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Characterization and expression analysis of growth regulating factor (GRF) family genes in cucumber

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Abstract: The growth regulating factor (GRF) family is a conserved class of transcription factors involved in various biological processes in plants. However, there have been only a few studies of the *GRF* family genes in cucumber, *Cucumis sativus* (Cs). In this study, we identified and characterized 8 *CsGRF* genes in cucumber. Two highly conserved domains, QLQ and WRC, were identified to be present in all CsGRF proteins. In addition, three less conserved domains (FFD, TQL, and GGPL) were also detected in some CsGRF members. Based on phylogenetic analysis, the *GRF* genes from cucumber, *Arabidopsis*, tomato, rice and maize could be classified into 10 groups, and CsGRFs were clustered closer with the *GRF* genes from dicots (*Arabidopsis* and tomato) than with those from monocots (rice and maize). Promoter analysis revealed that the *CsGRF* genes were involved in cucumber growth and development as well as in responses to various hormones and stresses. Transcriptome data showed that the *CsGRF* genes have distinct expression patterns in different tissues, especially in ovaries and leaves. Expression profiling analysis indicated that all *CsGRF* genes were responsive to salt and drought stress treatments. These results demonstrate that the cucumber *GRF* gene family may function in organ development and plant stress responses.

Key words: cucumber; growth regulating factor (GRF); gene family; expression analysis; abiotic stress

INTRODUCTION

GRFs are plant-specific transcription factors (TFs) widely distributed in the genomes of all known seed plants [1]. In previous studies, *GRF* family genes were identified in different plant species, such as Arabidopsis [2], rice [3], maize [4], Chinese cabbage [5], cucumber, melon and watermelon [6], Chinese pear, poplar and grape [7], citrus [8], common bean [9], oilseed rape [10], tea plant [11], and tomato [12]. These reports showed that all of the GRF proteins harbor two types of N-terminal domains, named QLQ (Gln, Leu, Gln) and WRC (Trp, Arg, Cys). The QLQ domain can act as a transcriptional coactivator by interacting with the SNH domain of GRF interacting factors (GIFs) to form plant-specific transcriptional complexes [13,14]. The WRC domain contains a nuclear localization signal and a zinc finger DNA binding motif, which is capable of binding to DNA [2,15]. In addition, some GRF proteins also contain less-conserved TQL (Thr, Gln, Leu), FFD (Phe, Phe, Asp) and GGPL (Gly, Gly, Pro, Leu) domains in their C-terminal regions [9, 11, 12].

GRF proteins play crucial roles in various biological processes, such as the development of leaf [2,13,16], root [17,18], stem [15], and floral organ [19-21], as well as seed formation [22,23] and hormone signaling [15,24]. As the first identified *GRF* gene, *OsGRF1* was observed to be responsive to gibberellin (GA) and involved in stem growth in rice [15]. RNA interference silencing of *OsGRF3*, *OsGRF4* and *OsGRF5* resulted in dwarfism and delayed growth and inflorescence formation [25]. Higher expression of

OsGRF4 modulated tissue and organ size, resulting in significantly larger grain, longer panicle and lower seed shattering, and enhanced grain yield [22,24,26-28]. In *Arabidopsis*, *grf* mutants exhibited smaller and narrower leaves, while overexpression of some *GRF* genes resulted in larger leaves than wild-type [2,16,29]. Ectopic expression of *BrGRF8* in *Arabidopsis* also resulted in increases of the sizes of the leaves and other organs by regulation of cell proliferation [5].

GRF proteins also function in responses to various abiotic stress conditions such as salt, drought, heat and cold [12,30]; however, the roles of plant *GRF* family genes in stress responses remain elusive. Although a previous study reported 8 *GRF* family genes in cucumber [6], to date there has been no report about the molecular and expression characterization of the cucumber *GRF* gene family. In this study, we examined the phylogenetic relationships and promoter sequences of cucumber *GRF* genes, as well as their expression profiles in different tissues and in response to different abiotic stresses. The results indicated that *GRF* genes might have differential functions during tissue development and responses to various abiotic stresses, and they provide a foundation for further elucidation of the functions of GRF members in cucumber.

MATERIALS AND METHODS

Identification and protein properties of CsGRFs

The sequences of *Arabidopsis* and rice GRF proteins were downloaded from TAIR (http://www.Arabidopsis.org/) and TIGR (http://rice.plantbiology.msu.edu/), respectively, according to previous studies [2,3]. A BLASTP search was performed using these protein sequences in the Cucumber (Chinese Long) Genome Database (http://cucurbitgenomics.org/organism/2) to identify putative GRF proteins. After removal of redundant sequences, the remaining putative GRF protein sequences were subjected to online database searches of SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) to confirm the conserved QLQ and WRC domains. Finally, the candidate GRF proteins were named based on their distributions on the seven cucumber chromosomes according to a previous study [6]. The ProtParam tool (http://web.expasy.org/protparam/) was used to analyze the theoretical molecular weight (MW), isoelectric point (pI), and grand average of hydropathicity (GRAVY) of the deduced CsGRF proteins.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of GRF protein sequences from cucumber, *Arabidopsis*, and rice was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [31] and displayed using the Genedoc software. For a phylogenetic analysis of the plant *GRF* gene family, the full-length GRF protein sequences from cucumber, *Arabidopsis*, tomato, rice, and maize were aligned with Clustal Omega and a phylogenetic tree was then generated using the MEGA 7.0 software by the neighbor-joining method with 1,000 bootstrap replicates [32].

Analysis of promoter regions

To investigate the *cis* elements in the promoter regions of *CsGRF* genes, 1000-bp upstream sequences of the transcriptional start site of the *CsGRFs* were chosen to identify the *cis* elements in the putative promoter regions using the PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) [33].

Expression profiling of *CsGRFs* by transcriptome data

Genome-wide transcriptome data from different tissues were downloaded from a public repository database (https://www.ncbi.nlm.nih.gov/sra/?term=sra046916). The tissue expression profiles of the *CsGRF* genes were retrieved and analyzed according to previous studies [34,35] and displayed as scaled and centered fragments per a kilobase-exon model per million mapped reads (FPKM) of values by the pheatmap package in R software.

Plant materials and treatments

Two-week-old seedlings of cucumber (*Cucumis sativus* L. cv. Chinese long No. 9930) were subjected to salt and drought stress conditions, which were described in our previous study [36]. The leaf tissues were collected at 0, 3, 6, and 12 h, frozen immediately in liquid nitrogen and stored at -80°C until RNA isolation.

RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA from seedlings samples was extracted using Trizol reagent (Tiangen, China) according to the manufacturer's instruction. The first-strand cDNA was synthesized using the SuperscriptIII RNase H-Reverse Transcriptase kit (Invitrogen, USA) following the manufacturer's instructions. qRT-PCR was performed in triplicate using the SYBR Green Master Mix (Tiangen, China) as described previously [36]. The cucumber actin gene (*CsAct3*) was used as an internal control and the relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method [37], and the expression of control plants (without treatment, 0 h) was normalized to 1. The primers used for qRT-PCR are listed in Table S1.

RESULTS

Identification and characterization of GRF proteins in cucumber

Eight candidate cucumber GRF proteins (CsGRFs) were identified and named according to a previous study [6]. Chromosome 2 and 3 contained 3 *CsGRF* genes each, whereas one *CsGRF* gene was observed in chromosomes 1 and 6 (Table 1). The *CsGRF* genes varied in length from 1129 bp (*CsGRF2*) to 4403 bp (*CsGRF4*), and encoded proteins ranged from 319 (CsGRF2) to 672 (CsGRF3) amino acids in length. Analysis of the physical and chemical characteristics of CsGRF proteins showed that the CsGRFs had MW ranging from 36.81 (CsGRF2) to 71.21 (CsGRF3) kDa, the pI ranged from 7.04 (CsGRF3) to 9.59 (CsGRF5), and GRAVY was from -1.052 (CsGRF2) to -0.547 (CsGRF5) (Table 1). The GRAVY values of CsGRF proteins were lower than 0, indicating that all the CsGRFs were hydrophilic proteins.

Nearly all the CsGRF proteins contained one QLQ domain and one WRC domain in the N-terminal region, whereas CsGRF5 contained one QLQ domain and two WRC domains (Table 1). To investigate the conserved domains in GRF proteins, we performed a comparative analysis of the full-length deduced amino acid sequences of GRF proteins from cucumber, *Arabidopsis* and rice. As shown in Fig. 1A, the Q-L-Q residues were highly conserved in nearly all the GRF proteins from cucumber, *Arabidopsis* and rice, with the exception of AtGRF9, which contained F in place of L. This phenomenon was also observed in the GRF proteins of *Solanum lycopersicum* [12] and *Camellia sinensis* [11]. A zinc finger motif (CCCH) was present within the WRC domain in all CsGRF, AtGRF and OsGRF proteins. In addition, three short stretches of amino acid residues termed as FFD, TQL and GGPL domains were also found in the C-terminal region of some GRF proteins in cucumber, *Arabidopsis* and rice (Fig. 1B and C), and these domains might serve as transactivation domains [1], suggesting that they may have specific functions.

Phylogenetic analysis of GRF proteins from different plant species

To further elucidate the phylogenetic relationships of GRF family proteins in cucumber and other plant

Table 1. A complete list of 8 CsGRFs identified in this study.

Genes	Locus name	Chromosome	Location (5′-3′)	Protein physicochemical characteristics				gDNA size (bp)	Domain (Start-End)	
				length (amino acids)	MW (kDa)	pI	GRAVY		QLQ	WRC
CsGRF1	Csa1G595880.1	1	22512341–22513837	333	37.82	9.38	−0.801	1165	7–43	77–119
CsGRF2	Csa2G000170.1	2	123959–125295	319	36.81	8.95	−1.052	1129	7–43	80–122
CsGRF3	Csa2G354030.1	2	16402951–16406142	672	71.21	7.04	−0.568	3071	195–231	266–308
CsGRF4	Csa2G432230.1	2	22551475–22556300	337	37.09	7.73	−0.695	4403	30–66	93–135
CsGRF5	Csa3G124990.1	3	7591313–7595750	502	55.04	9.59	−0.547	2969	83–119	155–197; 391–433
CsGRF6	Csa3G651860.1	3	25711809–25713614	343	38.28	7.17	−0.806	1427	10–46	77–119
CsGRF7	Csa3G751470.1	3	29246154–29248293	416	45.26	8.74	−0.711	1800	77–111	151–193
CsGRF8	Csa6G496380.1	6	24156268–24159569	572	62.05	7.20	−0.620	2977	155–191	225–267

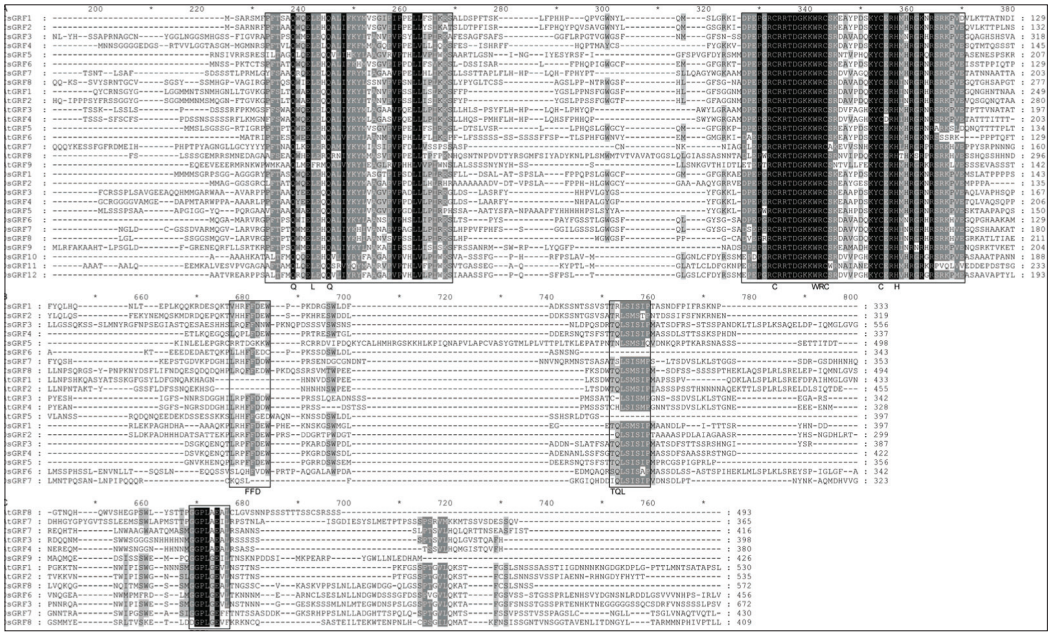

Fig. 1. Multiple sequence alignment of the GRF proteins from cucumber, *Arabidopsis* and rice. Identical amino acids are shown against a black background. **A** – QLQ and WRC domains of the CsGRF, AtGRF and OsGRF proteins. **B** – FFD and TQL domains of CsGRFs, AtGRF1-5, and OsGRF1-7 proteins. **C** – GGPL domains of CsGRF3, CsGRF7, CsGRF8, AtGRF1-4, AtGRF7, AtGRF8 and OsGRF6-9 proteins. These domains are framed by black lines.

species, we constructed a phylogenetic tree with the GRF protein sequences from four other plant species including *Arabidopsis* (9 AtGRFs), rice (12 OsGRFs), tomato (13 SlGRFs), and maize (14 ZmGRFs). Phylogenetic analysis suggested that all the 56 GRF proteins were clustered into ten groups (a-j), and each group had 2-8 members with high bootstrap (Fig. 2). Of these, only two groups (a and j) contained GRFs from both monocot and dicot species. All of the 8 CsGRFs were distributed in 5 groups and clustered together with GRF members from *Arabidopsis* and tomato. For example, CsGRF3 and CsGRF8, which were clustered with 2 AtGRFs, 2 SlGRFs and 1 OsGRF, belonged to group a (Fig. 2). Group j was comprised of CsGRF5, which was clustered with 1 *Arabidopsis*, 2 tomato, 1 rice and 2 maize GRF proteins (Fig. 2), while groups d, f and h, which were composed of other CsGRFs, only contained GRFs from dicot species.

Analysis of *cis element*s in the promoter regions of *CsGRF* genes

Cis-acting regulatory elements are important molecular switches involved in the regulation of gene transcription during plant growth and development as well as in the responses to various hormones and stresses [38]. To identify the presence of *cis* elements in the promoter regions of *CsGRF* genes, a 1.0-kb promoter region of each *CsGRF* gene was retrieved and a comprehensive *cis* element analysis was performed using PlantCARE. The *cis* elements related to plant stress, hormone responses and developmental processes are shown in Fig. 3 and Table S3. Numerous *cis* elements related to plant stress and hormone response were identified, implying that *CsGRF* genes might be involved in response to various stresses and hormones. Essentially, 3 TC-rich repeats and 4 TCA elements, which might account for plant responses to various defenses and salicylic acid (SA), were present in the promoters of *CsGRF4* and *CsGRF8*, respectively. In addition, several *cis* elements related to developmental processes such as meristem-specific regulation (CAT-box and CCGTCC-box), circadian control (circadian), zein metabolism regulation (O2-site), cell cycle regulation (MSA-like), and endosperm-specific elements (GCN4_motif and Skn-1_motif) were found in some *CsGRF* promoter regions (Fig. 3 and Table S3), implying that *CsGRF* genes have development-specific expression.

Expression analysis of *CsGRF* genes in cucumber tissues

To better understand the functions of *CsGRFs* in growth and development of cucumber, their FPKM values of transcriptome data in different developmental tissues (ovaries, tendrils, roots, stems, leaves, male and female flowers) were retrieved and analyzed according to our previous study [34]. The results showed that some *CsGRFs* were strongly transcribed in ovaries. For example, *CsGRF1* and *CsGRF8* had preferential accumulation of transcripts in unexpanded ovaries, and *CsGRF5* displayed the highest expression in fertilized ovaries, while *CsGRF3* was the most abundantly transcribed in unfertilized ovaries (Fig. 4). In addition, the highest expression of *CsGRF2* was observed in both fertilized and unfertilized ovaries, but it was not detected in unexpanded ovaries. Additionally, *CsGRF4* and *CsGRF6* exhibited the highest expression in leaves, and relatively lower expression in unfertilized and fertilized ovaries. In particular, the expression of *CsGRF7* was only detected in roots (Fig. 4). These results indicated that CsGRFs might be involved in the development of specific tissues and growth periods in cucumber.

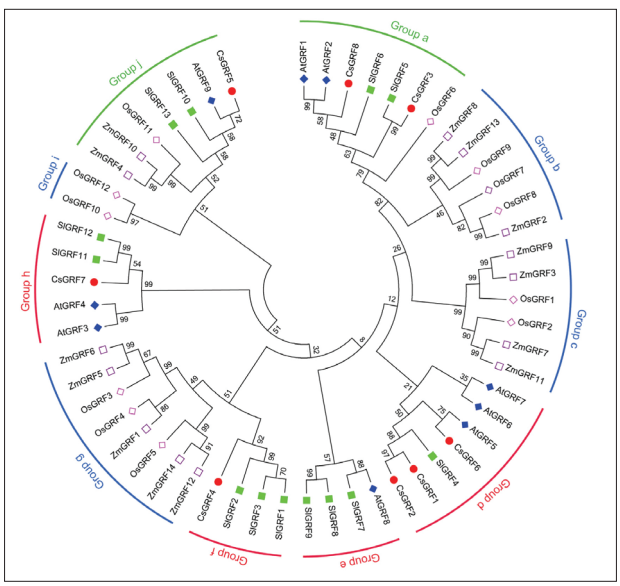

Fig. 2. Phylogenetic tree of GRF proteins from cucumber, *Arabidopsis*, tomato, rice and maize. The phylogenetic tree was constructed using the neighbor-joining method as implemented in MEGA 7.0 with the full-length GRF protein sequences. The GRF proteins can be classified into 10 groups (a-j). Bootstrap values from 1,000 replicates and the protein IDs are displayed in Table S2.

Expression profiles of *CsGRF* genes in response to salt and drought stresses

To examine the potential functions of *CsGRF* genes in response to abiotic stress, the transcript levels of these genes were determined in seedlings under salt and drought stress conditions. Under salt treatment, the expression of *CsGRF5*, *CsGRF6* and *CsGRF8* markedly decreased at 3 h, while that of *CsGRF7* increased (Fig. 5A). However, nearly all *CsGRFs* were downregulated at 6 h and 12 h, with the exception of *CsGRF6*, whose transcription level showed a drastic increase (Fig. 5A). After 3 h of drought treatment, the expression of *CsGRF3* and *CsGRF8* sharply decreased, while that of other genes had no significant change (Fig. 5B). In addition, the expression of nearly all *CsGRF* genes significantly decreased under drought treatment at

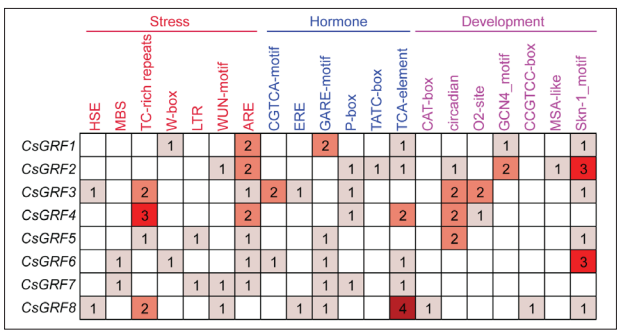

Fig. 3. Distributions of stress-, hormone-, and development-related *cis* elements in cucumber *GRF* gene promoters.

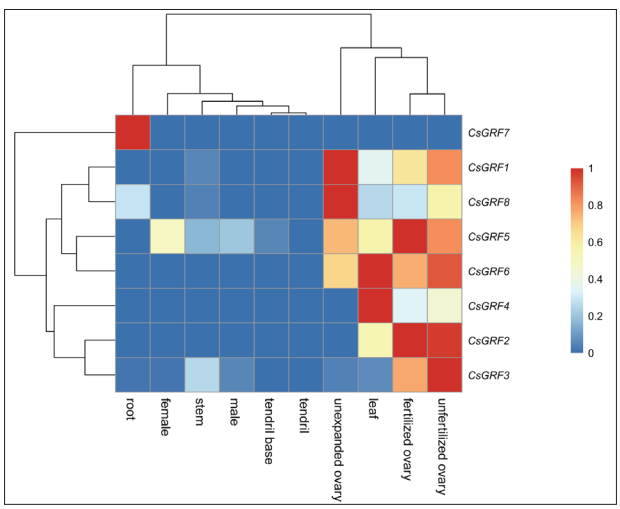

Fig. 4. Expression patterns of *CsGRF* genes in different developmental tissues of cucumber. The spectrum from blue to red shows the relative expression level of each gene from 0 to 1.

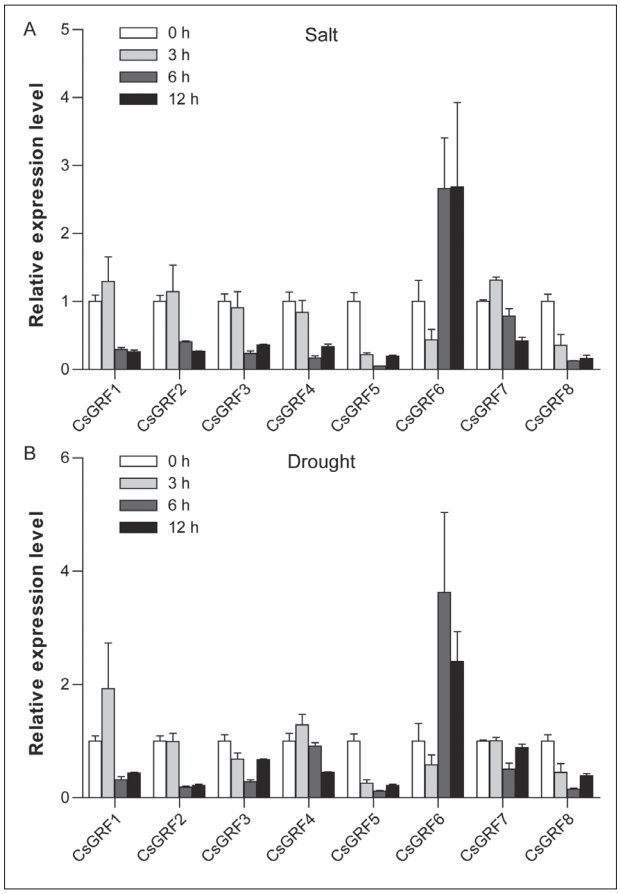

Fig. 5. Expression of *CsGRF* genes in response to salt (**A**) and drought (**B**) stress treatments. Values presented are means of three independent experiments, with error bars indicating standard deviations.

12 h and 24 h, while that of *CsGRF6* was remarkably induced (Fig. 5B). These results showed that *CsGRF* genes were involved in responses to salt and drought stresses.

DISCUSSION

In this study, we conducted a comprehensive analysis of the *GRF* family genes in cucumber and we identified 8 *CsGRFs*. The number of *GRFs* was smaller than that in some other plant species, such as Arabidopsis (9 members) [2], citrus (9 members) [8], pear (9 members) [7], rice (12 members) [3], tomato (13 members) [12], maize (14 members) [4], whereas it was larger than that in tea plant (6 members) [11]. This phenomenon may be attributed to gene duplication events of the *GRF* gene family. For example, one segmental duplication (*BdGRF3* and *BdGRF6*) was identified among the *GRF* genes in *Brachypodium distachyon* [39]. Four *Arabidopsis GRF* genes and 8 pear *GRF* genes were found in the duplication regions of their genomes [7], while no *CsGRF* genes were located in the duplication region of cucumber genome.

All CsGRF proteins contain one QLQ domain and one WRC domain, except for CsGRF5, which contains one QLQ domain and two WRC domains, similar to GRF12 in Chinese cabbage [5], GRF10 in tomato [12] and GRF6 in tea plant [11]. In addition, the FFD and TQL domains are conserved in nearly all CsGRF proteins with the exception of CsGRF5 and CsGRF6 which lack the FFD and TQL domains, respectively. Moreover, CsGRF3, CsGRF7, CsGRF8 and some GRF members from *Arabidopsis* and rice shared a GGPL domain in their C-terminal regions, which is consistent with the results of previous studies [5,11]. These results suggest that CsGRF proteins are evolutionarily conserved in plants. In addition, phylogenetic analysis showed that the GRF proteins from cucumber, *Arabidopsis*, tomato, maize and rice can be divided into 10 groups, with only 2 groups (group a and j) containing clusters of GRFs from both monocot and dicot species, indicating that these GRF proteins may have evolved either before (groups a and j) or after (groups b-i) the divergence of monocots and dicots. Moreover, CsGRFs seem to have a closer relationship with GRF proteins in dicots (*Arabidopsis* and tomato) than with those in monocots (rice and maize). For example, most of the CsGRFs were clustered in the groups with dicot species only. Phylogenetic analysis showed that CsGRF6 and AtGRF5 belong to the same group (group d); interestingly, both lack the TQL domain. Although CsGRF3, CsGRF5 and CsGRF8 were located in the groups with monocot species only, they also clustered together with GRFs from tomato and *Arabidopsis*.

GRF proteins are plant-specific TFs involved in normal plant growth, such as leaf, stem, flower and seed development [5,8,14,19,25]. In this study, the expression patterns of the *CsGRF* genes in different tissues showed some spatial differences, indicating their diverse roles in growth and development of cucumber. For example, the *CsGRF* genes, except *CsGRF4* and *CsGRF7*, were expressed in different growth periods of ovary development and shared a common *cis*-element Skn-1_motif,

which is required for endosperm expression. Another *cis* element involved in endosperm expression, the GCN4_motif, was also found in the promoter regions of *CsGRF1* and *CsGRF2*. In *Arabidopsis*, *GRF* genes are highly expressed in developing pistils, and overexpression of miR396 results in pistil abnormality through directly mediated cleavage of its *GRF* targets, which disrupts the formation of the GRF/GIF complex [21]. A recent report also demonstrated that the GRF-GIF duo regulates the meristematic and pluripotent competence of carpel margin meristems and the archesporial cell lineage in *Arabidopsis* [19]. These results indicate that some *CsGRF* genes may play essential roles in ovary development. In addition, *CsGRF6* showed the highest expression in leaves, and clustered together with *AtGRF5* in the same cluster of group d. AtGRF5 is required for the development of appropriate leaf size and shape and promotes cell proliferation in leaf primordia [16]. Hence, *CsGRF6* and *AtGRF5* may have similar functions in leaf development.

The expression of *GRF* genes has been reported to be regulated by various abiotic stresses [9,12,30]. In this study, all *CsGRF* genes exhibited differential accumulation or downregulation in response to salt and drought stresses, indicating that they may play essential roles in the response to abiotic stress. However, most *CsGRF* genes were downregulated, which is different from the responses of *SlGRF* genes to salt and drought stresses [12]. A previous study showed that AtGRF7 acts as a repressor of a wide range of osmotic stress-responsive genes, presumably by preventing growth inhibition under normal conditions [30]. Hence, the downregulated *CsGRF* genes under salt and drought stresses may play negative roles in the stress response. In addition, some closely related genes may have similar expression patterns in response to salt and drought stresses. For example, *CsGRF1, CsGRF2* and *SlGRF4* were in the same cluster of group d; *CsGRF1* and *CsGRF2* were sharply downregulated by salt and drought stresses at 6 h and 12 h. Similar expression patterns were also observed for *SlGRF4* under salt and drought treatments, implying similar functions of these genes in response to salt and drought stresses. It is worth noting that the expression of *CsGRF6* was significantly affected by salt and drought treatments, and *CsGRF6* harbors a MBS *cis* element that might be involved in drought-stress tolerance [40]. In addition, the promoter in *CsGRF6* was also predicted to possess a W-box, which is the cognate *cis* element for WRKY proteins and is widely present in the promoter region of stress-related genes [41, 42]. These results suggest that *CsGRF6* may act as a positive regulator involved in salt and drought stress. Further functional characterization of the stress-responsive *CsGRF* genes will lay the foundation for a better understanding of the potential mechanisms against abiotic stresses in cucumber.

CONCLUSIONS

We performed a detailed analysis of the phylogenetic relationships and *cis* elements in the promoter regions, and of the expression of *CsGRF* genes in cucumber. Our results provide the foundation for a better understanding the roles of *GRF* genes in responses to environmental stresses and help reveal the functions of this important gene family in specific developmental processes.

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Supplementary Data

Supplementary Table S1. Primers used for qRT-PCR in this study.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Zhou%20et%20al_2844_Table%20S1.doc
Supplementary Table S2. GRF protein IDs used for phylogenetic tree analysis.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Zhou%20et%20al_2844_Table%20S2.doc
Supplementary Table S3. The predicted promoter elements of CsGRF genes.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Zhou%20et%20al_2844_Table%20S3.xls