



Cloning and Primary Functional Analysis of *PeGRF1* in *Phyllostachys edulis*

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Abstract To explore the role of Growth regulating-factor (GRF) in the growth of bamboo shoots, one GRF gene was isolated from moso bamboo (*Phyllostachys edulis*) by RT-PCR. Bioinformatics methods were used for gene sequence analysis, and the expression pattern of the GRF gene in bamboo shoots at different development stages was analyzed by qRT-PCR. Simultaneously, ectopic expression in *Arabidopsis* was conducted to validate the gene function. The result showed that the isolated gene from moso bamboo was 1 164 bp, encoding 387 amino acids, which was named as *PeGRF1*. Protein sequence analysis showed that *PeGRF1* had the complete typical domains (WRC and QLQ) of GRF family. The phylogenetic analysis demonstrated that *PeGRF1* were clustered closer to the GRFs of monocotyledonous plants such as *Oryza sativa*, indicating they had close relationship. *PeGRF1* expressed predominantly in young bamboo shoots. Meanwhile, the expression level of *PeGRF1* was significantly higher during rapid growth stage than that during slow growth stage of bamboo shoots, and the expression level of *PeGRF1* in the upper and middle of bamboo shoots was significantly higher than that in the base. Overexpression of *PeGRF1* could increase the plant height of transgenic *Arabidopsis*. Taken together, our results demonstrated that *PeGRF1* was involved in development of bamboo shoots, which provided references for elucidating the biological functions of GRF genes in bamboo.

Keywords *Phyllostachys edulis*; GRF gene; Functionl analysis; Stem growth

The Growth regulating factor (GRF) is a plant-specific transcription factor, and that playing an important role in regulating plant growth and development (Omidbakhshfard et al., 2015; Yuan et al., 2017; He et al., 2018). The *OsGRF1* of *Oryza sativa* is the first member of GRF gene family was identified, and it perform a regulatory role in rice stem growth by GA (Van der Knaap et al., 2000). At present, more and more GRF gene families have been identified. For example, the *Oryza sativa* genome harbours at least 12 GRF genes and the *Arabidopsis thaliana* genome harbours at least 9 GRF genes (Kim et al., 2003; Choi et al., 2004). In recent years, with the continuous publication of plant genome, many non-model plants GRF gene families have also been identified and analyzed, such as *Brassica rapa* (Wang et al., 2014), *Citrus sinensis* (Liu et al., 2016), *Camellia sinensis* (Wu et al., 2017) and *Brassica juncea* (Zhang et al., 2020). GRF proteins consist of two conserved domains, the QLQ and WRC domains, in the N-terminal regions. The QLQ domain acts as a transcriptional co-activator, while the WRC domain comprises a functional nuclear localization signal and a zinc-finger motif that functions in DNA binding, which play an important role in the implementation of biological functions of GRF genes (Cao et al., 2016; He et al., 2018).

Previous studies have shown that the GRF genes are strongly expressed in actively growing and developing tissues, and thus participate in the regulation of the growth and development of plant, such as stem elongation (Van der Knaap et al., 2000), leaf development (Kim et al., 2003), flower development (Lee et al., 2014) and root growth (Bao et al., 2014). Overexpression of *AtGRF1*, *AtGRF2* or *AtGRF5* results in larger leaves in *Arabidopsis* (Kim et al., 2003; Horiguchi et al., 2005); meanwhile, overexpression of *OsGRF1* lead to plant height in *Arabidopsis* (Van der Knaap et al., 2000). In addition, overexpression of *ZmGRF10* results in smaller leaves, decreased cell number and increased cell size (Wu et al., 2014). These results indicated that the function of GRF gene was diverse.

Stem is the main structural of the plant, supporting the above-ground tissues or organs of the plant, and its growth largely effect the size of the plant. In grasses, the stem is composed of nodes and internodes. Bamboo belongs to the subfamily Bambusoideae of the Poaceae family, as an important group of forest, many bamboo species can complete their height growth in the short term. For example, the moso bamboo can grow more than 10 m in 2 months (Yuan et al., 2015), the *Dendrocalamus hamiltonii* height-growth lasts for about 3 months (Li et al., 2010). In order to explore the mechanism of the rapid growth of bamboo, there are many studies about moso bamboo from various aspects, such as morphological (Cui et al., 2012), physiology and biochemistry (Sun et al., 2018), molecular regulation (Bu et al., 2020). The moso bamboo genome harbours at least 18 GRF genes, and among which at least one GRF1 homologous gene exists (He et al., 2018). Therefore, Based on previous studies, a GRF gene, *PeGRF1*, was cloned from moso bamboo, and its expression pattern in the rapid growth stage of bamboo shoots was preliminarily analyzed in this study. Meanwhile, ectopic expression in *Arabidopsis* provided reference for the functional analysis of moso bamboo GRF gene in the future.

1 Results

1.1 Molecular characterization of *PeGRF1* in moso bamboo

A cDNA fragment of 1 200 bp was obtained from moso bamboo (*Ph. edulis*) leaves using the primers, which was consistent with the expected size (Figure 1). The sequencing results showed that the isolated gene from moso bamboo was 1 164 bp, which was 99.57% consistent with that of PH01087379G0010 in the BambooGDB database. The gene encoded 387 amino acids with a calculated molecular mass of 42.3 kDa and a theoretical isoelectric point of 7.66, named *PeGRF1*.

The secondary structure of *PeGRF1* protein was predicted by SOMPA software, and the results showed that the random coil was about 67.44%, the extended strand was about 11.89%, and the α helix and β turn were about 18.09% and 2.58%, respectively. There are similar in the N and C terminal regions between *PeGRF1* and *OsGRF1* proteins, but it is different in their middle region.

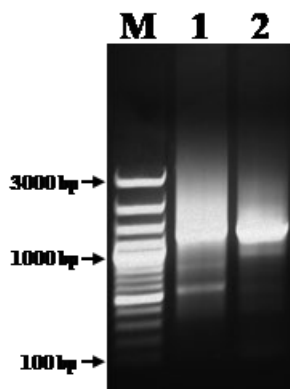


Figure 1 Cloning of Moso bamboo *PeGRF1*
Note: M: 100 bp Ladder III; 1~2: PCR products

1.2 Alignment and phylogenetic analysis of *PeGRF1*

The deduced amino acid sequences of *PeGRF1* were further examined for homology analysis with other known GRF1 sequences using BLASTP program in NCBI. The result showed that *PeGRF1* was more than 75% homologous with those of other monocots, such as *Oryza sativa*, *Brachypodium distachyum* and *Hordeum vulgare*. And about 55% homologous with those of other dicots, such as *Arabidopsis thaliana*, *Brassica rapa* and *Populus trichocarpa*. Similar to other GRF, *PeGRF1* had the complete typical domains (WRC and QLQ) of GRF family (Figure 2).

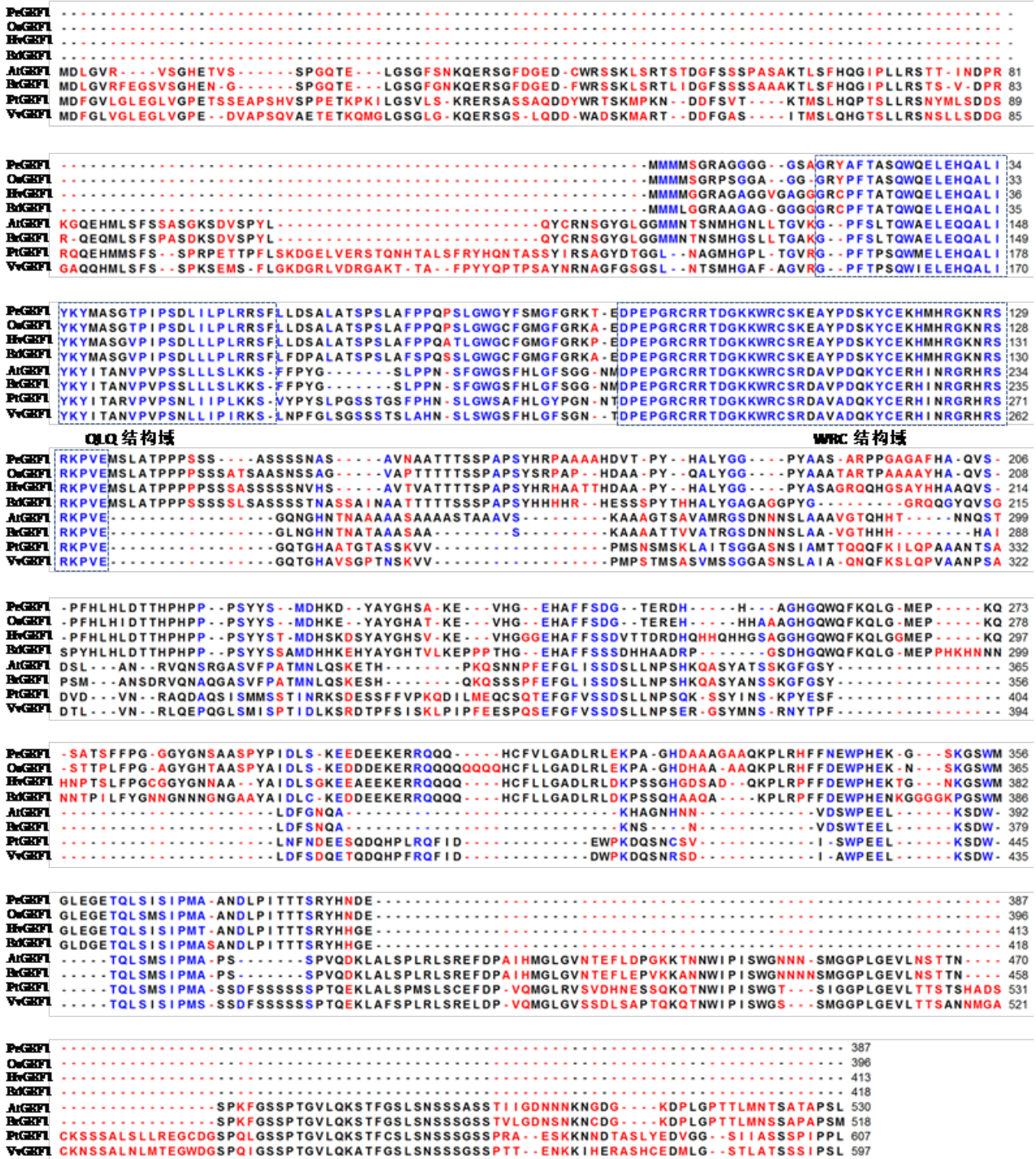


Figure 2 Alignment of the deduced amino acid sequences of GRF1 from *Ph. edulis* and other plants

Note: At: *Arabidopsis thaliana*; Bd: *Brachypodium distachyum*; Br: *Brassica rapa*; Hv: *Hordeum vulgare*; Pe: *Phyllostachys edulis*; Pt: *Populus trichocarpa*; Os: *Oryza sativa*; Vv: *Vitis vinifera*

To investigate the phylogenetic relationship of GRF1 among different species, a phylogenetic tree was constructed based on the amino acid sequence of PeGRF1 and GRF from other plants. In the phylogenetic tree, GRF from monocots and dicots were clustered into two clades (Figure 3). Furthermore, the PeGRF1 and OsGRF1 were in the same clades, PeGRF1 homology with the OsGRF1 was the highest (84%), indicating that they are closely relationship.

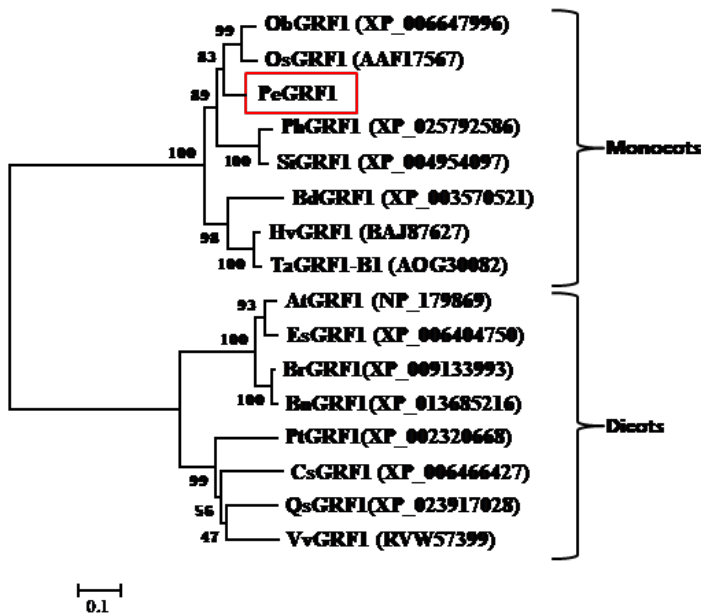


Figure 3 Phylogenetic tree constructed based on GRF1 protein sequences

Note: At: *Arabidopsis thaliana*; Bd: *Brachypodium distachyum*; Bn: *Brassica napus*; Br: *Brassica rapa*; Cs: *Citrus sinensis*; Es: *Eutrema salsugineum*; Hv: *Hordeum vulgare*; Pe: *Phyllostachys edulis*; Pt: *Populus trichocarpa*; Ob: *Oryza brachyantha*; Os: *Oryza sativa*; Ph: *Panicum hallii*; Qs: *Quercus suber*; Si: *Setaria italica*; Ta: *Triticum aestivum*; Vv: *Vitis vinifera*

1.3 Expression analysis of *PeGRF1*

The expression patterns of *PeGRF1* in different tissues of moso bamboo were analyzed according to the RPKM value of PH01087379G0010 in moso bamboo transcriptome data. It is found that RPKM value of *PeGRF1* in leaves, early panicles, advanced panicles, roots, rhizomes, 20-cm and 50-cm bamboo shoots were 2.42, 1.83, 1.52, 4.12, 5.92, 55.33 and 72.14, respectively. This showed that *PeGRF1* are expressed in all tissues, however, the expression levels of *PeGRF1* are difference in different tissues. *PeGRF1* expressed predominantly in young bamboo shoots, such as 20 cm and 50 cm bamboo shoots.

The expression patterns of *PeGRF1* in different development stage of bamboo shoots were analyzed using qRT-PCR. The results demonstrated that the expression levels of *PeGRF1* increased significantly along with the shoot growth, and reached the highest expression level in 3.0 m bamboo shoots. The expression levels of *PeGRF1* in 6.7 m bamboo shoots decreased slightly compared with that of 3.0m bamboo shoots, but it was still significantly higher than that of 0.2 m bamboo shoots (Figure 4A). Further analysis showed that *PeGRF1* had the highest expression in the upper internodes and nodes of 3.0 m and 6.7m bamboo shoots, and it was significantly higher than that of the middle and the basal (Figure 4 B; Figure 4C).

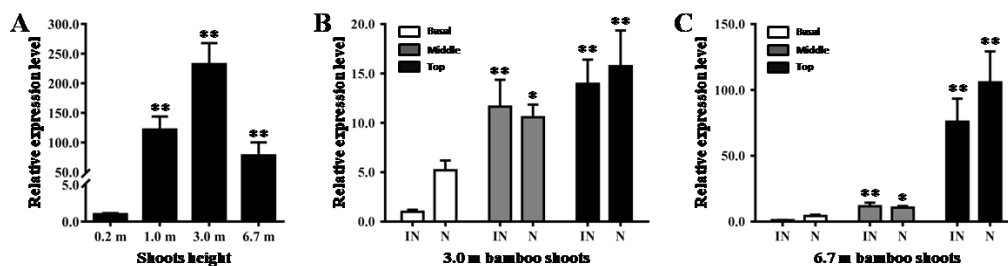


Figure 4 Expression pattern analysis of *PeGRF1*

Note: A: Basal of bamboo shoots with different heights; B-C: Different parts of 3.0 m and 6.7 m bamboo shoots; IN: Internode; N: Node; A single and two asterisks indicate the significance of the difference is $p < 0.05$ and $p < 0.01$

1.4 Generation of *PeGRF1* transgenic Arabidopsis plants and phenotypic analysis

To investigate whether heterologous expression of *PeGRF1* is functional, overexpression vector of *PeGRF1* was constructed and transformed into Arabidopsis (Col-0). After hygromycin resistance screening and verified by PCR, two transgenic lines (L4 and L8) were obtained (Figure 5A). In addition, the transcript levels of *PeGRF1* in L1 and L8 transgenic lines were determined using semi quantitative RT-PCR. The results showed that *PeGRF1* was successfully introduced into the Arabidopsis genome and expressed successfully (Figure 5B). It was found that the plant height of the *PeGRF1* transgenic plants increased significantly compared with the Arabidopsis (Col-0) (Figure 6), indicating that the overexpression of *PeGRF1* affect the growth of transgenic plants.

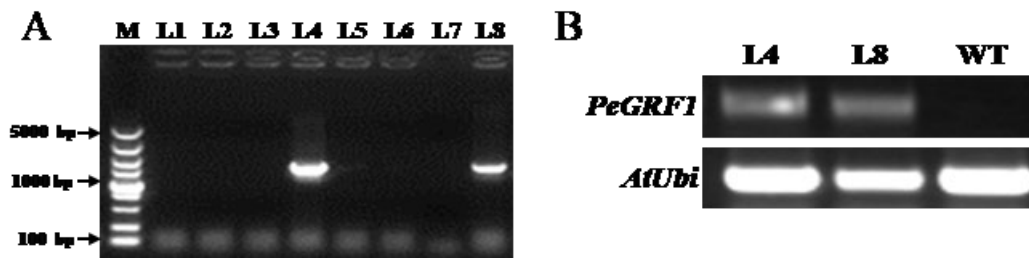


Figure 5 Identification of transgenic Arabidopsis (A) and expression analysis of *PeGRF1* in transgenic lines (B)
 Note: L1~L8: Suspected transgenic Lines; WT: Wild type (Col-0); M: DL5000 DNA Maker

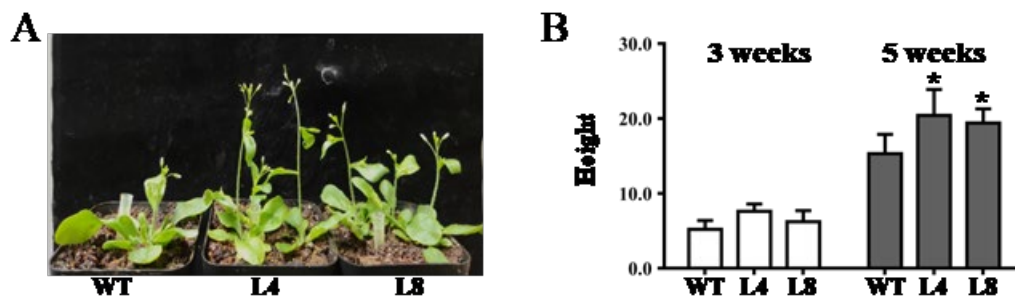


Figure 6 Analysis of phenotype (A) and height (B) in *PeGRF1* transgenic Arabidopsis
 Note: L4 and L8: Transgenic Lines; WT: Wild type (Col-0); Asterisks indicate significant difference ($p < 0.05$)

2 Discussions

GRF gene is a plant specific transcription factor gene family, and its related studies are increasing in recent years. In previous research, our teams have identified and analyzed 18 GRF genes from moso bamboo by bioinformatics, meanwhile, which tissue expression pattern also analyzed (He et al., 2018). Based on previous studies, a GRF gene, *PeGRF1*, was cloned from moso bamboo, with a length of 1 164 bp, encoding 387 amino acids, and belonging to the GRF gene family. According to the results of *PeGRF1* homology analysis and phylogenetic relationship, *PeGRF1* had high homology and closely relationship with GRF1 from monocots, such as *Oryza sativa*, *Hordeum vulgare*, moreover, had low homology with GRF1 from dicot, such as *Arabidopsis thaliana*, *Brassica rapa*. This indicates that the evolution of the GRF1 in monocots and dicot plants may be differences.

It has reported that the *GRF* genes are strongly expressed in actively growing and developing tissues, but weakly expressed in mature tissues or organs (Kim et al., 2003; Liu et al., 2016; He et al., 2018), In other words the expression level of *GRF* gene in tissues or organs is related to the development stage of that. For example, the expression levels of *CsGRF* genes were higher in tender leaves and consistently downregulated during tea plant leaf development (Wu et al., 2017). Bamboo shoots are the tender stage of the stem of bamboo, and the most active parts in the growth and development of various tissues or organs of bamboo. In this study, *PeGRF1* was mainly expressed

in bamboo shoots, which was consistent with the expression patterns of other plant GRF genes (Kim et al., 2003; Liu et al., 2016), meaning that *PeGRF1* play the roles in the development of moso bamboo shoots.

The expression patterns of *PeGRF1* in the 0.2, 1.0, 3.0 and 6.7 m bamboo shoots were analyzed, and found that *PeGRF1* higher expressed in the 1.0 and 3.0 m bamboo shoots, which was similar to the expression patterns of *GRF* genes families. The plant height growth process of moso bamboo can be divided into four stages: the initial stage, the ascending stage, fast growth stage and maturity stage. Bamboo shoots after Unearthed, which growth from the basal to the top internode, and showed the pattern of "slow-fast-slow". The 0.2 m bamboo shoots, which just unearthed and in the initial stage of height growth process, with slow growth and less active of tissues, so the *PeGRF1* low expressed. The 1.0, 3.0 and 6.7 m bamboo shoots are in the ascending and fast growth stages, the rapid growth of 1.0 and 3.0 m bamboo shoots mainly caused by the rapid elongation of the basal internode, which have the high activity, and the expression levels of *PeGRF1* increased significantly. However, the basal internode of the 6.7 m bamboo shoots has basically completed the height growth, which in the "mature stage", so it activity has decreased. At the same time, *PeGRF1* expression decreased. This suggests that *PeGRF1* may be involved in the rapid growth of moso bamboo shoots. In addition, at fast growth stages, 3.0 m and 6.7 m bamboo shoots, the growth and development activity of the upper internode of the shoots was significantly higher than that of the middle and basal internode, and the expression level of *PeGRF1* in the upper internode was higher than that of the middle and basal internode. The nodes of the bamboo shoots have intercalary meristem, which is an important reason for the elongation of bamboo shoots. Compared with the internodes, the expression level of *PeGRF1* in the nodes of bamboo shoots is higher.

GRF transcription factors play an important role in regulating plant growth and development. For example, overexpression of *AtGRF1* or *AtGRF2* results in larger leaves and cotyledons in *Arabidopsis* (Kim et al., 2003), Overexpression of *BnGRF2* and *BrGRF8* in *Arabidopsis* also leads to larger leaves, and Overexpression of *PagGRF15* in poplar leads to larger leaves (Liu et al., 2012; Wang et al., 2014; Zhou et al., 2019). However, overexpression of *ZmGRF10* results in smaller leaves by decreased cell number and increased cell size (Wu et al. 2014). This indicating that *GRF* gene has both positive and negative regulatory functions. In the present study, we found that *PeGRF1* affected plant height, and overexpression of *PeGRF1* can increase the plant height in *Arabidopsis*. Combined with the previous results that *PeGRF1* expressed predominantly in young bamboo shoots, meanwhile, the expression levels of *PeGRF1* were significantly higher during rapid growth stage than that during slow growth stage of bamboo shoots, we deduced that *PeGRF1* plays an important role in regulating bamboo shoots growth and development.

In this study, the full-length cDNA of *PeGRF1* of moso bamboo was cloned, and its expression pattern in the rapid growth stage of bamboo shoots was preliminarily analyzed. Meanwhile, ectopic expression in *Arabidopsis* provided reference for the functional analysis of moso bamboo GRF gene in the future.

3 Materials and Methods

3.1 Plant materials

To examine the expression levels of *PeGRF1* in moso bamboo shoots at different development stages, the basal parts of bamboo shoots with different heights (0.2, 1.0, 3.0, and 6.7 m) were collected from the Nanchang Botanical Garden of Jiangxi Academy of Forestry in April to May 2018. In addition, the nodes and internodes of the basal, middle and upper parts with the 3.0 m and 6.7 m moso bamboo shoots were collected, respectively. Each sample was three Biological replicates, frozen immediately in liquid nitrogen and stored at -80°C.

3.2 Total RNA Isolation and cDNA synthesis

The total RNA was extracted using the plant total RNA extraction kit (TaKaRa MiniBEST Plant RNA Extraction Kit, TaKaRa, Dalian, China) according to the manufacturer's instructions. High quality total RNA was selected and used for the synthesis of cDNA by a reverse transcription system (PrimeScript™ 1st Strand cDNA Synthesis Kit, TaKaRa, Dalian, China).

3.3 Gene isolation and bioinformatics analysis

To identify the putative *GRF1* homologue sequence in moso bamboo, the BLAST search was performed using the known sequences of *OsGRF1* (AF201895) as queries in the database of BambooGDB. Primers were designed according to the *GRF1* homologue sequences (PH01087379G0010) in moso bamboo. *GRF1*-001: 5'-ATGATGATGATGAGCGGTCGC-3' and *GRF1*-501: 5'-TCACTCATCGTTGTGGTAGCGG -3'. PCR amplification reactions were performed in a volume of 25 μ L, which contained 3.0 μ L of cDNA, 2.5 μ L of 10 \times LA Buffer, 2.5 μ L of dNTPs Mixture (2.5 μ mol/L each), 1.5 μ L of forward and reverse primer (10 μ mol/L), 0.3 μ L of LA Taq DNA polymerase (5 U/ μ L) and 13.7 μ L of ddH₂O. PCRs were performed with an initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperature of 65°C for 30 s, and extension at 72°C for 90 s; followed by a final extension at 72°C for 10 min. The PCR products were inserted into the pMD19-T vector, and subsequently confirmed by sequencing.

Sequence homology was searched using BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignments of *PeGRF1* and *GRF1*s from other plants were conducted using DNAMAN. Phylogenetic tree was constructed using MEGA 7.0 software. The open reading frame (ORF), molecular weight (MW), and isoelectric point (pI) parameters of *PeGRF1* were calculated by the online program ExPasy (<http://www.expasy.org/tools/>).

3.4 *PeGRF1* expression analysis

The expression pattern of *PeGRF1* was assessed using quantitative real-time PCR (qRT-PCR), which performed on a qTOWER system, and with specific primers *GRF1*-002 and *GRF1*-502 (*GRF1*-002: 5'-CTCTCCGGCACTTCTCAAC-3' and *GRF1*-502: 5'-GTAGCGGGAAGTGGTGGTAA-3'), *PeNTB* was also amplified as the reference gene (Fan et al., 2013). The qRT-PCR procedure consisted of 95°C for 5 min and 40 cycles of 95°C for 10 s, and 62°C for 10 s. The reaction volume was 10.0 μ L containing 5.0 μ L of 2 \times SYBR Green 1 Master, 1.0 μ L cDNA, 0.3 μ L of primer (5.0 mM, each) and 3.4 μ L ddH₂O. The relative expression level of *PeGRF1* was calculated with $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

In addition, to investigate the expression of *PeGRF1* genes in the different tissues and developmental stages of shoots, RNA-seq data generated from leaves, early panicles, advanced panicles, roots, rhizomes, 20 cm shoots and 50-cm shoots were downloaded from the NCBI Short Read Archive (SRA) (Peng et al., 2013) and used for gene expression analysis.

3.5 Construction of plant expression vectors and transformation

The ORF sequences of *PeGRF1* was amplified using the primer pairs of *GRF1*-003 and *GRF1*-503, which harbored *Bam*H I and *Xba* I restriction sites in forward and reverse primers respectively. The fragments were subcloned into the corresponding sites of modified pCAMBIA1301 vector, and construction of plant expression vector pCAMBIA1301-*PeGRF1*. After the validation of integrity by sequencing, the vector pCAMBIA1301-*PeGRF1* was introduced into *Agrobacterium tumefaciens* EHA105 by electroporation, and the positive clones were selected for transform Arabidopsis plants.

Transformation Arabidopsis plants by the floral dip method. T₀ generation transgenic Arabidopsis plants were selected using 1/2 MS medium with 30 mg/L hygromycin. T₁ generation resistant lines were obtained, and then which DNA extracted for PCR validation. Finally, T₂ transgenic lines were selected and used in subsequent research.

Authors' contributions

This work was carried out in collaboration between authors. Lou Yongfeng designed and carried out the experiments, analyzed the results and wrote the manuscript; Song Xiaochen and Sun Huayu contributed experiments and analysis. Gao Zhimin conceived the idea, designed experiments, analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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