WIND1 was functionally characterized as activating shoot regeneration [79], exhibiting tissue-specific expression in callus [80] and participating in cell dedifferentiation as well as abiotic stress signaling, such as wounding [58, 79]. In promoter sequences of FvERF-06 and FvERF-61, abundance MeJA-responsiveness and anoxic specific inducibility cis-elements were found that revealed FvERF-06 and FvERF-61 may also be involved in response to abiotic stress. In addition, there were high transcript levels of FvERF-20 and FvERF-23 only in the development of fruit, which suggested they potentially determine the quality of fruit.

AP2 genes acted primarily in the regulation of floral development and determine the third whorl as the floral organ develops as petals or stamens [81]. In *Arabidopsis*, AP2 genes were identified to be involved in floral development, including sepals and petals [82, 83]. Theissen et al. found that overexpression of AP2 would lead to the production of double flowers, while silencing of AP2 function would result in stamens replacing petals [84]. Hence, the regulatory behaviors of AP2 in floral morphology of strawberry cannot be ignored. Some AP2 genes of strawberry, FvAP2-01, FvAP2-04 and FvAP2-05, were widely expressed in different tissues of the flower. In particular, FvAP2-01 was more preferentially expressed in the perianth, receptacle and carpel than the anther. Its homologous gene, RcAP2, can up-regulate the number of rose petals under low temperature. Thus, FvAP2-01 may take part in the formation of petals and sepals [85]. In addition, other than FvRAV-02, RAV genes in strawberry were barely expressed during plant growth and development, indicating the RAV family potentially has defensive responsibilities.

Potential roles of FvAP2/ERF in response to biotic and abiotic stressors

A series of AP2/ERF genes from various plants confer tolerance to multiple abiotic and biotic stresses when expressed ectopically in different plants [86, 87]. In this study, many stress response cis-acting elements, such as MeJA-responsiveness and anoxic specific inducibility, were frequently identified in the promoter regions of FvAP2/ERF genes (Figure 6), which demonstrated AP2/ERF genes play key roles in stress responses of strawberry. For instance, FvERF-08 has eight MeJA-responsiveness elements, and as shown in Figure 6, after 1 d of MeJA treatment, the transcription level of FvERF-08 peaked (Figure 9). Furthermore, RNA-seq data showed that six AP2/ERF genes were up-regulated after MeJA treatment, while after salicylic acid treatment, the FvAP2/ERF gene was insensitive to stresses, which could be caused by few salicylic acid responsiveness elements in the promoter (Figures 6 and 9). As the main plant hormones, JA and SA both play essential roles in regulating physiological mechanisms and mediating plant defense mechanisms against stress. Therefore, JA and SA are often used as simulated signals of plant stress. For example, low temperature can induce JA synthesis, and exogenous application of MeJA also increased endogenous JA accumulation in banana fruit [88]. Previous studies found JA was the central signaling compound in responses to necrotrophic pathogens and wounding, whereas in response to biotrophic pathogens, SA was the main signaling compound [89]. Thus, six up-regulated AP2/ERF genes in strawberry may be involved in abiotic and biotic stress responses related to MeJA, but FvAP2/ERF genes were barely induced by salicylic acid.

Crown rot induced by *Phytophthora cactorum* is one of the most common diseases of strawberry and hinders strawberry yield all around the world [67], which results in stunting and wilting of the plants as well as rotten fruits. In the defense mechanism of strawberry, seven AP2/ERF genes were up-regulated significantly, and four genes were down-regulated. It is worth mentioning that strongly up-regulated genes, ERF-25 and ERF-55, both belong to group III of the ERF family, and genes of this group primarily play defensive roles in plants according to identified genes in Table I. Thus, ERF-25 and ERF-55 are probably involved in responses to pathogens. Previous studies found StERF3 and GmERF5 are both involved in the defense mechanism of *Phytophthora* and salt stress in potato [87] and soybean [90], respectively. However, similar behaviors were not found in the AP2/ERF family of strawberry, which revealed the functional differentiation of AP2/ERF genes in strawberry may be different from that in potato and soybean.

Strawberry is sensitive to osmotic stress conditions, in particular salt stresses. Evidence from other species demonstrated that AP2/ERF genes are involved in regulating defense-related genes under salt stresses [91]. In this respect, 22 genes were obviously up-regulated, and 6 genes were down-regulated in the root of strawberry under salt stress (Figure 9), consistent with earlier studies that AP2/ERF genes showed diverse functions in response to salt stress [92]. Among these genes of strawberry, it is worth noting that the transcriptional level of RAV-02 was 28 times higher than that before treatment. Similar changes occurred in RAV genes in soybean, pepper and rice. In soybean, all genes from the RAV family were down-regulated other than GmRAV-03, which was up-regulated, and additional investigation revealed GmRAV-03 could strengthen the transgenic plants' tolerance to high salinity [93]. Following high salinity, CARAV1 and OsRAV2 (from pepper and rice, respectively) were up-regulated, and transgenic

plants were highly insensitive to the osmotic stress [94, 95]. Thus, the better salt stress confrontation strategies in strawberry may be due to the regulatory properties of the RAV-02 protein.

Conclusions

We performed a systematic analysis of the AP2/ERF family in strawberry and identified 117 AP2/ERF genes that were characterized and further grouped into three families AP2 (17), ERF (94), RAV (5) as well as soloist (1), according to the gene structure, phylogenetic tree and conserved domains. Moreover, through searching earlier published RNA-seq data, the expression profiles of FvAP2/ERF genes in various tissues, developmental stages, biotic stress as well as environmental pressures were gained. These results, combined with identified AP2/ERF genes in other species, revealed that AP2/ERF in strawberry is involved in the regulatory network of plant growth, reproduction and responses to stressors. In conclusion, our findings provide valuable clues to gain better insight into each FvAP2/ERF gene in different types of biological development.

Declarations

List of abbreviations

MeJA: methyl jasmonate; SA: salicylic acid; HMM: Hidden Markov Model; MEME: Multiple Em for Motif Elicitation; NJ: Neighbor joining; RPKM: Reads Per Kilobase per Million mapped reads;

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All RNA-seq data used in this article can be found in NCBI(accession numbers: SRR3743193–3743198, PRJNA508389, SRR7157737, SRR7157738 and PRJNA575936,). Others generated or analyzed during this study are included in this published article and the additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZK Li was responsible for the main part of data analysis and experimental design. JQ Chen, CM Chen and ZN Wang participated in the data analysis of RNA-seq. YH Hong and AY Zheng took part in sequence analysis of the promoter. QX Chen was responsible for manuscript revision. All the authors have commented, read and approved the final manuscript.

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Tables

Table I AP2/ERF genes whose biological function has been reported

Family	Gene	Involve biology progress	Species	References ^a
AP2 family	RAP2.2	hypoxia stress	Arabidopsis	1
	CitAP2.10	valencene synthesis	Orange	2
ERF family group I	AtERF6	drought stress	Arabidopsis	3
	SlERF5	drought and salt stress	Tomato	4
	LeERF4	fruit ripening	Tomato	5, 6
ERF family group II	AtERF5	Pathogen resistance	Arabidopsis	7
	SlERF36	Photosynthesis and growth regulation	Tomato	8
	pti6	Hormone and stress response	Tomato	9
	PpERF3	ABA biosynthesis	Peach	10
	AdERF9	Fruit ripening	Kiwifruit	11
ERF family group III	AtERF7	Absciscic acid response	Arabidopsis	12
	NtERF5	Disease resistance	Arabidopsis	13
	MdERF2	ethylene synthesis	Apple	14
	PpEBB	Bud Break	Pear	15
	pti4	Disease resistance	Tomato	16
	LeERF1	Fruit ripening	Tomato	17
	CitERF71	Fruit ripening	Sweet Orange	18
	MaERF11	Fruit ripening	Banana	19
	MsERF11	salt tolerance	Alfalfa	20
	AtERF4	Ethylene, jasmonic acid, and absciscic acid response	Arabidopsis	21, 22
ERF family group IV	AtERF98	ascorbic acid synthesis	Arabidopsis	23
	TSRF1	Pathogen resistance	Tomato	24
	pti5	Disease resistance	Tomato	16
	AtERF1	Disease resistance	Arabidopsis	25
	ABR1	Absciscic acid response	Arabidopsis	26
ERF family group VI	SlERF6	carotenoid and ethylene synthesis	Tomato	27
	LeERF2	Ethylene response and seed germination	Tomato	28
	JERF1	Salt tolerance	Tomato	29
	JERF3	Salt tolerance	Tomato	30
	CaERFLP1	Salt tolerance and disease resistance	Peper	31
	CaPF1	Freezing tolerance, disease resistance	Peper	32
	HRE1	anaerobic responses	Arabidopsis	33
	HRE2	anaerobic responses	Arabidopsis	33
	RAP2.2	Ethylene response and pathogen resistance	Arabidopsis	34
	ABI4	Absciscic acid response, sugar signaling	Arabidopsis	35, 36
ERF family group VII	WIND1	cell dedifferentiation	Arabidopsis	37
ERF family group VIII	WXP1	Wax accumulation	Alfalfa	38
ERF family group IX	MdDREB1	ethylene synthesis	Apple	39
	PpCBF1	Bud Break	Peach	40
	DDF1	Salt tolerance and GA biosynthesis	Arabidopsis	41
	TINY	Growth regulation	Arabidopsis	42
	CBF2	delays leaf senescence and extends plant longevity	Arabidopsis	43
	PpERF2	ABA biosynthesis and cell wall	Peach	44

^a1[22]; 2[23]; 3[24]; 4[25]; 5[26]; 6[27]; 7[28]; 8[29]; 9[30]; 10[31]; 11[32]; 12[33]; 13[34]; 14[35]; 15[36]; 16[37]; 17[38]; 18[39]; 19[40]; 20[41]; 21[42]; 22[43]; 23[44]; 24[45]; 25[46]; 26[47]; 27[48]; 28[49]; 29[50]; 30[51]; 31[52]; 32[53]; 33[54]; 34[55]; 35[56]; 36[57]; 37[58]; 38[59]; 39[55]; 40[60]; 41[61]; 42[62]; 43[63]; 44[64].

Figures

Figure 2

The locations of the AP2/ERF family genes on the strawberry chromosomes. The blue, orange, pink and purple colors represent ERF, RAV, AP2 and soloist genes, respectively. Each little green box indicates a duplication event.

Figure 3

Phylogenetic relationships, gene structure and architecture of conserved protein motifs in AP2/ERF genes of strawberry. a The phylogenetic tree was constructed based on the sequence of the FvAP2/ERF proteins by MEGA X software. Details of clusters are shown in different colors. b Gene structure of FvAP2/ERF genes. Green boxes indicate CDS; yellow boxes indicate the B3 domain; pink boxes indicate the AP2 domain; blue boxes indicate UTR. Black lines indicate introns, and the number indicates the phases of

corresponding introns. c The motif composition of FvAP2/ERF proteins. The twenty-five motifs are displayed in different colored boxes. The sequence information for motifs is provided in Additional file 2.

Figure 4

Schematic representations of the chromosomal distribution and interchromosomal relationship of strawberry AP2/ERF genes. Gray lines indicate all syntenic blocks in the strawberry genome, and the red lines indicate duplicated AP2/ERF genes pairs.

Figure 5

Synteny analysis of the AP2/ERF gene family between strawberry and five representative plant species. Gray lines in the background indicate the collinear blocks within the strawberry and other plant genomes, while the blue lines highlight the syntenic ERF gene pairs, the orange lines highlight the syntenic RAV gene pairs, the pink lines highlight the syntenic AP2 gene pairs, and the purple lines highlight the syntenic soloist gene pairs.

	MeJA-responsiveness	abscisic acid responsiveness	gibberellin responsiveness	salicylic acid responsiveness	gibberellin-responsive element	other auxin responsive	low temperature responsiveness	drought responsiveness	defense and stress responsiveness	anoxic specific inducibility	wound-responsive element	meristem expression	seed-specific regulation	endosperm expression	endosperm-specific negative expression	root specific	circadian control	palisade mesophyll cells	flavonoid biosynthetic genes regulation	cell cycle regulation	phytochrome down-regulation	expression	light response	MYB1H1 binding site
ERF-01	2	2		1	1	1	1																	7
ERF-02	2	1		1																				11
ERF-03	4	5		2	2	1	3																	11
ERF-04	4	1	1	1	3	1																		11
ERF-05	6	1	1	1	4	1	2	4																6
ERF-06	4	4	1	1	1	1	1																	17
ERF-07	4	2	1	1	2																			15
ERF-08	6	5		1		2																		14
ERF-09	8	4			1	1	1	2			2													8
ERF-10	2	2	1	1			1																	9
ERF-11	2	3					1																	11
ERF-12	4	1	1	1	3	5																		12
ERF-13	8	2		1	1																			8
ERF-14	6	1	1			3	1	4																6
ERF-15	4	1																						8
ERF-16	8	4	1	1	1	1	4																	2
ERF-17	6	2	2			1	1	1																1
ERF-18	2	1		2		3	1	1																8
ERF-19	4	2			1	4	1	3																16
ERF-20	4	3		1		2	3	1																1
ERF-21	6					4		1	1															12
ERF-22	5	1		1	1	1	5																	11
ERF-23	1				2	2	1	2																12
ERF-24	2	1	1	2	1		3	1																12
ERF-25	11			1	1	1	2																	2
ERF-26	2	7			2	1	2																	1
ERF-27	4	7			3	2	5																	6
ERF-28	2	2	1	1	1	1	2	1																14
ERF-29	4	2			1	3	1	1																12
ERF-30	6			1	2	1	1	2																7
ERF-31	8	3	2	1		1	1	1																9
ERF-32	4	3		3		1	1																	9
ERF-33	4	5	2	2		1	2	1	3															2
ERF-34	2	5			1	2	1	2	4															1
ERF-35	1	1		1	2	1	1	1	3															4
ERF-36	4	2	1			2																		9
ERF-37	3			1	1	1	2	5																13
ERF-38	2			1	1	4																		17
ERF-39	6	5	1	2	2	1	2																	13
ERF-40	4	4		2		1	1	3																9
ERF-41	6	4	1		1	2	1																	11
ERF-42	2	1	1	1	3	1	1	6																8
ERF-43	12	1				1	6																	16
ERF-44	6	1		2		1	1	2																11
ERF-45	6				2	1	2																	14
ERF-46	2	11	1	2		1	2																	2
ERF-47	2	1	1	2	1	2	1	2																13
ERF-48	2	1		2	1		1	6																11
ERF-49	1	2				2	3	1																11
ERF-50	4		2	2	1	1	5	1																1
ERF-51			1			1	6																	7
ERF-52	2	4			3	1	4																	12
ERF-53	3	1		4	1	1		2	1															11
ERF-54	2	2		1	2	1	2	1																7
ERF-55	1	4		1	1	1	1	5																11
ERF-56	1	2	3	1	1	2	1																	1
ERF-57	6	7		1	2	1	1	2																11
ERF-58	8	3			1	1																		1
ERF-59	8		1	1	1	2																		20
ERF-60	2	7	1		1	2	1	1																2
ERF-61	6	2		1	1		4																	14
ERF-62	4	9	1	1	2	2	3	2	1															17
ERF-63	2	6																						8
ERF-64	6	1		1		1	2	1	1															21
ERF-65	6	7			1	1	2																	4
ERF-66	4	2	2	1	1	1	2																	1
ERF-67	2	5		1		1	3																	8
ERF-68	4	1	3			1	1	1																17
ERF-69	8	8		1		1	1	3																17
ERF-70	2	4			1	2	2	7																17
ERF-71	8	1	1	1	2																			1
ERF-72	4	1	1	1	2	1																		11
ERF-73	6	6		1	1	1	2	2																14
ERF-74	4	3	1	1	1	1	3	1																13
ERF-75	2	4				1	3																	8
ERF-76	4		1	1	1	1	1	2	1	2	1													11
ERF-77	1	4	3	2		1	2	1	3															12
ERF-78	4	2		1	1	1	2	1	5															14
ERF-79	2		2	1	1	1	1	6																6
ERF-80	4	5		3			2	2																17
ERF-81	6	2	1	1	1			1																9
ERF-82	12	2			1	1	3																	8
ERF-83	2	2		1	2	1	4																	12
ERF-84					2	1	2	2																13
ERF-85						3																		7
ERF-86		1	1			6		1	1															9
ERF-87	12	12			3	1	5																	21
ERF-88	2	12	1	1	2	1	3																	16
ERF-89	6	5		1	1	1	2																	1
ERF-90	4	8		1	1		5																	18
ERF-91	4	2	2			1	2	1																13
ERF-92	8	5		2	1	1	2	1																9
ERF-93	4	1			2	2	1	4																4
ERF-94					2		1	2	2															4

	MeJA-responsiveness	abscisic acid responsiveness	gibberellin responsiveness	salicylic acid responsiveness	gibberellin-responsive element	other auxin responsive	low temperature responsiveness	drought responsiveness	defense and stress responsiveness	anoxic specific inducibility	wound-responsive element	meristem expression	seed-specific regulation	endosperm expression	endosperm-specific negative expression	root specific	circadian control	palisade mesophyll cells	flavonoid biosynthetic genes regulation	cell cycle regulation	phytochrome down-regulation	expression	light response	MYB1Hv1 binding site
AP2-01		2	3	2				1	1	1					1		1					13		
AP2-02		1		2				2	1	2				1				1				5		
AP2-03	4			1		1	3			1												1		
AP2-04	2						1		1	1								1				1		
AP2-05	2	3			1	1		2	1		1	1										11		
AP2-06	2	2	1		2		1	2		1												11		
AP2-07	6	1	1					2		2												16		1
AP2-08	2	4							1	1				2								13		
AP2-09	2	2					1	2		1		2	2				1					11		
AP2-10	2	2	1							3		3	2						1			9		
AP2-11	2	1				1	1			2				2								12		
AP2-12	2	4					1			3												14		
AP2-13	2	3			1	1		2	1	2												7		
AP2-14	2	3		1		1			1	1		1		1								9		
AP2-15	2			1	1	1	1	2		5	1	1			1							3		
AP2-16	2	1	2	1	1							1										18		
AP2-17	2	2		1		1	3	2		3		1					1					11		
RAV-01	2	2						1	1	3												11		
RAV-02		3		1	2	3				1													1	1
RAV-03		2	3	1			1		1	3		2		1								8		1
RAV-04		6		1		1				1		1										14		
RAV-05		5	2					1		4									1			12		
SOL.OIST		2	1	1		1	1			4									1			9

Figure 7

Expression pattern of identified FvAP2/ERF in root, leaf and flower (microspore, pollen, perianth, receptacle, carpel and anther).

Figure 8

Expression pattern of identified FvAP2/ERF in early-stage fruit development (achene: embryo, ovule, seedling, ghost and wall; receptacle: cortex and pith.) and fruit development and ripening (green stage, white stage, turning stage and red stage).

Figure 9

Expression profiles of AP2/ERF genes in response to various abiotic and biotic stress treatments.

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