Cloning and analysis of DlERF23 gene in flower induction

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Abstract

Irregular flowering is a serious problem in longan production. Identifying the flower induction-related genes and analyzing their regulation mechanism is the key to solve this problem. The APETALA2/ethylene responsive factor (AP2/ERF) superfamily members are transcription factors (TFs) that regulate diverse developmental processes, including flowering time, and stress responses in plants. However, there is still no research about AP2/ERF involved in the regulation of longan flower induction. In the present study, a AP2/ERF TF member DlERF23 was cloned from longan (Dimocarpus longan). It has a typical AP2 domain with the coding sequence (CDS) of DlERF23 is 552 bp in length and encodes 184 amino acids. The molecular weight of DlERF23 protein was 20.41 kda and the theoretical isolectric point (PI) was 7.69. The amino acid sequence of DlERF23 protein had the highest similarity with CsERF23 (XP_006478313.1) of Citrus sinensis and CcERF23 (XP_006441807.2) of Citrus clementina. The results of qRT-PCR showed that the relative expression level of DlERF23 gene in pericarp was higher, followed by stem, leaf, flower and flower bud. Meanwhile, DlERF23 gene significant down-regulated in the early stage of flower induction in ‘Sijimi’ (SJ) longan and up-regulated in the late stage of flower induction in ‘Shixia’ (SX). The results of transient expression of Arabidopsis protoplasts showed that the fluorescence signal was mainly concentrated in the nucleus. Moreover, overexpression of DlERF23 in Arabidopsis promoted early flowering. These results provide useful information for revealing the biological roles of DlERF23 in longan and increase our understanding of the AP2/ERF superfamily members in fruit trees.

Keywords: DlERF23; flowering; longan; transgenic Arabidopsis

Introduction

The AP2/ERF superfamily is the largest and one of the most important transcription factors (TFs) family which plays important roles in plant growth, development, fruit ripening and biotic and abiotic stress responses in plants (Jiang *et al*., 2022). AP2/ERF superfamily was early confirmed in Arabidopsis thaliana with 147 members (Feng *et al*., 2005). Then, AP2/ERF family was gradually identified in diverse species, such as rice (Nakano *et al*., 2006), banana (Lakhwani *et al*., 2016), cotton (Liu *et al*., 2017), pear (Li *et al*., 2018), pineapple (Zhang *et al*., 2021), and Actinidia eriantha (Jiang *et al*., 2022). Based on the number of AP2 domain and the
specific B3 DNA-binding domain, AP2/ERF family can be divided into five subfamilies, APETELA2 (AP2), ABI3/VP1 (RAV) containing both of AP2 and B3 domain, DREB (dehydration-responsive element-binding protein), ERF (ethylene-responsive factor) subfamily and other proteins (Soloist). Additionally, based on the sequence similarity of their AP2 domain, DREB subfamily can be further grouped into A1 to A6, and ERF subfamily can be grouped into B1- B6 as well (Sakuma et al., 2002).

Generally, AP2/ERF family proteins play important roles in the response to external biological and environmental stresses, including water deficit, drought (Dossa et al., 2016), high salinity (Zhang et al., 2009), low and high temperatures (Dubouzet et al., 2003; Ito et al., 2006), hormone response (Zarei et al., 2011), and disease response (Zhang et al., 2009). Additionally, previous studies also showed that AP2 family proteins have important functions in the regulation of developmental processes, such as seed development (Jofuku et al., 2005), and embryo development (Boutilier et al., 2002). There are plenty of evidences that AP2/ERF TFs cooperated other floral meristem genes to regulate floral organ development or flowering (Irish et al., 1990; Bowman et al., 1993; Zeng et al., 2021). For instance, during the flower development of Dendrobium officinale, the expressions of two DoAP2 genes (DoAP2-8 and DoAP2-10) were down-regulated and another two AP2 genes (DoAP2-2 and DoAP2-3) were up-related (Zeng et al., 2021). TOE1, one of the AP2-like TFs, could control flowering by regulating FT expression (Du et al., 2020). Overexpression of miR172, whose target is AP2-like gene, resulted in the double flower phenotype in roses (François et al., 2018).

Longan (Dimocarpus longan) is an important tropical/subtropical evergreen fruit tree which is widely cultivated in Southeast Asia, especially in China. Obtaining a stable annual fruit yield is the key factor that affecting the healthy development of longan industry (Jue et al., 2019). However, the adverse environmental conditions, such as spring frost and high temperature and moisture could lead to irregular flowering of longan and cause an unstable production (You et al., 2012). Therefore, the key to solve this problem is to identify the flower induction-related genes and analyze their regulation mechanism. Previous study has identified 125 longan AP2/ERF superfamily members in longan, and found some AP2/ERF members regulated early somatic embryogenesis and developmental processes in longan seed, root, and flower, and responded to exogenous hormones (Zhang et al., 2020). However, the detailed functional analysis for AP2/ERF superfamily members involving in the regulation of longan flower induction is still limited. In this work, DlERF23 was isolated and its potential function was investigated by overexpressing in transgenic Arabidopsis plants. Our findings indicated that DlERF23 plays important roles in the flowering induction of longan.

**Materials and Methods**

**Plant material**

The longan cultivars of 'SJ' and 'SX', which display opposite flowering phenotypes, were grown in an experimental orchard located in Mazhang district (110°16′ E, 21°10′ N), Zhanjiang, Guangdong Province, P. R. China. Three different developmental flower bud samples were obtained during different periods from November in 2016 to February in 2017: the dormant apical bud period (T1), the floral primordia differentiation period (red bud) (T2), and the floral organ formation period (T3). Three biological replicates from three different trees were used for each sample. Nine ‘SJ’ longan tissues, including flower, flower bud, leaf, pericarp, pulp, root, seed, stem, and young fruit (the fruit that 60 days after flowering) which were collected from November in 2016 to April in 2017 were used for tissue expression analysis. All samples were frozen immediately in liquid nitrogen and stored at -80 °C.
RNA extraction, qRT-PCR and DlERF23 gene cloning

Total RNA from different longan tissues was extracted by using the plant RNA extraction Kit (Huangyueyang, Beijing, China) according to the manufacturer’s instructions, and the quality of the RNA were detected as described of our previous study (Jue et al., 2019). The cDNA synthesis was performed with the PrimeScript First-Strand cDNA synthesis Kit (TaKaRa, Bio, China). The base sequence and amino acid sequence information of the DlERF23 gene (Dlo_015669.1) were obtained from the longan genome database (NCBI Sequence Read Archive, SRA315202) (Lin et al., 2017). Primer Premier 5.0 was used to design the primers ERF23-S and ERF23-A for DlERF23 gene cloning based on the ORF sequence of the DlERF23 gene. LightCycler® 480 Real-Time PCR System (Roche, Germany) and SYBR Green II PCR Master Mix (Takara, Dalian, China) were used for qRT–PCR experiment. The amplification program was performed as described in our previous study (Jue et al., 2018). The longan Actin1 gene (Dlo_028674) was used as an internal control for normalization. The gene-specific primers used in this study are listed in Table S1. The relative expression levels of the candidate genes were calculated by the 2^{−ΔΔCt} method. The analysis included cDNA from the three biological samples for each tissue, and all the reactions were run in triplicates.

Sequence alignment and bioinformatic analysis

The BioXM 2.6 software (http://cbi.njau.edu.cn/BioXM/) was used to calculate the MW, the number of amino acids, the ORF, ORF length, and isoelectric point (pI) of DlERF23 protein. The domain of DlERF23 protein was predicted by using Simple Modular Architecture Research Tool (SMART; http://smart.emblheidelberg.de/). Multiple sequence alignment of the DlERF23 protein was performed by using Clustal X version 1.83. Based on this alignment, a phylogenetic tree of DlERF23 was constructed by using the neighbor-joining (NJ) method with MEGA 11, with 1000 bootstrap replicates (Tamura et al., 2021).

Subcellular localization analysis of DlERF23

The full coding sequence (CDS) of DlERF23 gene without the termination codon was amplified using primers ProDlERF23-S and ProDlERF23-A (Table S1). The amplification conditions were: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 40 seconds, 35 cycles (denaturation and extension); extend at 72 °C for 10 min and store at 4 °C. Plasmids pBWA(V)HS-osgfp (negative) and pBWA(V)HS-DlERF23-osgfp were constructed and introduction into Agrobacterium tumefaciens strain GV3101, separately. Then, the pBWA(V)HS-osgfp vector and pBWA(V)HS-DlERF23-osgfp vector were transfected into Arabidopsis thaliana protoplasts by PEG mediated method (Yoo et al., 2007). After dark incubation at 28 °C for 24–48 h, fluorescence signals in protoplasts were examined by using the Olympus FluoView FV1000 confocal microscope software (Olympus, Tokyo, Japan).

Transformation of DlERF23 into Arabidopsis and phenotype analysis

The full-length CDS of DlERF23 was cloned and inserted into the BamHI and SacI sites in pBI121 under the control of the CaMV35S promoter to construct the overexpression vector. Then the pBI121-DlERF23 and pBI121 constructs were introduced into Agrobacterium strain GV3101 for Arabidopsis transformation using the floral dip method (Clough et al., 2005). Ten transgenic plants overexpressing DlERF23 were obtained. Seeds were collected and sown on Murashige and Skoog (MS) agar medium containing 25 μg·mL\(^{-1}\) hygromycin for selection. Two T3 generation homozygous lines (OE6 and 9) were used for further functional analysis. The Arabidopsis ecotype Columbia (Col-0) plants used for transformation were maintained in our laboratory. The flowering time and number of rosette leaves of the transgenic, WT, and empty vector-transformed Arabidopsis plants (which were used as controls) were recorded or measured under long-day (LD) conditions.
Results

*Isolation and sequence analysis of DlERF23 in longan*

By using 'SJ' leaves cDNA as the template and ERF23-S/ERF23-A as the specific primers, a PCR fragment with a size about 500bp was amplified (Figure 1). After sequencing, we found the nucleotide similarity between *DlERF23* isolated from 'SJ' and *DlERF23* sequence (dlo_015501.2) of 'SX' longan is 99.27%. There are three different bases: the base at position 171 changes from C to G; the base at position 303 changes from C to A; the base at position 361 changes from A to C; the base at position 378 changes from C to T (Figure 2A). Meanwhile, there is one different amino acid: the amino acid at position 121 changes from K (lysine) to Q (glutamine) (Figure 2B).

![Figure 1. PCR amplification of DlERF23 gene in longan](image)
Differences between base sequence (A) and amino acid sequence (B) of DlERF23 gene from 'SJ' and 'SX' longan (DlERF23 (SX)). The red arrows represent differential bases or amino acids.

The cDNA length DlERF23 was 552 bp, encoding a 184 amino acid protein, with a pI value of 7.69, and a MW value of 20.41 kDa. There are no signal peptides and transmembrane structures in DlERF23 protein. Amino acid sequence analysis showed that DlERF23 contains an AP2/ERF domain and is a typical member of the AP2/ERF family (Figure 3).

**Phylogenetic analysis of DlERF23**

To study the phylogenetic relationships between DlERF23 and other plant AP2/ERF proteins, a phylogenetic tree was created by using MEGA 11.0. As shown in Figure 4, DlERF23 is clustered with WRKY of other woody plants, and is closest to CsERF23 (XP_006478313.1) of *Citrus sinensis* and CcERF23 (XP_006441807.2) of *Citrus clementina*. Whereas there is a distant genetic relationship between DlERF23 and the AP2/ERF members of model plants Arabidopsis or dicotyledon maize (Figure 4).
Figure 4. Phylogenetic tree analysis between longan DlERF23 and similar sequences in GenBank

Tissue-specific expression of DlERF23

By using nine 'SJ' longan tissues, including flower, flower bud, leaf, pericarp, pulp, root, seed, stem, and young fruit, the tissue-specific expression of DlERF23 was analyzed. The result of qRT-PCR shown that the expression of DlERF23 can be detected in the nine tissues, with the highest expression displayed in the pericarp, which is 20 times that of the young fruit, followed by the stem, leaf, flower and flower bud, and the lowest expression in the young fruit (Figure 5). This result indicates that the expression of DlERF23 gene is tissue-specific and may specifically regulate the development of longan pericarp, young fruit, flower and other organs.

Figure 5. Relative expression levels of DlERF23 in different longan tissues
Significant differences among samples were assessed at p < 0.05 level by Student's t-test; different letters indicate significant difference between samples

Expression during different flowering development of DlERF23

The expression of DlERF23 in three flower induction stages of 'SX' and 'SJ' longan was detected. The result showed that DlERF23 shown the opposite expression pattern with a significant down-regulated in the early stage of flower induction in 'SJ' and an up-regulated in the late stage of flower induction in 'SX' (Figure 6).
**Figure 6.** Relative expression levels of *DlERF23* in ‘SX’ and ‘SJ’ during different flower development stages. Significant differences among samples were assessed at p < 0.05 level by Student’s t-test; different letters indicate significant difference between samples.

**Subcellular localization of DlERF23**

In order to determine the subcellular localization of DlERF23 protein, recombinant plasmid pBWA (V) HS-DlERF23-osGFP was generated and introduced into *Arabidopsis* protoplasts. As shown in Figure 7, under excitation at a wavelength of 480 nm, the fluorescence signal from the DlERF23-GFP fusion protein was mainly detected in the nucleus under confocal laser scanning microscopy, whereas the GFP control was detected in both the nucleus and cytoplasm, without clear localization. These results demonstrated that DlERF23 is a nuclear protein.

**Figure 7.** Subcellular localization of the DlERF23-GFP fusion protein in Arabidopsis protoplasts. GFP: Green fluorescent protein; Chloroplast: Chloroplast autofluorescence; Bright: Bright field; Merged: Fusion of two kinds of fluorescence; Scale bar= 10 μm.
Overexpression of DlERF23 promoting plant flowering

To further investigate the function of DlERF23, the overexpression vector of DlERF23 was constructed and introduced it into Arabidopsis (Col-0). Meanwhile, WT plants and WT plants transformed with the pBI121 empty vector were used as negative controls and positive controls, respectively. Two homozygous T3 transgenic lines were randomly selected from the seven independent transgenic lines for phenotype analyzing. Compared with the control plants, the transgenic plants flowered earlier (Figure 8A and 8B). The flowering time ranged from 20.6 to 21.2 d in the transgenic plants, and from 26.9 to 28.1 d in the control plants (Figure 8A and 8B). The average number of rosette leaves in the WT plants is 8.5 which is significantly lower than transgenic (between 11 and 12) (Figure 8C).

Figure 8 Flowering phenotype of DlERF23 transgenic Arabidopsis. (A) Phenotypes of WT Arabidopsis, DlERF23-overexpressing Arabidopsis and transgenic pBI121 empty vector Arabidopsis; (B) Flowering time analysis; (C) Analysis of rosette leaf number
Discussion

AP2/ERF superfamily is the largest TFs family which plays important roles in plant growth, development, biotic and abiotic stress responses, and regulating floral organ development or flowering in plants (Jiang et al., 2022). However, the research of AP2/ERF superfamily members involving in the regulation of longan flower induction is still limited.

Previous studies showed that all of the AP2/ERF superfamily members have the conservative AP2 domain (Zhang et al., 2021). Consistent with these studies, we found DlERF23, belonged to DREB subfamily, contain one AP2 domain, was more closely related to ERF members from woody plants in evolutionary relationships, especially for citrus species. AP2/ERF superfamily members appear to be deferentially expressed in different tissues and organs. For instance, among the ninety-seven AP2/ERF members of Pineapple (Ananas comosus L. Merr), eight-five AcAP2/ERF genes were expressed in at least one tissues, and AcAP2/ERF57/48/71/06/95 (DREB subfamily) almost had no expression in all the four tested tissues, including the leaf, fruit, flower and root (Zhang et al., 2021). Similarly, 173 of 214 ZmAP2/ERF genes from maize (Zea mays L.) were examined in at least one tissues, whereas 41 genes were not detected in any tissues (Zhang et al., 2022). In the present study, the expression level of DlERF23 varied in different tissues with the highest expression in the pericarp, followed by the stem, leave, flower and flower bud, and the lowest expression in the young fruit. As the expression level of genes can reflect their role in the development of plant organs and tissues (Rishmawi et al., 2014), our result suggesting that DlERF23 may specifically participate in the development of fruit and flower organs.

AP2/ERF superfamily members involved in flower organ development and flower induction in plants. For instance, approximately 95.00% of PpcAP2/ERF genes from Chinese cherry (Prunus pseudocerasus) were expressed in the three dormancy stages (Zhu et al., 2021). In this study, we found that DlERF23 shown the opposite expression pattern with a significant down-regulated in the early stage of flower induction in 'SJ', a perpetual flowering (PF) genotype, which flowers and bears fruits throughout the year and does not require special external environmental conditions, and an up-regulated in the late stage of flower induction in 'SX', seasonal flowering (SF), which flowers and bears fruits once a year and requires favorable conditions such as a period of low temperature (vernalization) (Jue et al., 2021). This result indicates that DlERF23 gene may be involved in the induction of longan flower. To better understand the function of DlERF23, the DlERF23 overexpression Arabidopsis lines were conducted. Many studies shown that overexpression different plants AP2/ERF genes resulted delay or promote flowering. For example, overexpressed rice AP2/ERF-N22 gene in Arabidopsis would delay flowering by 1 week as compared to WT (Mawlong et al., 2015). Rice LATE FLOWERING SEMI-DWARF (LFS) encodes an AP2/ERF TFs that promotes flowering under non-inductive LD conditions (Shim et al., 2022). Consistent with these studies, overexpressed DlERF23 in Arabidopsis, the transgenic plants flowered earlier ranged from 6 to 7 d than the control plants. This result indicates that DlERF23 gene may be positive regulating plant flowering. As one kind of TFs, AP2/ERF members mainly through cooperated with other floral meristem genes to regulate plant flowering. For example, AtERF98 could regulate flowering in Arabidopsis by activating the function through the EDLL activation motif (Tiwari et al., 2012). TOE1, one of the AP2-like TFs, could control flowering by regulating FT expression (Du et al., 2020). However, since the mechanism of AP2/ERF participating in the regulation of plant flowering is relatively complex, further research need to perform to clarify its regulatory mechanisms.
Conclusions

In the present study, we identified a DIERF23 gene from longan, and analyzed its gene and protein features by bioinformatics approaches. DIERF23 is a typical DREB subfamily of AP2/ERF TFs, and possesses one typical AP2 domain. Expression pattern analysis showed that DIERF23 is preferentially expressed in fruit and flower organs, and displayed opposite expression pattern in ‘SJ’ and ‘SX’ during three flower induction stages. Meanwhile, overexpressing DIERF23 in Arabidopsis, the transgenic lines shown an early flowering phenotype. This study provides useful information for identifying DIERF23 function during flower induction.

Authors’ Contributions

Conceptualization: DJ; Data curation: CR; Formal analysis: DJ and XS; Funding acquisition: DJ; Investigation, Methodology, Resource, Software, and Visualization: DJ and XS; Writing-original draft: DJ; Writing-review and editing: DJ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References


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