Article

Genome-Wide Identification and Expression Analysis of Growth-Regulating Factor Family Genes in Sunflower(*Helianthus annuus* L.)

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Abstract: Growth-regulating factor (GRF) is a plant-specific transcription factor family, which is involved in nearly all of the central developmental processes in plants. However, little is known about GRF family genes in cultivated sunflower. In this study, 17 GRF genes were identified and characterized from sunflower genome. Their gene structures, conserved motifs, chromosomal distributions and phylogenetic relationships were analyzed. The expression patterns of these genes were detected in various tissues of sunflower inbred line SK02R, which revealed that the 10 seed-specific GRF genes may play important roles during seed development in sunflower. Additionally, transcripts changes of the GRFs under two major abiotic stresses and phytohormones showed that most of the detected GRFs were reduced significantly by GA3 treatment, and other treatments(ABA, NaCl and PEG6000) differently regulated various sunflower growth-regulating factors at different time points. MiR396 target analysis indicated that there may exist a complicated homeostasis between miR396 and its targets GRF and WRKY transcription factor genes in cultivated sunflower. The phylogenetic and expression analyses of the GRF gene family in sunflower would be useful for further cloning and function exploration of the HaGRF genes.

Keywords: sunflower; growth-regulating factor; expression pattern; abiotic stress

1. Introduction

Transcription factors(TF) are major regulators of gene expression, which could be classified into various families mainly according to their conserved functional domains in eucaryotic organisms[1]. The growth-regulating factor (GRF) family is a plant-specific TF family, of which the first member named *OsGRF1* was identified in rice[2]. As many whole genomes of plants have been published, the GRF family has been identified in various plant species, including *Arabidopsis thaliana*, *Brassica rapa*, *Nicotiana tabacum*, *Solanum tuberosum*, *Oryza sativa*, *Zea mays*, *Arachis hypogaea* and *Camellia sinensis*[3-10]. Recent studies have gradually reported that the TF family members are involved in almost all of the central developmental and some stress-responsive processes in plants[11].

GRF TF proteins contain two highly conserved domains named QLQ and WRC in their N-termin regions and variable C-termin. The QLQ domain-contained region functions as an indispensable element for protein interaction[2]. The plant-specific WRC-contained region is predicted to be a nuclear localization signal and DNA binding domain of these TFs in plants[2,3,12]. The expression of GRF genes is generally more in growing tissues than that in mature zones, which is accordance with their function in regulating cell proliferation and expansion[9,13,14]. GRF-interacting factors (GIF) are a group of transcriptional co-activators, which form a complex with GRFs and regulate cell proliferation in developping of leaf and reproductive organs[14-17]. As an abiotic stress-induced miRNA，miRNA396 targets many GRF transcripts, and then negectively regulates expression of these GRF genes[18-21]. In contrast, it has been reported that some GRFs are

induced by abiotic stresses or ABA treatment[5,6] . These may suggest that there would be furthermore complicated regulation network involved in GRF TFs, miRNA396 under abiotic stresses. Genome-wide identification and expression profiles analysis of GRFs would facilitate the revelation of GRF regulatory mechanism on plant development and abiotic stress responses.

For the relative wide adaption to various bad environmental conditions such as soil salinity and drought, cultivated sunflower(*Helianthus annuus* L.) is one of the most important oil crops worldwide, and has been researched as a model for understanding solar tracking of plants for decades[22,23]. Recently, a high-quality assembled genome gave more detailed evolutionary and genomic architecture informations of sunflower[24]. This data made it viable to identify and characterize important gene families from whole genome of domesticated sunflower. In this study, 17 GRF family genes were identified from HanXRQ-SUNRISE (https://www.heliagene.org/HanXRQ-SUNRISE/)[24]. We analyzed their gene structure, conserved motif and phylogenetic relationships with newly assembled lettuce, sweet wormwood, arabidopsis, chinese cabbage, grape, rice and maize. Expression patterns of these GRFs in eight tissues/organs were investigated using both RNA-Sequencing(RNA-Seq) and qRT-PCR. RNA-Seq analysis was also performed to obtain their expression responses to salt and simulated drought stresses at different time points within 24 hours. Furthermore, after treating with GA3 and ABA, expression levels changes of these genes were also analyzed simultaneously.

2. Results

2.1. Identification and characterization of GRF genes in cultivated sunflower

To find all the GRFs in sunflower, QLQ(PF08880) and WRC(PF08879) domain search against all proteins were performed in the sunflower genome (https://www.heliagene.org/HanXRQ-SUNRISE/). A total of 17 proteins containing both of the two conserved domains were identified. Blastp search using the 9 Arabidopsis GRF proteins as requries resulted in no further hits. As showed in Table 1, these 17 GRF genes were named based on their chromosomal distribution and location from top to bottom. The 17 GRF genes encode proteins ranging from 155(*HaGRF2*) to 508 (*HaGRF12*) amino acids in length, and from 17.27(*HaGRF2*) to 55.47(*HaGRF12*) kDa in molecular weight respectively. MiR396 target analysis indicated that 14 of these 17 GRFs mRNA contained miRNA396 target position in cultivated sunflower. HaGRFs were unequally distributed on 12 of the 17 chromosomes, with 3 members on the chromosome(chr)03, 2 on chr06 and chr13, only one on chr(01,02,05,07,09,10,11,12,15) respectively(Table 1 and Figure S1). The other characteristics of the individual GRF genes, including gene Locus_tag, ORF length, chromosomal location, number of exons and isoelectric point (pI), are also given in Table 1.

Table 1 Informations of HaGRF genes

Gene name	Locus_tag	ORF length(bp)	Location		Exon	MW(kDa)	Length(aa)	pI	miRNA
			Start	Stop					
HaGRF1	HanXRQChr01g0017671	720	105818726	105836216	4	26.38	239	9.60	Y
HaGRF2	HanXRQChr02g0047741	468	126398457	126399122	2	17.27	155	8.67	N
HaGRF3	HanXRQChr03g0082181	1059	133413450	133416500	3	39.10	352	8.58	Y
HaGRF4	HanXRQChr03g0082231	633	133460237	133461415	3	23.03	210	9.66	Y
HaGRF5	HanXRQChr03g0085261	969	143049925	143051234	4	35.16	322	8.38	y

HaGRF6	HanXRQChr05g0133051	1473	20408183	20410282	4	53.03	490	8.41	Y
HaGRF7	HanXRQChr06g0166741	1173	6661782	6664364	4	42.81	390	6.40	Y
HaGRF8	HanXRQChr06g0178171	975	46439440	46442161	3	37.19	324	7.27	Y
HaGRF9	HanXRQChr07g0192351	1065	42946365	42948208	4	39.01	354	7.59	Y
HaGRF10	HanXRQChr09g0276951	981	208226201	208227356	3	36.79	326	8.51	Y
HaGRF11	HanXRQChr10g0319661	612	245432914	245433781	4	23.47	203	9.49	N
HaGRF12	HanXRQChr11g0323531	1527	12861091	12866787	4	55.47	508	7.07	y
HaGRF13	HanXRQChr12g0354071	1002	347847	349919	3	38.10	333	8.17	y
HaGRF14	HanXRQChr13g0405031	1068	104381671	104387290	3	39.18	355	8.63	y
HaGRF15	HanXRQChr13g0414361	975	152931243	152933083	3	35.63	324	8.04	y
HaGRF16	HanXRQChr15g0493101	1152	143314681	143316747	4	41.93	383	9.56	N
HaGRF17	HanXRQChr00c0041g05	1515	10979	13270	4	54.50	504	8.45	Y

2.2. Gene structure, protein structure, and phylogenetic analysis of GRF genes

These GRF gene structures were analyzed using the GSDS online suite and schematically illustrated based on their gene names. As showed in Figure 1, the number of GRF gene exons ranges from 2 to 4, with 8 members possessing 3 exons, 8 possessing 4 exons and only the shortest *HaGRF2* containing 2 exons. The *HaGRF1* gene possesses a 16.6 kb super intron, with a 7814bp LTR retrotransposon. REpeat detector analysis showed that there were two repeat regions in this intron, with 11281bp and 1367bp in length respectively. *HaGRF17* was not located to any one of the 17 chromosomes, and none of the 17 GRFs located on chr17 of XRQ genome, whereas the gene sequence of *HaGRF17* was completely similar to that of *HaGRF17-like1*(Ha412v1r1_00g106720) in Ha412 genome(Figure S2). Additionally, a 75bp length of inron fragment in *HaGRF17* and *HaGRF17-like1* was found to be an individual exon in CDS of *HaGRF17-like2*(Ha412v1r1_17g006360) (Figure S2). These findings indicated that *HaGRF17* may be located on the 17th chromosome based on the assembly result of Ha412 genome, *HaGRF17-like1* and *HaGRF17-like2* transcripts may be the different products of *HaGRF17* gene in sunflower. As most of the GRFs reported in other plant species, the conserved WRC(motif1) and QLQ(motif2) domains were located in the N-termini of the 17 sunflower GRF proteins(Figure 2 and Figure S3). The HaGRF3, HaGRF6, HaGRF8, HaGRF12, HaGRF13, HaGRF14, HaGRF16, HaGRF17 proteins shared a short stretch of amino acid residues termed FFD(motif5) domain, HaGRF3, HaGRF8, HaGRF12, HaGRF13, HaGRF14, HaGRF17 shared a TQL(motif8) domain, and HaGRF1, HaGRF5, HaGRF6, HaGRF7, HaGRF9, HaGRF12, HaGRF15, HaGRF16, HaGRF17 shared a GGPL(motif3) domain in their C-termini(Figure 2). To further investigate the phylogenetic relationships of GRFs between sunflower and other plant species in/out of compositae family, 13 GRFs were also identified in lettuce and sweet wormwood genomes, respectively. A phylogenetic tree was generated, including the 17 HaGRFs in sunflower, 13 GRF proteins in lettuce, 13 members in sweet wormwood, 9 in arabidopsis,12 in rice,14 in maize,17 in chinese cabbage and 9 in grape. All the GRFs were divided into eight subgroups based on clade and evolution of these plant species in the phylogenetic tree(Figure 3). Subgroup C1, C2,

C4 and C7 only contained GRFs from dicot species, and subgroup C6 only contained monocot species. GRFs from Other subgroups such as C3,C5 and C8 contained GRFs members from both dicot and monocot species.The 17 sunflower GRFs were classified into six of the eight subgroups, except C6 and C8 subgroups. HaGRF5, HaGRF7, HaGRF9 and HaGRF15 which contained both motif3(GGPL) and motif7 were clustered into subgroup C1. HaGRF1, HaGRF6, HaGRF12, and HaGRF17 contained both motif3(GGPL) and motif6 were divided into subgroup C3. HaGRF8 and HaGRF13 in subgroup of C5 contained motif4，motif5 and motif8. HaGRF3, HaGRF14 contained motif5, motif8, motif9 and motif10 were clustered in subgroup C7.

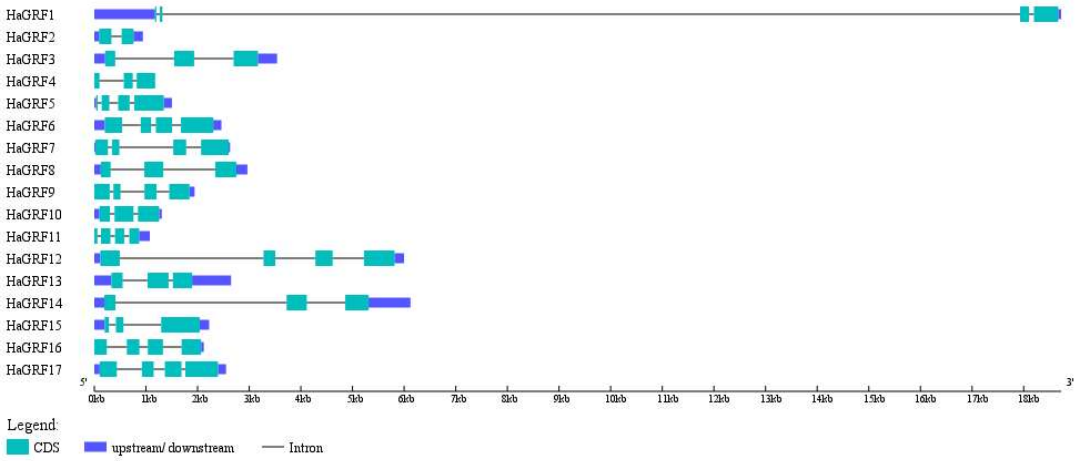

Figure 1 Structures of the 17 *HaGRF* genes. Structural analyses of the *HaGRF* genes were performed using the gene structure display server. The exons and introns are represented by green boxes and black lines, respectively. The blue boxes are upstream/downstream regions.

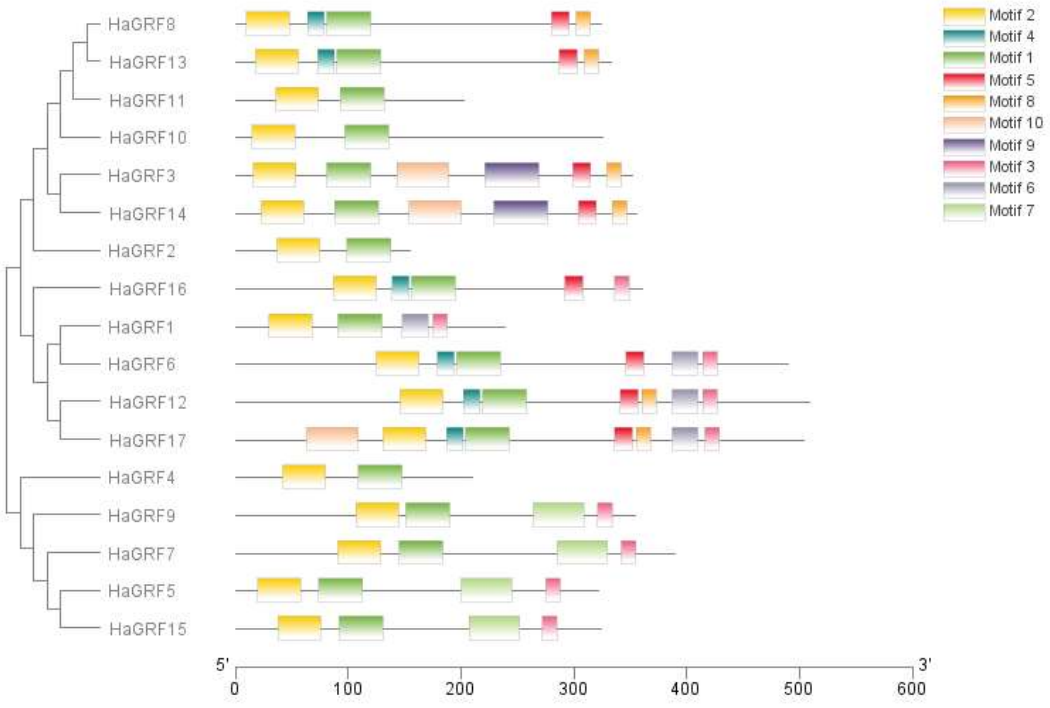

Figure 2 Conserved motifs of the HaGRF proteins. The conserved motifs of HaGRF proteins were identified using Multiple Em for Motif Elicitation (MEME). 10 predicted motifs are shown in different colors.

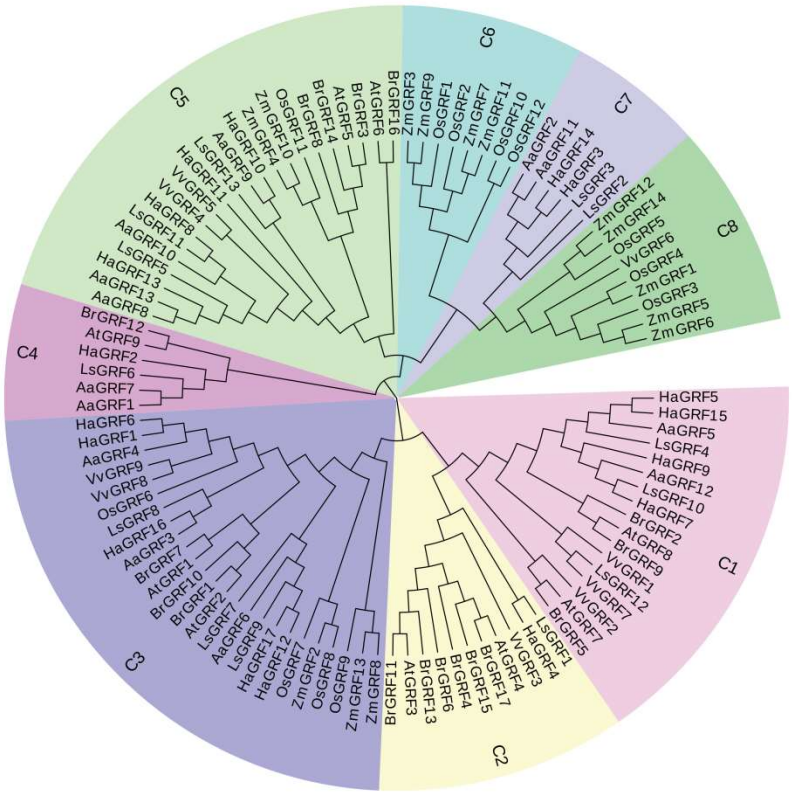

Figure 3 Phylogenetic tree of GRF proteins from sunflower(Ha), lettuce(Ls), sweet wormwood(Aa), arabidopsis(At), chinese cabbage(Br), rice(Os), maize(Zm) and grape(Vv) were constructed using the neighbor-jointing method with 1000 bootstrap replications. Eight clades(C1 to C8) were marked in different colors.

2.3. Expression parttern of GRF genes in different tissues

Previous studies indicated that the gene expression pattern is associated with its function. Expression profiles of sunflower GRF genes were analyzed in eight different sunflower tissues/organs such as root, young leaf, mature leaf, shoot, seed, stamen, pistil and ligule using qRT-PCR. 15 of the GRF genes could be detected in at least one of the eight sunflower tissues/organs. According to the results, 10 of the GRF genes(*HaGRF2*, *HaGRF3*, *HaGRF7*, *HaGRF8*, *HaGRF9*, *HaGRF10*, *HaGRF12*, *HaGRF14*, *HaGRF15* and *HaGRF16*) were highly expressed in seed(Figure 4). The expression levels of *HaGRF6* were relatively high in four tissues(seed,root,young leaf, pistil), *HaGRF17* were highly expressed in seed, root, stamen and pistil, *HaGRF5* in seed and root, *HaGRF11* in stamen, *HaGRF13* in pistil(Figure 4). These tissue-specific expression of HaGRF genes indicated that this family genes may specifically play important roles in seed, leaf and/or root development, respectively. It is worth noting that *HaGRF3*, *HaGRF8*, *HaGRF9*, *HaGRF10*, *HaGRF12*, *HaGRF14*, *HaGRF15* and *HaGRF16* showed the highest expression level in seed, indicating that these GRF genes may play important roles in seed developmental process of cultivated sunflower.

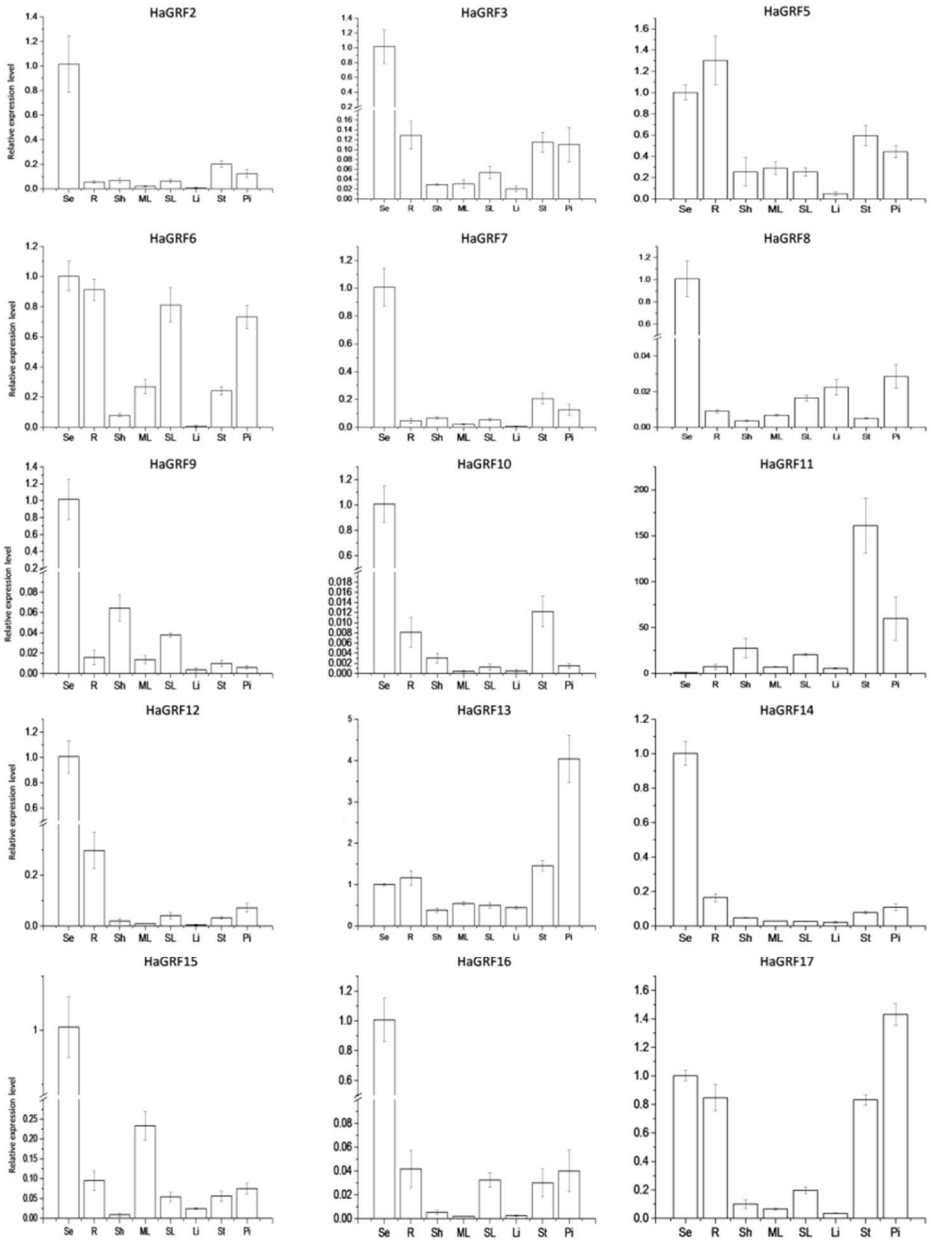

Figure 4 qRT-PCR analysis of HaGRF genes expression in eight different tissues, including seed(Se), root(R), Shoot(Sh), mature leaf(ML),Young leaf(SL), Ligule(Li), Stamen(St), Pistil(Pi). Relative expression levels are shown as means±SD.

2.4. Expression of GRF genes in response to ABA and GA3 treatments

After treating with ABA, the expressions of *HaGRF3*, *HaGRF5*, *HaGRF8*, *HaGRF12* and *HaGRF13* genes were up-regulated by 2-fold or more at different time points compared to the control. 6 hours after treating, the expressions of *HaGRF8*, *HaGRF12* and *HaGRF13* reached the highest level. In contrast, the expressions of *HaGRF2*, *HaGRF6*, *HaGRF7* and *HaGRF14* genes were suppressed by ABA. The expression changes of other sunflower GRF genes were not significant in response to ABA(Figure 5). GA3 treatment induced expression of *HaGRF3* and suppressed that of other 12 GRF genes significantly. After 24-hour treatment, *HaGRF5*, *HaGRF6*, *HaGRF9*, *HaGRF13* and *HaGRF14* expression levels returned to at least half of the control(Figure 6).

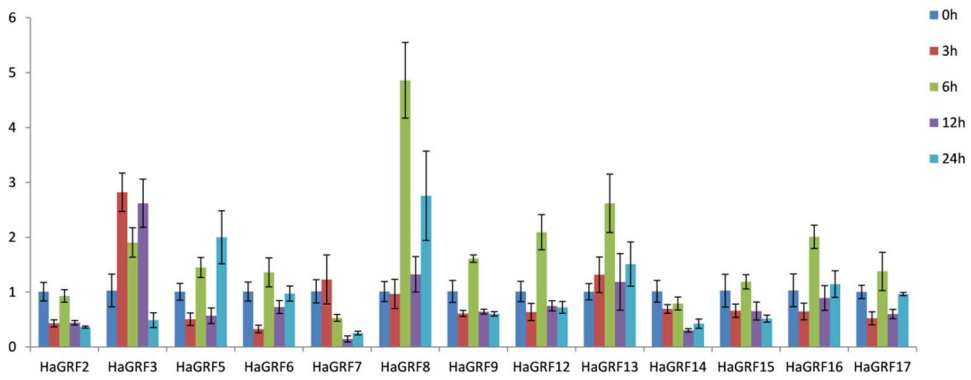

Figure 5 Expression patterns analysis of HaGRF genes treated with ABA by qRT-PCR. Relative expression levels are shown as means±SD.

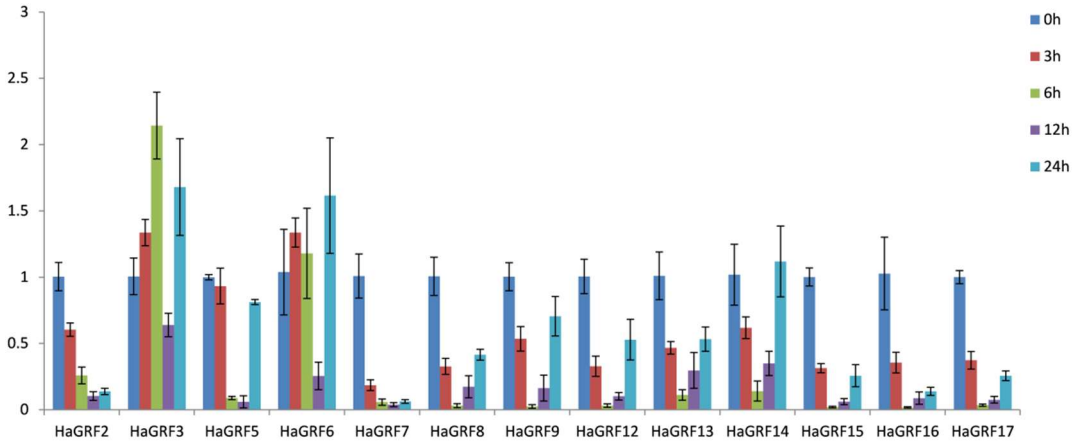

Figure 6 Expression profiles of HaGRF genes treated with GA3 by qRT-PCR. Relative expression levels are shown as means±SD.

2.5. Expression of GRF genes under salt and PEG treatments

To examine the responses of GRF genes to salinity and drought in sunflower, qRT-PCR analysis were performed to investigate expression patterns of GRF genes under NaCl and PEG treatments. Expression of *HaGRF2*, *HaGRF5*, *HaGRF7*, *HaGRF9*, *HaGRF12*, *HaGRF14* and *HaGRF15* increased significantly at 6-hour and 12-hour points after treating with NaCl. Three HaGRF members of subgroup C3, including *HaGRF6*, *HaGRF16*, *HaGRF17* were down-regulated compared to the control at 3-hour point(Figure 3 and Figure 7). qRT-PCR data also revealed that expression of *HaGRF2*, *HaGRF5*, *HaGRF7*,*HaGRF9* and *HaGRF15* genes were induced by PEG treatment at different time points, whereas that of *HaGRF6*, *HaGRF16*, *HaGRF17* were repressed at 6 and 12-hour points by this simulated drought(Figure 8). All of the four HaGRF genes in Subgroup C1 and *HaGRF2* the only HaGRF member of subgroup C4 were significantly induced by both salt and PEG treatment. In contrast, expression of *HaGRF6*, *HaGRF16*, *HaGRF17* in C3 were reduced by the two abiotic stresses(Figure 3 to Figure 8). These findings indicated that HaGRFs in C1 and C3 clades may function diversely in adapting or maintaining development of leaves under abiotic stresses such as salinity and osmotic stress in cultivated sunflower.

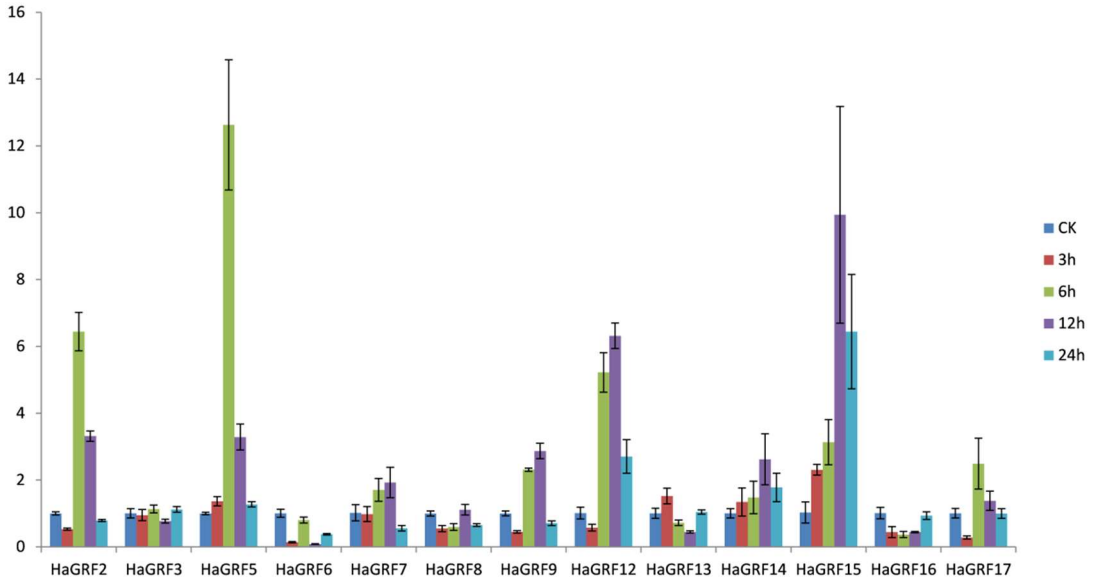

Figure 7 Expression profiles of HaGRF genes under NaCl treatment. To calculate the relative expression level, the expression of each GRF gene under control (0h) was set as 1. The results were represented as mean±SD.

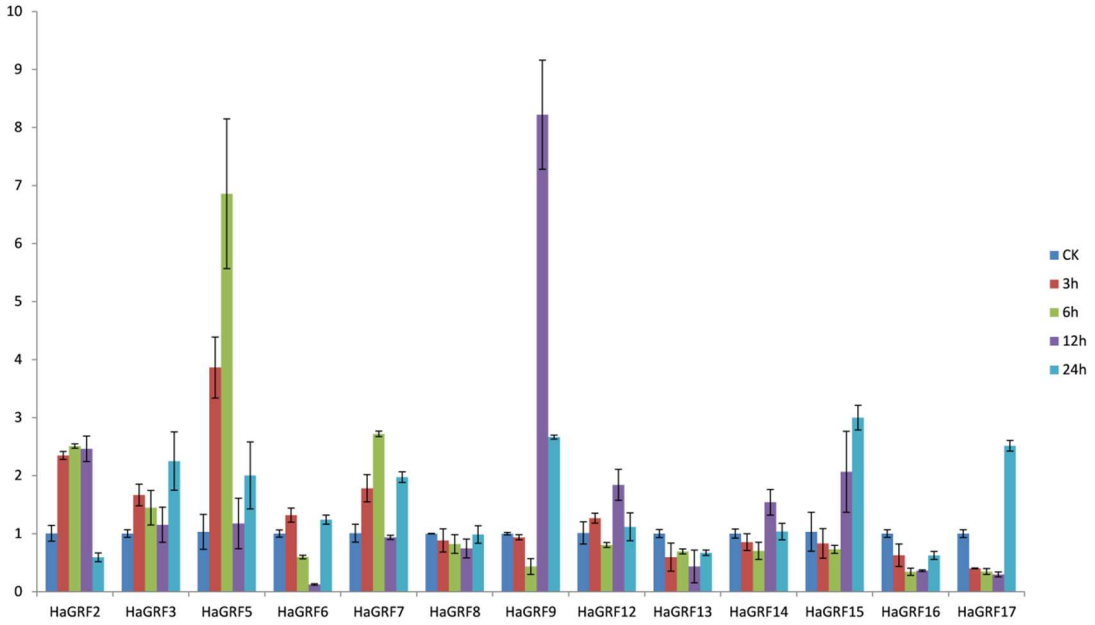

Figure 8 Expression patterns analysis of HaGRF genes under drought by qRT-PCR. To calculate the relative expression level, the expression of each GRF gene under control (0h) was set as 1. The results were represented as mean±SD.

3. Discussion

As plant-specific transcription factors, GRFs were previously reported for their roles in almost all of the important development processes such as root, stem, root, leaf, flower development, seed formation, and in response to various hormones and abiotic stresses[25]. The GRF family has been identified experimentally or in silico in various land plant species, including *Arabidopsis thaliana*, *Brassica napus*, *Glycine max*, *Nicotiana tabacum*, *Solanum tuberosum*, *Oryza sativa*, *Zea mays* and *Camellia sinensis*[5,6,25]. In this study, we identified 17 GRFs in sunflower mainly based on the newly reported XRQ genome. *HaGRF17*(HanXRQChr00c 0041g0571301) was not assembled to any one of the 17 chromosomes, and none of the 17 GRFs located on the 17th chromosome in XRQ genome. Blastn analysis revealed that the gene sequence of *HaGRF17* was completely similar to that of predicted gene *HaGRF17-like1* in Ha412 genome. Additionally, A gene *HaGRF17-like2* shared high similar nt sequence with *HaGRF17*. A 75bp length of additional nucleotides was found as an individual exon in CDS of *HaGRF17-like2*(Figure S2). RNA-seq data mapped on these two genes showed that the 75bp nucleotides could be detected in leaf and stem under drought stress[24]. These results indicated that transcripts of *HaGRF17-like1*(or *HaGRF17* in XRQ genome) and *HaGRF17-like2* may be the different products of alternative mRNA editing in sunflower. Considering most of the GRFs exon number ranged from 3 to 4, HaGRF17 may be the only GRF with 5 exons, and only *HaGRF2* with 2 exons.

Many studies had reported that the GRF family genes were differentially expressed in various tissues/organs of other plant species[5,6,9,26-28]. Our tissue-specific expression analysis indicated that 10 of the 17 GRFs were expressed in developing seed specifically, and other 3 were also highly expressed in root, stamen or silique(Figure 4). It has been reported that transcripts levels of *BnGRF2a* and *BnGRF2b* in ovules of the high-oil seeds were higher than that of in low-oil seeds，and over-expression of *BnGRF2a* increased seed mass and oil yield compared to the control[29]. Our tissue-specific expression analysis sugguested that these seed specifically or highly expressed GRF genes may play more important roles on seed development, and then affect seed weight or oil content in cultivated sunflower. Further biological function exploration and regulatory mechanism illumination of these seed highly expressed HaGRFs would be helpful for increasing oil yield of cultivated sunflower.

Not all of the predicted GRF genes were detected effectively by qRT-PCR. Expression of *HaGRF1* and *HaGRF4* could not be detected in any one of the eight tissues by qRT-PCR. It has been reported that more than 75% of the sunflower genome consisted of long terminal repeat retrotransposons (LTR-RTs)[24]. Gene structure analysis revealed that there was a 7814bp LTR retrotransposon in the 16.6 kb super intron of *HaGRF1*, which may impaired expression of this gene. Most of reported GRF genes contain both the QLQ and WRC domains in other plant species[25], whereas Blastp results showed that there were 4 predicted proteins only containing QLQ domain, and 8 only possessing WRC domain in XRQ genome database. It is now unclear whether these genes function as identified GRFs or not.

Previous studies had found that many GRF genes were induced by GA3 in some plant species[4,5], but a few others were not being affected[3,30], and even some were suppressed simultaneously[6]. Our data showed that GA3 treatment slightly induced expression of *HaGRF3*, but suppressed that of other 12 GRFs in young leaf(Figure 6). It has been known that GA3 plays a positive role in cell proliferation， and then regulates expansion of young leaf[31]. KNOX homeodomain proteins have been reported to suppress expression of gibberellins biosynthetic genes such as *AtGA20ox1* in Arabidopsis[32] and *Ntc12* in the tobacco shoot apical meristem[33]. As repressors of KNOX genes, GRFs promote cell division[34]. These results suggest that some GRF genes may function in maintaining or promoting cell division by a feedback regulation mechanism, in which the GRF genes positively regulate the production of gibberellins, and then excessive GA3 in turn down-regulate GRF gene expression, maintaining gibberellins homeostasis in sunflower.

As a central regulator of abiotic stress signalling network, ABA regulates expression of abiotic stress responsiveness genes in most upland plants[35]. Previous studies had found that ABA treatment regulated expression of GRF genes in other plants[5,6]. Here, our data showed that

expression levels of some GRF genes were induced or repressed when exposed to salt and simulated drought, but not regulated significantly by ABA(Figure 5 and Figure 7-8). These results suggest that HaGRF genes are involved in abiotic stress responses via ABA-dependent and ABA-independent signaling pathways at least at transcript level. *HaGRF2*, the only HaGRF member of subgroup C4 was significantly induced by both salt and PEG treatment. *AtGRF9* clustered in C4 negatively regulate leaf growth by activating expression of the bZIP TF OBP3-RESPONSIVE GENE 3 (*ORG3*)[36]. These facts indicated that *HaGRF2* may also function in reducing leaf size under salt and drought stresses in sunflower. miRNAs are critical regulators of many development and abiotic stress signaling processes in plants. It has been reported that the miR396 control many organs growth via post-transcriptional regulating GRF genes, and the miR396/GRF module is conserved in plants[25,37-39]. miR396 and the targets GRF genes established a homeostasis by negatively regulating each other's expression[40]. Sequence analysis revealed that 14 HaGRF genes have the miR396 target sites，which confirmed the existence of this regulatory module in sunflower. Additionally, a predicted WRC domain-contained gene *HanXRQChr03g0093741* and three sunflower WRKY genes also possess miR396 target sites. It has been reported that high temperature induced expression of *HaWRKY6* and repressed hamiRNA396, and further evidence indicated that miRNA396 regulated *HaWRKY6* in sunflower[41]. As members of an important abiotic stress responsive transcription factors family in plant species[42,43], the three WRKY genes may be involved in the homeostasis between miR396 and the target HaGRF genes under abiotic stresses such as salinity, drought, high temperature in sunflower.

4. Materials and Methods

4.1. Identification of GRF family genes in sunflower

The Sunflower (Helianthus annuus L.)genome from HanXRQ-SUNRISE (https://www.heliagene.org/ HanXRQ-SUNRISE/) was used to identify HaGRF genes. Protein files of lettuce(Lactuca sativa L.) and sweet wormwood(Artemisia annua L.) were downloaded from NCBI genome(https://www.ncbi.nlm.nih.gov/genome).The Hidden Markov Model profiles of the GRF family domains PF08880 and PF08879 were downloaded from Pfam(http://pfam.xfam.org/) and used as queries to search for candidate GRF genes by hmmer 3.2.1(http://hmmer.org/download.html).Furthermore, all the candidate genes were analyzed to confirm the presence of the conserved QLQ(PF08880) and WRC(PF08879) domains using SMART (http://smart.embl-heidelberg.de/), and CDD(Conserved Domain Database) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

4.2. Chromosome location and phylogenetic analysis

The physical positions of HaGRF genes were extracted from annotation gff3-file in HanXRQ-SUNRISE(https://www.heliagene.org/HanXRQ-SUNRISE/) and mapped to 17 chromosomes in the sunflower genome using MapChart 2.3. For phylogenetic analysis, full-length GRF amino acid sequences of sunflower, lettuce, sweet wormwood, arabidopsis, chinese cabbage, grape, rice and maize were aligned using Clustal X 2.1 software, and MEGA 5.1 was used to construct a neighbor-joining (NJ) tree using the Bootstrap method with 1000 replicates..

4.3. Gene structures and protein motifs analysis

The Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/) online tool was used to construct diagrams of HaGRFs exon-intron structures. Conserved motifs of putative HaGRF proteins were predicted with Multiple EM for Motif Elicitation (MEME: http://meme-suite.org/) with default settings for motif width (between 6 and 50 wide) and site distribution (zero or one occurrence per sequence). The maximum number of motifs was set as 10.

4.4. Plant materials and treatments

All the samples of tissues/organs, such as root,young leaf,mature leaf, shoot, stamen, pistil, seed, ligule were collected from sunflower line SK02R at the field in Northwest A&F University, Shaanxi, China. For stress and hormone treatments, the seeds of SK02R were germinated on wet filter paper for 3 days at 25 °C and then grown in MS solution in plant growth chambers at a temperature regime of 25/22 °C, light intensity of 15000 lx and photoperiod of 14 hour light/10 hour dark. After the expansion of four true leaves, the plantlets were subjected to different abiotic stress and hormone treatments. The plantlets were cultivated in MS solution supplemented with 200 mM NaCl for the salt treatment, 15%(v/v) polyethylene glycol 6000 for the drought treatment, sprayed with 0.2 mM ABA for the ABA treatment and sprayed with 1 mM gibberellic acid for GA3 treatment. The leaves of treated plantlets were harvested at 0, 3,6,12 and 24 hour.All of the harvested samples were frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted.

4.5. Expression pattern analysis of HaGRFs using qRT-PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions(Invitrogen, Carlsbad, CA, USA). qRT-PCR was conducted in a StepOneTM Real-Time PCR system (Applied Biosystems). The RT-qPCR was carried out in a 25 μL reaction volume containing 12 μL 2 × SuperReal PreMix Plus, 0.5 μL 50 × ROX Reference Dye, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer(10 μM), 2.0 μL cDNA (100 ng/μL), and 4.5 μL RNase-free ddH_2O, following the instructions of the SuperReal PreMix Plus (SYBR Green) kit (Tiangen,Beijing, China). The thermal cycling conditions were polymerase activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and elongation at 72 °C for 32 s. Acin was selected as the reference gene to normalize gene expression data in real-time RT-PCR. The results were then analysed via the $2^{-\triangle\triangle CT}$ method. Three technical replicates were performed with each of the three biological replicates.

5. Conclusions

In the present study, we identified and characterized 17 GRF genes in sunflower genome. Their gene structure, conserved motif, phylogenetic relationship and chromosome location informations were provided. The expression profiles of these genes in different sunflower organs were obtained by qRT-PCR analysis, which indicated that 9 GRFs highly expressed in seed may play important roles in seed development. The expression data, together with available regulation relationships for GRF genes, suggested that HaGRF genes may function in maintaining or promoting cell division by a feedback regulation mechanism in sunflower. Their different responses to ABA and abiotic stresses(NaCl, PEG6000) gave us a cue that GRFs regulate leaf development by both ABA-dependent and ABA-independent pathways at transcript level under abiotic stresses. miR396 targets findings suggested that there may exist a homeostasis involving miR396 and its abiotic stress responsive targets(three WRKY genes and 15 WRC-contained genes) in cultivated sunflower. Further studies need to be done for illuminating their biological functions and regulation mechanisms in developmental and abiotic stress responsive processes in sunflower..

Additional file 1: Table S1. Sequence of GRF proteins from lettuce, sweet wormwood, arabidopsis, chinese cabbage, rice, maize and grape.

Additional file 2: Table S2. qRT-PCR primers used in this article.

Additional file 3: Figure S1. Distribution of 17 GRF genes on the 17 sunflower chromosomes.

Additional file 4: Figure S2. Multiple sequence alignment of *HaGRF17* (HanXRQChr00c0041g0571), Ha412v1r1_00g106720 and Ha412v1r1_17g006360.

Additional file 5: Figure S3. Alignment of the conserved regions of the 17 HaGRF proteins.

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Conflicts of Interest:

The authors declare no conflict of interest.

Abbreviations

TF	Transcription factors
GRF	growth-regulating factor
ABA	Abscisic acid
GA3	Gibberellin A3
PEG6000	polyethylene glycol 6000
CDS	Coding sequence
MEME	Multiple expectation maximization for motif elicitation
MS	Murashige and skoog
qRT-PCR	Quantitative real time PCR

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