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Full Length Article

Genome-Wide Characterization and Expression Analysis of the *Growth Regulating Factor* (*GRF*) Gene Family in Strawberry (*Fragaria vesca*)

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Abstract

As one of the transcription factors only found in plants, the growth regulating factor (GRF) gene family has been reported in some plant species, but information on this gene family in strawberries remains unclear. Here, *Fragaria vesca GRF* (*FvGRF*) genes were systematically studied, including chromosomal location, gene structure, conserved motif, phylogenetic, expression profiling, post-transcriptional regulation, and functional analyses. The identified 10 *FvGRFs* were phylogenetically classified into two groups and five subgroups. Of these, nine *FvGRFs* were distributed on the five chromosomes, while *FvGRF2* was located on the scf0512956. Motifs 2 and 1 corresponding to QLQ and WRC domains existed in all the FvGRF proteins. *FvGRFs* showed different expression patterns based on RT-qPCR analyses, for example, *FvGRF1*, *FvGRF3*, *FvGRF6* and *FvGRF8* were predominantly expressed in buds and blooming flowers, *FvGRF4* and *FvGRF5* were mainly expressed in young leaves, indicating that the roles of these genes are diverse and redundant in strawberry growth and development. Furthermore, *FvGRF2* and *FvGRF8* were experimentally validated to be the targets of strawberry miR396, suggesting the significance and conservation of miR396 in post-transcriptional regulation of *FvGRFs*. These results provide fundamental knowledge for further functional analyses of *FvGRFs* in strawberries. © 2021 Friends Science Publishers

Keywords: Growth regulating factor; Phylogenetic analysis; Expression profiles; Post-transcriptional regulation; functional analysis; Strawberry

Introduction

Growth regulating factor (GRF) is one of the transcription factors only found in plants and has important functions in the plant growth, development and the stress response (Omidbakhshfard *et al*. 2015). The first *GRF* gene (*OsGRF1*) was found in *Oryza sativa* which has been found to play an important role in regulating the length of stems (Knaap *et al*. 2000). Since then, the *GRF* gene family has been reported in other plant species, such as *Arabidopsis thaliana* (Kim *et al*. 2003), Chinese cabbage (*Brassica rapa*) (Wang *et al*. 2014), poplar (*Populus trichocarpa*) (Cao *et al*. 2016), oilseed rape (*Brassica napus*) (Ma *et al*. 2017), apples (Zheng *et al*. 2018), tobacco (*Nicotiana tabacum*) (Zhang *et al*. 2018), soybean (*Glycine max*) (Chen *et al*. 2019) and so forth. The members of the *GRF* gene family are few; for examples, nine *GRFs* are found in *A. thaliana*; 12, in *O. sativa*; 17, in *B. napus*; 20, in poplar; and 22, in *G. max*.

In the N-terminal regions, the GRF proteins have the conservative glutamine leucine glutamine (QLQ) and tryptophan arginine cysteine (WRC) domains (Choi *et al*. 2004). In *A. thaliana*, the QLQ conserved domain and GRF interacting factors (GIF) form a transcriptional co-activator (Lee *et al*. 2018), while the WRC domain consists of a functional nuclear localization signal (NLS) and a DNA-binding domain (Kim *et al*. 2003). The expression level of *GRF* genes is higher in young tissues or organs—like stem tips, flower buds, and young leaves—than in their mature counterparts (Ma *et al*. 2017). *GRF* genes play a critical regulatory role in the growth and development of these tissues or organs. For example, in *A. thaliana*, the overexpression of *AtGRF1* and *AtGRF2* made the leaf and cotyledon larger and the inflorescence stem bolting later (Kim *et al*. 2003). The overexpression of Chinese cabbage *BrGRF8* regulated the leaf and other organs size in transgenic *Arabidopsis* by the change of cell proliferation (Wang *et al*. 2014). In maize, the overexpression of

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ZmGRF10 decreased leaf size and plant height through the change of cell proliferation (Wu *et al*. 2014). In *O. sativa*, *OsGRF4* regulates grain shape, panicle length and seed shattering (Sun *et al.* 2016). In *B. napus*, *GRF2* was found to play a role in seed oil yield by the change of cell number and plant photosynthesis (Liu *et al*. 2012).

Additionally, another important molecular mechanism regarding *GRF* genes is the targets of microRNA396 (miR396) (Omidbakhshfard *et al*. 2015). It is well-known that the miR396-GRF regulatory module that operates in various developmental processes. For example, in *Arabidopsis*, miR396-targeted *AtGRFs* are critical for the development of leaves (Wang *et al*. 2011), and also regulates the cell transition from root stem to transit-amplifying (Rodriguez *et al*. 2015). MiR396 and GRF-GIF complex play an important role in controlling carpel number and pistil development (Liang *et al*. 2014). In *O. sativa*, *OsmiR396d*-targeted *OsGRFs*, together with *OsGIF1*, are associated with floral organ development (Liu *et al*. 2014). *OsmiR396* and its *OsGRF4* target control size and yield of grains (Duan *et al*. 2015; Li *et al*. 2016). *OsmiR396* and *OsGRF8* associate with *OsF3H* to mediate resistance to the brown planthopper by regulating flavonoid contents (Dai *et al*. 2019). However, the functions of *GRFs* and the miR396-GRF module are yet to be further investigated, especially in more economically important crops.

The *Fragaria* × *ananassa* Duch. (*F. ananassa*), with high nutritive and commercial value, is well-known as an octoploid hybrid of two wild octoploid species that have the same ancestor with the woodland strawberry —*Fragaria vesca*, a diploid (Shulaev *et al*. 2011). Therefore, the woodland strawberry is closely related with the cultivated strawberry in genetic terms (Shulaev *et al*. 2011), and its sequence is often used for a genome-wide analysis of genes. Information on *GRFs* in strawberries is currently limited. Although Omidbakhshfard *et al*. (2015) reported that 10 *GRF* genes were present in *F. vesca*, further information on this gene family in strawberries was lacking. Therefore, to get knowledge of the role of *GRF* genes in strawberries, the *GRF* gene family was systematically analyzed in woodland strawberry. Here, the molecular features, expression patterns and post-transcriptional regulation of *GRFs* in *F. vesca* were analyzed and their functions were predicted. The results provide valuable insight into the roles of *GRFs* in the regulation of strawberry plant growth and development.

Materials and Methods

Whole-genome identification and chromosomal distribution of *FvGRF* genes

First, the protein sequences of hypothetical GRF transcription factors in the *F. vesca* accession'Hawaii-4' were downloaded from the Plant Transcription Factor Database (PlantTFDB) (http://planttfdb.cbi.pku.edu.cn/), and were then used as a query to do BLAST-P searches with an e-value of e^{-10} in the strawberry genome (*F. vesca* Annotation Release 101) of the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/), as described previously by Wei *et al*. (2016). The gene with the highest similarity was then chosen, and the gene's location in chromosomes could be obtained from the NCBI database. Finally, conserved domains of FvGRFs were identified in the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The isoelectric points and the molecular weight of the amino acids of FvGRFs were obtained from the ExPasy website (http://web.expasy.org/protparam/).

Analysis of gene structure and motifs of FvGRFs

The genomic sequences and cDNA sequences of *FvGRFs* were compared using the online Gene Structure Display Server 2.0 (GSDS 2.0) software (http://gsds.cbi.pku.edu.cn/) to infer the exon and intron organization. The multiple alignments of the FvGRF protein sequences were done using the DNAMAN8 software (https://www.lynnon.com/). The conserved motifs of the amino acid sequences of FvGRFs were researched using the MEME database (http://meme-suite.org/tools/meme) with the width of optimum motif ≥ 6 and ≤ 100 as well as the maximum number of motifs =3. These were done based on the methods described by Wang *et al*. (2019) with a few minor modifications.

Phylogenetic analysis of amino acid sequences of GRFs from *F. vesca* and *A. thaliana*

The amino acid sequences of the AtGRF family members of *A. thaliana* were obtained from PlantTFDB. A phylogenetic tree for *F. vesca* and *A. thaliana* was constructed using the MEGA5.1 software (http://www.megasoftware.net) by the neighbor-joining (NJ) method with the Jones–Taylor–Thornton (JTT) model and 1000 bootstrap replications.

Real-time quantitative PCR (RT-qPCR)

The seeds of *F. vesca* 'Hawaii-4' were sown in polyethylene pots (bottom diameter 16 cm; top diameter 15 cm; height 11 cm) in a greenhouse at Qingdao Agricultural University. The roots, stems, young leaves, mature leaves, buds and blooming flowers of the *F. vesca* 'Hawaii-4' were collected for the expression analysis of *FvGRF* genes. All of the plant samples were stored at –80°C until use. Total RNA was extracted from the prepared samples using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa Bio, Japan) on the base of the manufacturer's instructions. First-strand cDNA synthesis and RT-qPCR were carried out with the HiScript® II One Step RT-PCR Kit and ChamQ™ SYBR® qPCR Master Mix (Vazyme, China), respectively. The reaction was performed on the BIO-RAD CFX96 sequence

detection system. The specific primers are shown in Table 1. *Actin* was used as a reference gene. The Mir-X miRNA qRT-PCR TB Green® kit (TaKaRa Bio, Japan) was used to assay for the expression of fve-miR396e in different organs or tissues of *F. vesca*. The primers are shown in Table 1. A 20 μL RT-qPCR reaction solution (cDNA template 2 μL, SYBR Green 10 μL, 10 μM forward and reverse primers 1 μL each, double-distilled water 6 μL) was applied. The amplification procedure was as follows: primary denaturing at 95°C for 30 s; 40 cycles denaturing at 95°C for 15 s and annealing at 60°C for 30 s; and elongating at 72°C for 30 s. The gene expression levels were evaluated by the $2^{-\Delta\Delta Ct}$ method (Li *et al*. 2019). Each reaction was repeated with three independent biological and technical replicates.

Statistical analysis

Statistical analysis was performed using SPSS with ANOVA (analysis of variance) (Version 19.0, IBM, USA). $P < 0.05$ was regarded as statistically significant.

Prediction and validation of miR396 target genes

All mature sequences of miR396 from *F. vesca* were downloaded from miRBase database (http://www.mirbase.org/). Target sites of miR396 in *FvGRF* genes were obtained from the online psRNATarget server (http://plantgrn.noble.org/psRNATarget/) with default settings. The maximum expectation was 3.0, and the target site accessibility evaluation by calculating unpaired energy (UPE) was 25. MiR396 cleavage sites in *FvGRF* genes were verified by the modified RNA ligase-mediated rapid amplification of 5′ cDNAs method (5′ RLM RACE) (SMARTer RACE 5′/3′ kit, TaKaRa Bio, Japan) (Li *et al*. 2019) based on the manufacturer's instructions. The nesting and nested primers (GSP and NGSP, respectively) were shown in Table 2. The primary PCR amplifications and the nested PCR amplifications were carried out as described previously by Li *et al*. (2019). The primary PCR amplifications were done with the nesting gene-specific primers GSP and the 5′ RACE Universal Primer Mix. The nested PCR amplifications were done with the nested gene-specific primers NGSP and the 5′ RACE Nested Universal Primer. The products of nested PCR amplification were purified, and then connected to the pMD-19T vector (TaKaRa Bio, Japan) to analyze DNA sequences (Sunny Bio, China).

Results

Identification and chromosome distribution of *FvGRF* genes

Totally, 10 *GRF* genes were identified in *F. vesca*; they were named from *FvGRF1* to *FvGRF10*, based on the gene ID in the NCBI database. High variation was in the coding sequence (CDS) lengths of these 10 *FvGRFs*. For example, *FvGRF4* was the longest at 1779 bp and *FvGRF3* was the shortest at 987 bp; the protein lengths were from 328 (FvGRF3) to 592 aa (FvGRF4). Moreover, the theoretical isoelectric point (*p*I) of the FvGRFs is from 6.09 to 9.25, and the molecular weight (Mw) is from 36.74 to 64.07 kDa, respectively (Table 3). Based on the available *FvGRF* gene distribution, the 10 *FvGRFs* were not evenly distributed across the five chromosomes and one scaffold. This is similar to the previous results in *Arabidopsis*, rice and Chinese cabbage (Choi *et al*. 2004; Wang *et al*. 2014). Both the LG2 and LG5 chromosomes have only one *FvGRF* gene each (*FvGRF5* and *FvGRF7*, respectively). While both the LG1 and LG6 chromosomes have two *FvGRF* genes each (*FvGRF1*, *FvGRF4* and *FvGRF3*, *FvGRF6*). The LG7 chromosomes had three *FvGRF* genes (*FvGRF8*, *FvGRF9* and *FvGRF10*) and the scf0512956 had one *FvGRF* gene, named *FvGRF2* (Table 3).

Table 1: qRT-PCR primers used for analysis of *FvGRFs* and fve-miR396e

Gene name	5'→3'
FvGRF1	forward: CCTCCTTGTTTTTGGACTCTGC
	reverse: TGCATGCTCATCCACCTCTTC
FvGRF2	forward: TTGATGGAGGCACAGCTACAC
	reverse: CTAACATTCACATTCACCATTCCAC
FvGRF3	forward: TCCAGACTCTTCCCTCATCACC
	reverse: GTATGCTTCCTTTGAACACCTCC
FvGRF4	forward: CTCCTCCTCCTGCTGATGC
	reverse: CTCTGATTGCGACGATTCTACC
FvGRF5	forward: GGAGTAAGCAGCAGTGTGGAGC
	reverse: ATGACCCTAACGAGGAAGGACTG
FvGRF6	forward: ATCTACTACCACCACCACCGC
	reverse: CAGCCAGCATGTACCTGAATATC
FvGRF7	forward: CTGTTCCTCCCGAGCTCTTG
	reverse: CACTTCTTGCCATCTGTCCTG
FvGRF8	forward: GATCAAAGACGTGACGGTGG
	reverse: AGAGAGGTTGAGTTGTGATGATGAG
FvGRF9	forward: CTGCTCCGTTTCAGCTTGTG
	reverse: GGAACTACATCCCTTCTACACCTC
FvGRF10	forward: GGTAACAGTACTGGGAATCTGATGG
	reverse: AGCACCTCCATTTCTTGCCATC
Actin	forward: TGGGTTTGCTGGAGATGAT
	reverse: CAGTAGGAGAACTGGGTGC
fve-miR396e	forward: TTCCACAGGCTTTCTTGAACT

Table 2: Primers used for analysis of fve-miR396e-directed cleavage of targets

Gene name	5' RACE (5'→3')
FvGRF2	GSP:GTGACCTCTGACTCTGTAGACCTTGGC
	NGSP:TGGTTAGAAACAGCAACAGAGGCG
FvGRF8	GSP:CACTCTTGCTCTGAACGCTGGCCG
	NGSP:CCGTACAATCCATCAATGAAAGAGTC

Gene structure analysis of *FvGRF* genes

The evolutionary relationship of gene members can be reflected by gene structures. Genes with similar gene structures tend to present in the same group. The number and location of the exons and introns of each gene can be

Table 3: Characteristics of *GRF* genes in *F. vesca* and *A. thaliana*

Name	Gene ID	Accession no.	Location	CDS (bp)	No. of aa	pI	Mw (kDa)
FvGRF1	101291561	XM_004287574.2	LG1:6644639-6642614	1110	369	8.4	41.54
FvGRF2	101291590	XM_011472589.1	scf0512956:463094-460771	1425	474	8.99	52.63
FvGRF3	101297752	XM_004303639.2	LG6:24416145-24414373	987	328	8.83	37.54
FvGRF4	101298840	XM_004289318.2	LG1:14704057-14700986	1779	592	6.09	64.07
FvGRF5	101299835	XM_004292721.2	LG2:20113962-20110483	1728	575	9.06	62.12
FvGRF6	101302177	XM_004302969.2	LG6:12933962-12931544	1104	367	8.75	40.26
FvGRF7	101303330	XM_011466751.1	LG5:19886658-19890251	993	330	9.12	36.80
FvGRF8	101310465	XM_004307858.2	LG7:20824850-20822348	1632	543	8.47	58.52
FvGRF9	101313153	XM_004306853.2	LG7:6608529-6605856	1338	445	9.25	48.30
FvGRF10	101313648	XM_004307789.2	LG7:20179705-20183028	1005	334	7.12	36.74
AtGRF1	816815	AT2G22840	LG2:9728480-9731301	1593	530	9.68	56.40
AtGRF2	829930	AT4G37740	LG4:17725337-17727909	1608	535	8.89	58.58
AtGRF3	818213	AT2G36400	LG2:15270088-15273115	1197	398	8.51	43.71
AtGRF4	824457	AT3G52910	LG3:19615977-19618507	1143	380	7.37	42.53
AtGRF5	820609	AT3G13960	LG3:4608076-4610497	1194	397	8.20	44.70
AtGRF6	815176	AT2G06200	LG2:2426176-2427355	735	244	8.80	28.21
AtGRF7	835447	AT5G53660	LG5:21794177-21796092	1098	365	8.18	40.41
AtGRF8	828515	AT4G24150	LG4:12535972-12539576	1482	493	6.93	54.61
AtGRF9	819156	AT2G45480	LG2:18745249-18747634	1290	429	8.18	48.61

Note: XM_, predicted model of mRNA; LG, linkage group; scf, scaffold; CDS, coding sequence; aa, amino acids; pI, theoretical isoelectric point; Mw, molecular weight

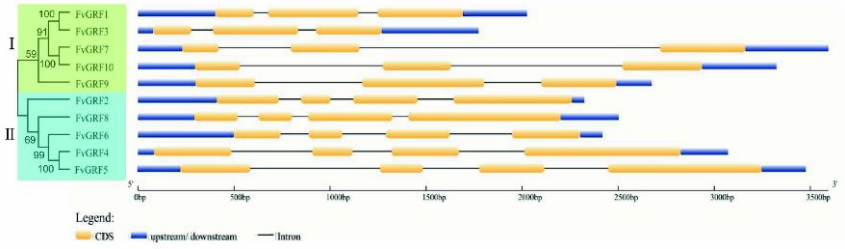

Fig. 1: Exon-intron structures of FvGRF genes and their phylogenetic relationships. The exon-intron structures of these genes were graphically displayed by the Gene Structure Display Server 2.0 using the cDNA sequence and genome sequence of *FvGRF* genes. The neighbor-joining (NJ) tree under the Jones-Taylor-Thornton (JTT) model was constructed using MEGA5.1 based on the full-length protein sequences of FvGRFs

elucidated through comparison of full-length cDNA sequences with the corresponding genomic DNA sequences (Kawaura *et al*. 2009). To understand the evolutionary relationship, we therefore analyzed the arrangement of the exons and introns of the *FvGRF* gene sequences using the GSDS 2.0 program. The results showed that *FvGRF1*, *FvGRF3*, *FvGRF7*, *FvGRF9* and *FvGRF10* belong to the I group and have three exons and two introns, of which *FvGRF1* and *FvGRF3*, *FvGRF7* and *FvGRF10* have closer relationships; *FvGRF2*, *FvGRF4*, *FvGRF5*, *FvGRF6* and *FvGRF8* belong to the II group and have four exons and three introns, of which *FvGRF4* and *FvGRF5* are clustered in a small clade (Fig. 1).

Conserved domains and motifs of FvGRF proteins

The previous studies have shown that the QLQ and WRC domains are present in the GRF proteins (Omidbakhshfard *et al*. 2015). Based on this information, the multiple sequence alignments and the conserved motifs of FvGRF proteins were analyzed. The results showed that motifs 2 and 1 corresponded to QLQ and WRC domains and existed in all the 10 FvGRF proteins (Fig. 2). Motif 3 was present in nine out of the 10 FvGRF proteins and was missed in the FvGRF9 (Fig. 2B). According to the phylogenetic tree, some FvGRF proteins belonging to a clade usually had similar motif structures; for example, FvGRF1/FvGRF3, FvGRF4/FvGRF5 and FvGRF7/FvGRF10 had similar motif structures (Fig. 2B).

Phylogenetic relationships of GRF proteins from *A. thaliana* and *F. vesca*

To gain knowledge about the evolutionary relationship of the strawberry *GRF* gene family, the full-length GRF protein sequences from *A. thaliana* and *F. vesca* were used to construct the phylogenetic tree. These GRF family genes were divided into two groups (I and II) and five subgroups (from G1 to G5 subgroups) (Fig. 3), which is similar to the previous results (Kim *et al*. 2003; Cao *et al*. 2016; Shang *et al*. 2018). The G4 and G5 subgroups belonged to the I group, and the G1, G2 and G3 subgroups were clustered in the II group. There were 8 and 11 GRF members in the I and II groups, respectively (Fig. 3). Furthermore, FvGRF2, FvGRF8, AtGRF7 and AtGRF8 were classified in the G1 subgroup and FvGRF4, FvGRF5, AtGRF1 and AtGRF2

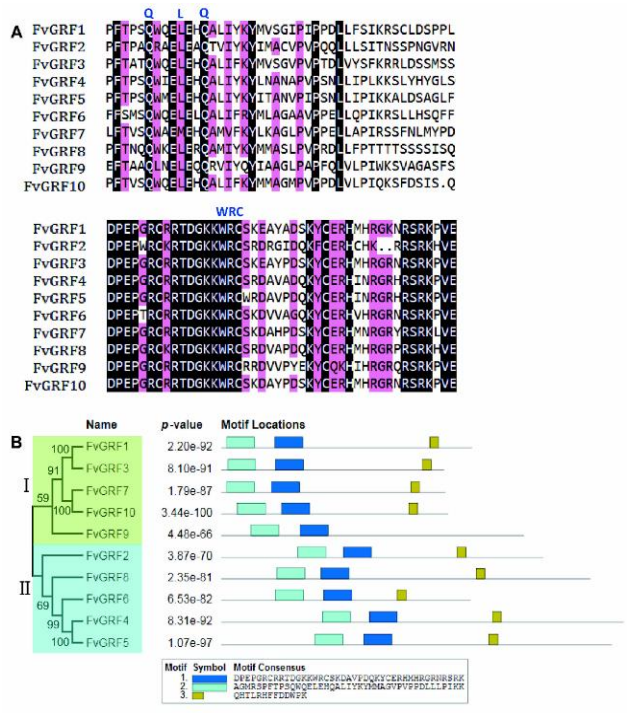

Fig. 2: Conserved domains and motif compositions of FvGRFs. Conserved domain (**A**), phylogenetic relationships and motif compositions (**B**) of FvGRFs. The multiple sequence alignments of FvGRF proteins were performed using the software of DNAMAN8. The neighbor-joining (NJ) tree under the Jones-Taylor-Thornton (JTT) model was constructed with 1000 bootstrap replications using MEGA5.1 based on the full-length protein sequences of FvGRFs. The conserved motifs of FvGRFs were predicted using the MEME Suite web server

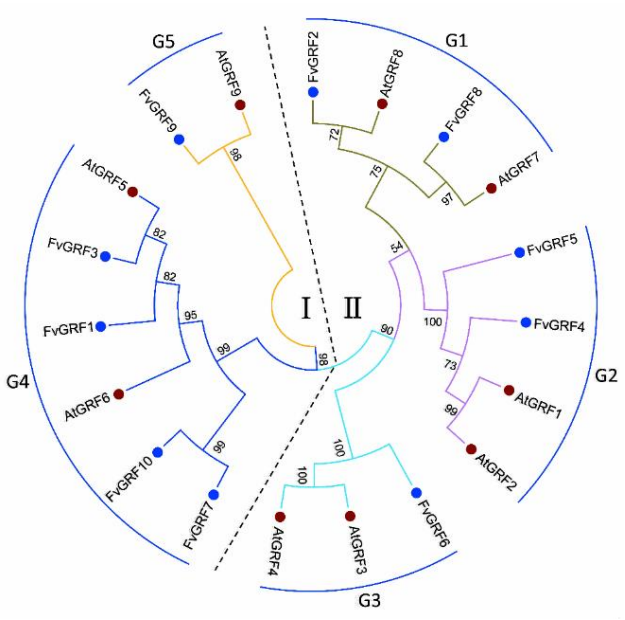

Fig. 3: Phylogenetic tree of GRF genes from *A. thaliana* and *F. vesca*. The multiple alignment of 19 full-length GRF protein sequences was performed by ClustalW program. The tree was generated using MEGA5.1 program by neighbor-joining method with the Jones-Taylor-Thornton (JTT) model and 1000 bootstrap replications. Gene groups were indicated with different colours, and were classified into two groups (I and II) and five subgroups (G1, G2, G3, G4 and G5)

were found in the G2 subgroup. The G5 subgroup only had FvGRF9 and AtGRF9 and the G3 subgroup consisted of three GRFs including AtGRF3, AtGRF4 and FvGRF6. The G4 subgroup was the largest group with six GRF proteins, comprising four FvGRF proteins (FvGRF1, FvGRF3, FvGRF7 and FvGRF10) and two AtGRF proteins (AtGRF5 and AtGRF6). Based on the phylogenetic tree, several pairs of orthologous genes were predicted, including FvGRF2/AtGRF8, FvGRF8/AtGRF7, FvGRF9/AtGRF9, and FvGRF3/AtGRF5 (Fig. 3).

Expression patterns of the *FvGRF* genes

The gene expression in space and time regulated the developmental progression and differentiation of distinct cell types (Brand *et al*. 2006). Therefore, an understanding of the expression pattern of a gene is crucial for the elucidation of its function. It has been known that *GRFs* play a crucial role in plant growth and development (Omidbakhshfard *et al*. 2015). To get insight into the function of *GRF* genes in strawberries, the expression levels of the *FvGRFs* in various organs or tissues of *F. vesca* were detected by RT-qPCR. The expression level in roots was considered one and the levels in other organs or tissues were given relative to root. The results indicated that almost all the *FvGRFs* (except for *FvGRF8*) were expressed in all the organs or tissues tested and exhibited different expression profiles (Fig. 4). Furthermore, *FvGRF1*, *FvGRF3*, *FvGRF6* and *FvGRF8* were predominantly expressed in buds and blooming flowers. *FvGRF4* and *FvGRF5* were mainly expressed in young leaves. *FvGRF2* had higher expression levels in young leaves and buds, whereas *FvGRF9* had higher expression in young leaves, buds and blooming flowers. The expression levels of *FvGRF7* were the highest in roots, close behind by similar in young leaves and blooming flowers, and *FvGRF10* exhibited similar expression levels in roots and blooming flowers, followed by similar in stems and young leaves compared with the levels in others. The analysis of gene expression patterns suggested that *FvGRFs* might be involved in the growth and development of these organs or tissues of strawberries.

Analysis of *FvGRFs* targeted by miR396

The miR396 and *GRF* regulatory network is evolutionarily conserved in plants and has been reported in *A. thaliana*, maize and rice (Wang *et al*. 2011; Zhang *et al*. 2015; Dai *et al*. 2019). However, there remains little information about the miR396 and *GRF* regulatory network in strawberries. To understand the miR396-mediated post-transcriptional

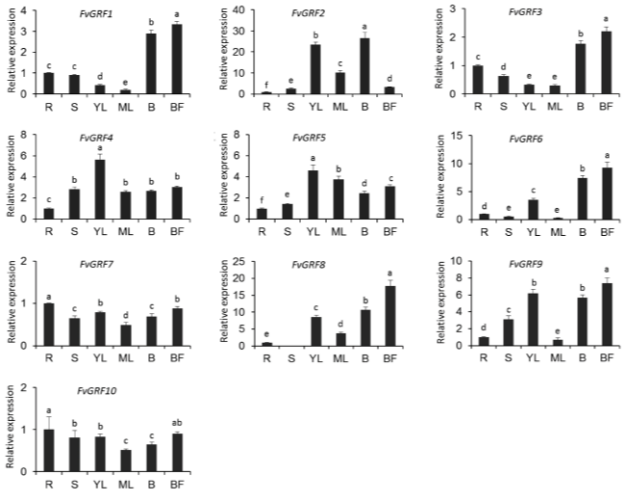

Fig. 4: RT-qPCR analysis of *FvGRF* genes in different organs or tissues of *F. vesca*. R: roots, S: stems, YL: young leaves, ML: mature leaves, B: buds, BF: blooming flowers. The expression level in roots was set to 1 and the levels in other tissues were given relative to this. The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method. ANOVA (analysis of variance) was calculated using S.P.S.S. (Version 19.0, IBM, USA). $P < 0.05$ was considered statistically significant. Data represent mean values of three replicates, error bars represent standard deviation, and different letters represent statistically significant differences using Duncan's test

regulation of *GRFs* in strawberries, the coding regions of all the 10 *FvGRFs* were searched for targets sites of miR396 via the online psRNATarget server. As a result, 10 of the *FvGRFs* were found to be the potential targets of miR396 (Table 4). Furthermore, *FvGRF2* and *FvGRF8* were experimentally validated to be cleaved by fve-miR396e using the 5′ RLM RACE (Fig. 5A–B). RT-qPCR analysis showed that fve-miR396e had the highest expression level in roots, the second highest in stems, the lowest in blooming flowers, and similar levels in young leaves and buds (Fig. 5C). Further investigation of the expression levels showed that fve-miR396e and its corresponding target genes *FvGRF2* and *FvGRF8* showed a significantly negative correlation (Table 5).

Discussion

Because of its small and sequenced genome, the diploid woodland strawberry (*F. vesca*), has recently emerged as a very good model for investigating significant genes in the rosaceae fruit crops (Darwish *et al*. 2015). It has been shown that *GRF* genes have important physiological function, such as in leaf and stem development (Kim and Lee 2006; Wang *et al*. 2014; Vercruyssen *et al*. 2015; Omidbakhshfard *et al*. 2018), flowering (Kim *et al*. 2003), seed and root development (Liu *et al*. 2012; He *et al*. 2015), and so forth. To fully understand the regulatory roles of GRF proteins in strawberries, 10 FvGRF proteins were identified and characterized on a genome-wide scale in *F. vesca* (Table 3) in this study. According to previous reports, the genome size of *F. vesca* and *A. thaliana* is 240 Mb and 125 Mb, respectively (Arabidopsis Genome Initiative 2000; Shulaev *et al*. 2011). The *F. vesca* genome is roughly double larger than the *A. thaliana* genome, but the number of *FvGRFs* in *F. vesca* is almost the same as that of *AtGRFs* in *A. thaliana* (10:9), suggesting that some genes may be disappeared during genome duplication (Shulaev *et al*. 2011; Darwish *et al*. 2015).

FvGRFs were classified into I and II groups based on phylogenetic analysis (Fig. 1 and 3). This is in line with a previous classification of *GRFs* from rice, cassava, etc. (Shang *et al*. 2018; Yashvardhini *et al*. 2018). Gene structure analysis showed that the *FvGRF* genes had three or four exons in the coding regions, and the II group of *FvGRFs* had more exons and introns than the I group (Fig. 1). This is consistent with the exon number in *AtGRFs*, with three or four exons in the coding regions (Choi *et al*. 2004). It indicated that the exon number of *GRFs* is highly conserved among *F. vesca* and *A. thaliana*. Conserved motif analysis showed that at least two GRF protein motifs existed in both the I and II groups of FvGRFs (Fig. 2). Similar results were found in *A. thaliana* and other plants (Wang *et al*. 2014). These results indicate the conservation of GRF protein sequences. The conservation of gene structures and protein sequences provide important basis for the classification and the functional prediction of FvGRFs. Together, these results prove that the classification of the *F. vesca* GRF family are credible. The similarity in gene structures between the *F. vesca* and *A. thaliana GRFs* indicates that there could be the same ancestors for these genes. At present, it is in accord with our knowledge of the plant evolutionary relationship that *F. vesca* and *A. thaliana* are dicotyledonous plants.

The phylogenetic analysis of genes is regarded as a very important basis for studying gene function. During plant evolution, in different species, genes with similar functions are usually strongly related to each other and are on the same branch in a phylogenetic analysis (Zhang *et al*. 2015). Therefore, we can predict the functions of unknown genes from known genes based on the phylogenetic analysis. Here, according to the phylogenetic relationship of 19 genes from *F. vesca* and *A. thaliana* (Fig. 3), we can infer the roles of the *FvGRFs* through *AtGRFs*. The functions of some *GRF* genes have been studied in the *A. thaliana*, for example, *AtGRF1* to *AtGRF3* regulate the development of leaves and cotyledons (Kim *et al*. 2003), *AtGRF1* and *AtGRF2* also delayed flowering (Kim *et al*. 2003) and *AtGRF4* demonstrates functional redundancy with from *AtGRF1* to *AtGRF3* (Kim and Lee 2006). Based on the phylogenetic tree, *FvGRF4* and *FvGRF5* with *AtGRF1* and *AtGRF2* were clustered in the G2 subgroup, *FvGRF6* with *AtGRF3* and *AtGRF4* was clustered in the G3 subgroup, therefore, *FvGRF4* to *FvGRF6* could have the same function to from *AtGRF1* to *AtGRF4*. *AtGRF5* also

Table 4: Prediction of miR396-mediated post-transcriptional regulation of FvGRFs

miRNA_Acc.	Target_Acc.	Expectation	UPE$	miRNA_start	miRNA_end	Target_start	Target_end	miRNA_aligned_fragment	alignment	Target_aligned_fragment
fve-miR396e	FvGRF7	0.5	19.347	1	21	334	354	UUCCACAGGCUUUCUUGAACU	::::::::::::::::::::	CGUUCAAGAAAGCUUGUGGAA
fve-miR396e	FvGRF8	1	15.768	1	21	553	573	UUCCACAGGCUUUCUUGAACU	::::::::::::::::::::	CGUUCAAGAAAGCAUGUGGAA
fve-miR396e	FvGRF2	1	13.689	1	21	646	666	UUCCACAGGCUUUCUUGAACU	::::::::::::::::::::	CGUUCAAGAAAGCAUGUGGAA
fve-miR396a/c-d	FvGRF1	3	15.562	1	21	348	369	UUCCACA-GCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF10	3	14.375	1	21	378	399	UUCCACA-GCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF6	3	22.542	1	21	564	585	UUCCACA-GCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF8	3	15.768	1	21	552	573	UUCCACA-GCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCAUGUGGAA
fve-miR396a/c-d	FvGRF3	3	18.209	1	21	351	372	UUCCACA-GCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF7	3	19.347	1	21	333	354	UUCCAC-AGCUUUCUUGAACUG	: ::::::::::::::::::	CCGUUCAAGAAAGCUUGUGGAA
fve-miR396a/c-d	FvGRF5	3	21.991	1	21	741	762	UUCCACA-GCUUUCUUGAACUG	::::::::::::::::::::	UCGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF2	3	13.689	1	21	645	666	UUCCACA-GCUUUCUUGAACUG	::::::::::::::::::::	ACGUUCAAGAAAGCAUGUGGAA
fve-miR396a/c-d	FvGRF9	3	15.938	1	21	459	480	UUCCACA-GCUUUCUUGAACUG	::::::::::::::::::::	ACGUUCAAGAAAGCCUGUGGAA
fve-miR396a/c-d	FvGRF4	3	20.696	1	21	765	786	UUCCACA-GCUUUCUUGAACUG	::::::::::::::::::::	UCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF2	3	13.689	1	21	645	666	UUCCACA-GCUUUCUUGAACUU	: ::::::::::::::::::	ACGUUCAAGAAAGCAUGUGGAA
fve-miR396b	FvGRF9	3	15.938	1	21	459	480	UUCCACA-GCUUUCUUGAACUU	: ::::::::::::::::::	ACGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF3	3	18.209	1	21	351	372	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF5	3	21.991	1	21	741	762	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	UCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF8	3	15.768	1	21	552	573	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCAUGUGGAA
fve-miR396b	FvGRF10	3	14.375	1	21	378	399	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF7	3	19.347	1	21	333	354	UUCCAC-AGCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCUUGUGGAA
fve-miR396b	FvGRF6	3	22.542	1	21	564	585	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF1	3	15.562	1	21	348	369	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	CCGUUCAAGAAAGCCUGUGGAA
fve-miR396b	FvGRF4	3	20.696	1	21	765	786	UUCCACA-GCUUUCUUGAACUU	::::::::::::::::::::	UCGUUCAAGAAAGCCUGUGGAA

Table 5: Correlation coefficients of relative expression levels between *FvGRFs* and *fve-miR396e*

Relative expression	Correlation coefficient
	fve-miR396e
FvGRF2	-0.54*
FvGRF8	-0.58*

*Correlation is significant at the 0.05 level (1-tailed)

plays a role in leaf development (Horiguchi *et al*. 2010). And *in situ* hybridization confirmed the *AtGRF5* was expressed in wild-type ovule primordia and its expression was significantly reduced in the *seu*/*ant* double mutant in later-stage gynoecia (Wynn *et al*. 2011). *FvGRF1* and *FvGRF3* with *AtGRF5* belonged to the G4 subgroup, therefore, *FvGRF1* and *FvGRF3* could share the similar function to *AtGRF5* according to their position in the phylogenetic tree. *AtGRF9* also contributes to regulating leaf size (Amin *et al*. 2018). Therefore, *FvGRF9* could play a role in leaf development according to its position with *AtGRF9* in the phylogenetic tree. *AtGRF7* to *AtGRF9* also shared the same functions in regulating leaf development (Liang *et al*. 2014). *AtGRF1* to *AtGRF9* (not including *AtGRF6*) caused *Arabidopsis* pistil abnormalities through post-transcriptional regulation of miR396 (Liang *et al*. 2014). Based on the phylogenetic tree, *FvGRF2* and *FvGRF8* with *AtGRF8* and *AtGRF7* were clustered in the G1 subgroup, therefore, *FvGRF2* and *FvGRF8* could play a significant role in regulating the leaf and/or flower development of strawberries. It suggests that some *FvGRFs* could perform overlapping and diverse function in the plant growth and development.

Comprehensive information on the tissue expression patterns of *GRF* genes would help to elucidate tissue development (Brand *et al*. 2006; Shang *et al*. 2018). Here, we found that almost all the *FvGRFs* (except for *FvGRF8*) were expressed in all the organs or tissues tested, with differential expression patterns, suggesting that *FvGRFs* may be overlap and diverse in function in strawberries (Mitchum *et al*. 2010). The *FvGRF4* and *FvGRF5* exhibited the highest expression level in young leaves (Fig. 4), suggesting that they might have prominent functions in the young leaf growth and development of strawberries. A previous study by Zhou *et al*. (2018) demonstrated that GRF15 is critical for leaf size in *Populus* species with large leaves. The *FvGRF7* was widely expressed in all the organs or tissues tested with the highest expression level in roots (Fig. 4), suggesting that it could take a big part in the growth and development of root in strawberries. For example, the *TaEXPB23* with root-specific expression in wheat can enhance root growth in tobacco (Li *et al*. 2015). The *FvGRF10* was higher expressed in roots, stems, young leaves and blooming flowers than in mature leaves and buds (Fig. 4), suggesting that this gene may be functionally redundant in strawberries. Fornari *et al*. (2013) found that *NF-YA3* and *NF-YA8* presented in vegetative and reproductive tissues, share the same role in early embryogenesis of *A. thaliana*. It supports our conclusion. The expression of the *FvGRF1*, *FvGRF2*, *FvGRF3*, *FvGRF6*, *FvGRF8* and *FvGRF9* genes was higher in buds and/or blooming flowers than in the other tested tissues (Fig. 4), suggesting that these genes could be crucial for the floral growth and development in strawberries. For example, AtMYB24 was found mainly expressed in flowers, especially in microspores and ovules, is associated with flower development in *Arabidopsis* (Yang *et al*. 2007). These results indicated that *FvGRFs* may have important function in the growth and development of strawberry organs or tissues. It is accordant with the results of phylogenetic analysis. The combination analysis of the expression profiles of *FvGRFs* and the phylogenetic relationships between *FvGRFs* and *AtGRFs* showed that the predicted functions of *FvGRFs* in strawberries were reasonable. These results would provide valuable information for further experimental

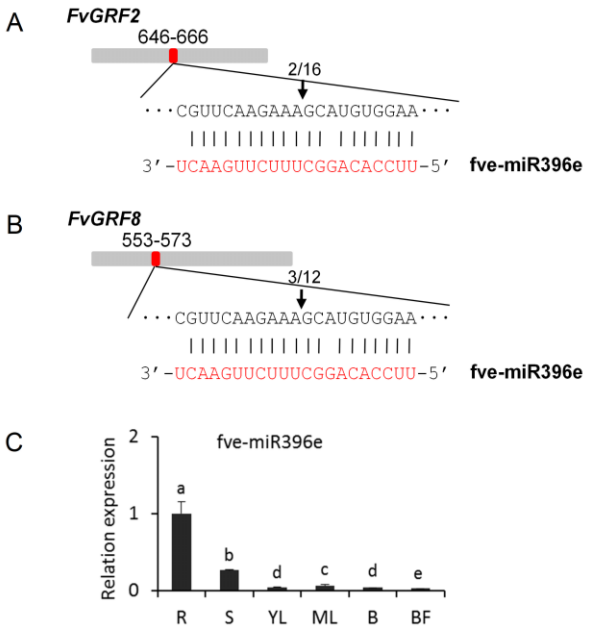

Fig. 5: *FvGRFs* targeted by miR396. (**A**) Experimental validation of fve-miR396e-mediated cleavage of *FvGRF2* using the modified RNA ligase-mediated rapid amplification of 5′cDNAs method (5′ RLM RACE). Grey lines represent coding sequences. miRNA complementary sites (red) with the nucleotide positions of *FvGRF2* coding region are indicated. The RNA sequence of each complementary site from 5′to 3′ and the predicted miRNA sequence from 3′to 5′are shown in the expanded regions. Vertical dashes indicate Watson-Crick pairing. Vertical arrows indicate the 5′ termini of fve-miR396e-mediated cleavage products, as obtained by 5′RACE, with the frequency of clones shown. (**B**) Experimental validation of fve-miR396e-mediated cleavage of *FvGRF8* using 5′RLM RACE. (**C**) Expression patterns of fve-miR396e in *F. vesca*. R: roots, S: stems, YL: young leaves, ML: mature leaves, B: buds, BF: blooming flowers. The expression level in roots was set to 1 and the levels in other tissues were given relative to this. The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method. ANOVA (analysis of variance) was calculated using SPSS (Version 19.0, IBM, USA). $P < 0.05$ was considered statistically significant. Data represent mean values of three replicates, error bars represent standard deviation, and different letters represent statistically significant differences using Duncan's test

validation of the functions of *FvGRFs* in strawberries.

MiRNAs play a vital role in plant physiological and developmental processes (James and Victor 2003). The miR396 family is conserved among plant species and is known to target the *GRF* gene family. In *Arabidopsis*, *GRF1* to *GRF9* (except for *GRF5* and *GRF6*) are the direct targets of miR396 (Liang *et al*. 2014). It is well known that the miR396-GRF network has important biological functions, such as in root development (Rodriguez *et al*. 2015), leaf development (Wang *et al*. 2011), flower development (Liang *et al*. 2014; Liu *et al*. 2014), grain size (Duan *et al*. 2015; Li *et al*. 2016), and so forth. In the present study, all of the 10 *FvGRFs* were found to be potential targets of fve-miR396 (Table 4), of which *FvGRF2* and *FvGRF8* were experimentally validated to have the cleavage sites of fve-miR396e using 5′ RLM RACE (Fig. 5). Furthermore, the expression levels of fve-miR396e were negatively correlated with those of its *FvGRF2* and *FvGRF8* targets (Table 5). A previous study by Xia *et al*. (2015) suggested that several *GRF* transcripts were regulated by fve- miR396 in *F. vesca* using a high-throughput approach, which supports our results. These results indicated that the fve-miR396-FvGRF network could play an important role in regulating the growth and development of *F. vesca*. Further analysis of biological functions using genetic engineering will be carried out to verify the roles of *FvGRFs* in the future.

Conclusion

In summary, 10 *FvGRFs* were identified —their sequence characteristics, gene structures and motif features, conserved domains, phylogenetic relationships, expression patterns in different strawberry organs or tissues, post-transcriptional regulation and functions were evaluated. *FvGRFs* could be mainly associated with leaf and flower development and were redundant in function in strawberries. Our findings will be offering a theoretical basis for further exploration of the functions of *GRF* gene family in strawberries.

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Author Contributions

HL and QL conceived the experiments, got the funding and revised the paper. XJ, PC and JL performed the experiments and analyzed results. XJ wrote the manuscript. All authors have read and agreed to publish this version of the paper.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The data will be made available on reasonable request to the corresponding author.

Ethics Approval

Not applicable.

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