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Research Article

Cloning and function analysis of *DlWRKY9* gene in longan (*Dimocarpus longan*)

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Abstract

WRKY is one of the largest plant transcription factors (TFs) which is widely involved in plant growth, development, and responses to stresses. In the present study, a WRKY TF DlWRKY9 was cloned from longan (*Dimocarpus longan*). The coding sequence (CDS) of *DlWRKY9* is 762 bp in length and encodes 253 amino acids. It has a typical WRKY domain and zinc finger structure which belongs to type IIa WRKY protein. The molecular weight of DlWRKY9 protein was 30.27kda and the theoretical isoelectric point (PI) was 5.24. It is an unstable hydrophilic protein. The secondary structure of DlWRKY9 protein consists of helical structure (17.39%), extended chain (8.70%) and other structures (turn and random coil) (73.91%). The amino acid sequence of DlWRKY9 protein had the highest similarity with DlWRKY9 (xp_006450293.1) of *citrus Clementina*. *DlWRKY9* gene promoter elements contain light, abscisic acid, gibberellin, jasmonic acid and other response elements. The results of qRT-PCR showed that the relative expression level of *DlWRKY9* gene in pericarp was higher, followed by young fruits and floral organs. Meanwhile, *DlWRKY9* gene specifically down-regulated in the early stage of flower induction in 'Sijimi' (SJ) longan. The results of transient expression of *Arabidopsis* protoplasts showed that the fluorescence signal was mainly concentrated in the nucleus. Moreover, overexpression of *DlWRKY9* in *Arabidopsis* promoted early flowering. These results provide useful information for revealing the biological roles of *DlWRKY9* in longan and increase our understanding of the WRKY family in fruit trees.

Keywords: *DlWRKY9*; flowering; longan; transgenic *Arabidopsis*

Introduction

Flowering is a crucial developmental process in the plant life cycle (Shabala *et al.*, 2014). Flowering at the right time and having the enough flowers is directly linked to production whenever seeds or fruits are harvested, especially for fruit tree (Jue *et al.*, 2019). To date, huge progress has been made in understanding the physiological and molecular mechanisms underlying flowering time in plants. The flowering molecular mechanisms of *Arabidopsis* is a complex gene regulatory network which containing at least six flowering

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pathways and several flowering-related genes, such as *flowering locus T* (*FT*), *CONSTANS* (*CO*), *flowering locus C* (*FLC*), and *LEAFY* (*LFY*) (Moon *et al*., 2005). Meanwhile, since TFs have the ability to regulate the expression of gene by binding its specific promoter regions, some TFs also play important roles during plant flowering by activating or inhibiting the expression of flowering-related genes (Hwang *et al*., 2019). For example, AtMYB33 can regulate *Arabidopsis* flowering by binding to a specific 8-bp sequence in the *LFY* promoter (Gocal *et a*l., 2001). In tomato (*Solanum lycopersicum*), silencing *SlMYB33* leads to delayed flowering by affects the expression of some genes controlling flowering, including *ANANTHA* (*AN*), *FALSIFLORA* (*FA*), *FLOWERING-PROMOTING FACTOR1* (*FPF1*) and *FLOWERING TIME CONTROL LOCUS A* (*FCA*) (Zhang *et al*., 2020). Overexpressing *NTL8*, one member of NAC (NAM, ATAF1/2, CUC2), the transgenic lines exhibited delayed flowering. Meanwhile, the expression of *FT* and its downstream genes was significantly reduced in the transgenic plants (Kim *et al*., 2007). Similar results also obtained in other TFs, such as MADS-Box TFs (Lee *et al*., 2013), CCAAT-Binding TFs (Cai *et al*., 2007), and WRKY TFs (Li *et al*., 2016; Ma *et al*., 2020b).

WRKYs are one of the largest TFs in plant (Rinerson *et al*., 2015). After the first WRKY gene was cloned in sweet potato, the WRKY gene family has been identified in several plants, such as *Arabidopsis thaliana* (Rushton *et al*., 2010), *Oryza sativa* (Ross *et al*., 2007), *Vitis vinifera* (Guo *et al*., 2014), longan (Jue *et al*., 2018), and Black raspberry (Wu *et al*., 2022). The main defining feature of WRKY proteins is their conserved WRKY domain (WDs) and a zinc-finger motif at the C-terminus of about 60 amino acids in length (Chen *et al*., 2017). Based on the number of WDs and the structure of zinc-finger motifs, WRKY proteins can be classified into three main groups: groups I, II, and III. Group I contain WRKY proteins with two WRKY domains including a C2H2 motif. Group II WRKY proteins containing a single WRKY domain with a C2H2 motif and can be further divided into five subgroups: IIa, IIb, IIc, IId, and IIe. The remaining WRKY proteins, containing a WRKY domain along with a C2HC motif, belong to group III (Ling *et al*., 2011; Phukan *et al*., 2016). An increasing number of studies revealed that WRKY TFs play a crucial role in plant growth and development, and response of plants to abiotic and biotic stress (Wani *et al*., 2021; Wu *et al*., 2022). In addition, previous studies shown that some WRKY TFs members participated in plant flowering regulation. For example, in *A. thaliana*, the lines overexpressed *Glycine soja WRKY20* (*GsWRKY20*), *Miscanthus lutarioriparius WRKY12* (*MlWRKY12*), *Chimonanthus praecox WRKY71* (*CpWRKY71*), *AtWRKY13* and *AtWRKY71* exhibited flowering earlier than the wild-type lines (Huang *et al*., 2019; Ma *et al*., 2020b). Overexpressing *AtWRKY13*, the transgenic lines shown a delay flowering phenotype (Li *et al*., 2016). In rice, under long-day (LD) condition, knock-down of *Dlf1* (*OsWRKY11*) expression exhibited early flowering (Cai *et al*., 2014).

Obtaining a stable annual fruit yield is the key factor that affecting the healthy development of longan industry (Jue *et al*., 2019). However, the irregular flowering of longan which triggered by many environmental conditions, such as spring frost and high temperature and moisture in winter, usually cause a unstable production (Wünsche and Ferguson, 2005; You *et al*., 2012). previous study shown that potassium chlorate (KClO3) had the ability to regulate longan tree flowering time and obtain a stable high yield (Zhang *et al*., 2016). However, the induction effect varies in different regions and varieties (Jue *et al*., 2019). Therefore, the key to solve this problem is to identify the flower induction-related genes and analyze their regulation mechanism. In our previous study, 55 *DlWRKY* genes were identified in longan genome (Jue *et al*., 2018). The expression pattern of these 55 *DlWRKY* genes in different longan tissues, flowering stages and the response to different abiotic stress were examined. We found some *DlWRKY* genes may be involved in the flower induction of longan, including *DlWRKY9*. However, the detailed functional analysis for *DlWRKY9* is still limited. In this work, *DlWRKY9* was isolated and its potential function was investigated by overexpressing in transgenic *Arabidopsis* plants. Our findings indicated that *DlWRKY9* plays important roles in the flowering induction of longan.

Materials and Methods

Plant materials

The longan cultivars of 'SJ' and 'SX' ('Shixia') were grown in the same orchard located in Mazhang district (110°16′ E, 21°10′ N), Zhanjiang, Guangdong Province, P. R. China. Three 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). For tissue expression analysis, nine 'SJ' longan tissues (flower, flower bud, leaf, pericarp, pulp, root, seed, stem, and young fruit) were collected from November in 2016 to April in 2017. The samples were frozen immediately in liquid nitrogen and stored at -80 °C.

RNA extraction, qRT–PCR and DlWRKY9 gene cloning

Total RNA was extracted from different longan tissues or WT and transgenic *Arabidopsis* lines by using a quick RNA Isolation (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). qRT–PCR was performed by using the LightCycler® 480 Real-Time PCR System (Roche, Germany) and SYBR Green II PCR Master Mix (Takara, Dalian, China). 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 (Jue *et al.*, 2018). 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 $2^{-\Delta\Delta Ct}$ method. The analysis included cDNA from the three biological samples for each tissue, and all the reactions were run in triplicates.

The cDNA extracted from 'SJ' leaves was used as template for *DlWRKY9* gene cloning. The primer for *DlWRKY9* gene cloning was designed by using primer 5.0 according the sequence of Dlo_015501.2 (Lin *et al.*, 2017) and listed in Table S1.

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 DlWRKY9 protein. The domain of DlWRKY9 protein was predicted by using Simple Modular Architecture Research Tool (SMART; http://smart.emblheidelberg.de/). The secondary and tertiary structures of DlWRKY9 protein was predicted by using predictprotein (https://predictprotein.org/) and SWISS-MODEL (https://swissmodel.expaty.org/). Transmembrane analysis and signal peptide analysis were performed by using CBS online tools (http://www.cbs.dtu.dk/services/TMHMM-2.0/ and http://www.cbs.dtu.dk/services/SignalP/). The PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used for searching the cis-acting elements. Multiple sequence alignment of the DlWRKY9 protein was performed by using Clustal X version 1.83. Based on this alignment, a phylogenetic tree of DlWRKY9 was constructed by using the neighbor-joining (NJ) method with MEGA 6.0, with 1000 bootstrap replicates (Tamura *et al.*, 2013).

Subcellular localization and transcriptional activation activity analysis of DlWRKY9

The full coding sequence (CDS) of *DlWRKY9* gene without the termination codon was amplified using primers ProDlWRKY9-S and ProDlWRKY9-A (Table S1). Plasmids pBWA(V)HS-osgfp (negative) and pBWA(V)HS-DlWRKY9-osgfp were constructed and introduction into *Agrobacterium tumefaciens* strain

GV3101, separately. Then, the pBWA(V)HS-osgfp vector and pBWA(V)HS-DlWRKY9-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 DlWRKY9 into Arabidopsis and phenotype analysis

The full-length CDS of *DlWRKY9* 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-DlWRKY9 and pBI121 constructs were introduced into *Agrobacterium strain* GV3101 for *Arabidopsis* transformation using the floral dip method (Clough, 2005). Seeds were collected and sown on Murashige and Skoog (MS) agar medium containing 25 $\mu g \cdot mL^{-1}$ hygromycin for selection. Seven transgenic plants overexpressing *DlWRKY9* were obtained and further confirmed by RT-PCR analysis. Two T3 generation homozygous lines (OE2 and 6) 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 DlWRKY9 in longan

By using 'SJ' leaves cDNA as the template and W9-S/W9-A as the specific primers, a PCR fragment with a size about 800bp was amplified (Figure 1). After sequencing, we found the nucleotide similarity between *DlWRKY9* isolated from 'SJ' and *DlWRKY9* sequence (dlo_015501.2) of 'Honghezi' longan is 99.88%. Only one base is different: the base at position 617 changes from T to C.

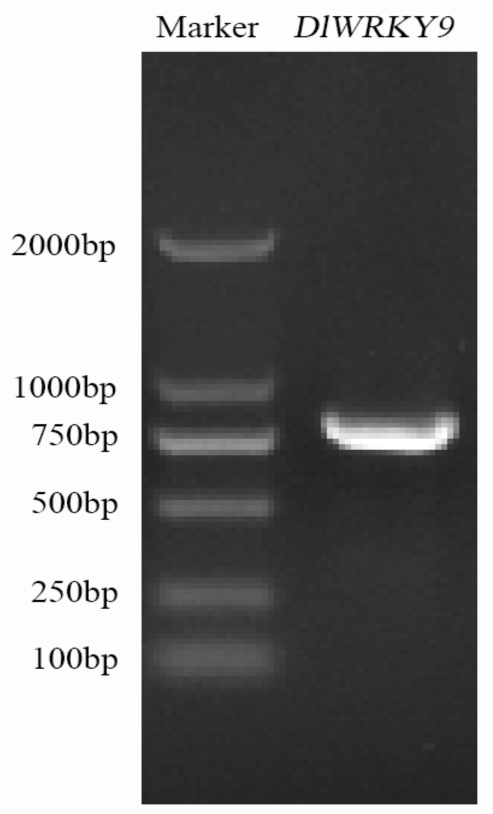
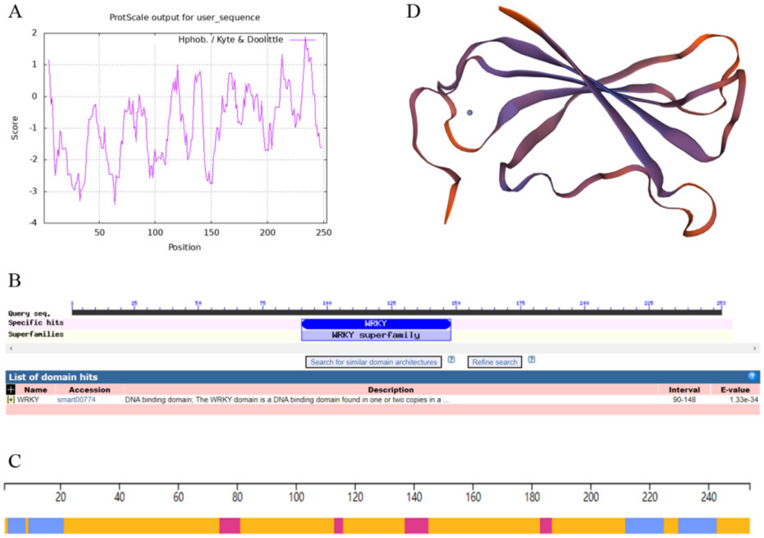


Figure 1. PCR amplification of *DlWRKY9* gene in longan

The cDNA length *DlWRKY9* was 762 bp, encoding a 253 amino acid protein, with a pI value of 5.24, and a MW value of 30.27 kDa. There are no signal peptides and transmembrane structures in DlWRKY9 protein. The instability coefficient of DlWRKY9 protein is 52.37, the aliphatic amino acid index is 56.64, and the average hydrophilicity index (gravy) is -0.880. Thus, DlWRKY9 is an unstable hydrophilic protein (Figure 2A). The SMART analysis showed that DlWRKY9 possessed one WRKY conserved domain and one C-X_{4-5}-C-X_{23}-HXH zinc finger structure (Figure 2B). This result indicated that DlWRKY9 protein belongs to Group IIa subgroup of WRKY TFs. Predictprotein and SWISS-MODEL analysis showed that the secondary structure of DlWRKY9 was composed by 17.39% helix, 8.70% extended strand, and 73.91% other structure (turn and random coil) (Figure 2C). Meanwhile, the predicted results of the tertiary structure of DlWRKY9 protein are consistent with the predicted results of the secondary structure (Figure 2D).



List of domain hits

Name	Accession	Description	Interval	E-value
[+] WRKY	smart00774	DNA binding domain; The WRKY domain is a DNA binding domain found in one or two copies in a ...	90-148	1.33e-34

Figure 2. Prediction of hydrophilicity/hydrophobicity and structure of DlWRKY9 protein
(A) Prediction of hydrophilicity / hydrophobicity of DlWRKY9 protein; (B) Prediction of the secondary structure of DlWRKY9 protein (Blue: Helix; red: Extended strand; yellow: other structure); (C) The secondary structure prediction results of DIWRKY9 protein; (D) Prediction of tertiary structure of DIWRKY9 protein

Phylogenetic analysis of DlWRKY9 with other DlWRKY9 proteins

To study the phylogenetic relationships between DlWRKY9 and other plant WRKY proteins, a phylogenetic tree was created. As shown in Figure 3, DlWRKY9 is clustered with WRKY of dicotyledonous plants, and is closest to CcWRKY40 (XP_006450293.1) of *citrus Clementina*. Whereas the relationship between DlWRKY9 and the WRKY of monocotyledonous plants is far, such as ZmWRKY40 (NP_001147760.1) of *Zea mays*. Thus, the result suggesting DlWRKY9 may have the highest similarity with CcWRKY40 of Citrus in evolution.

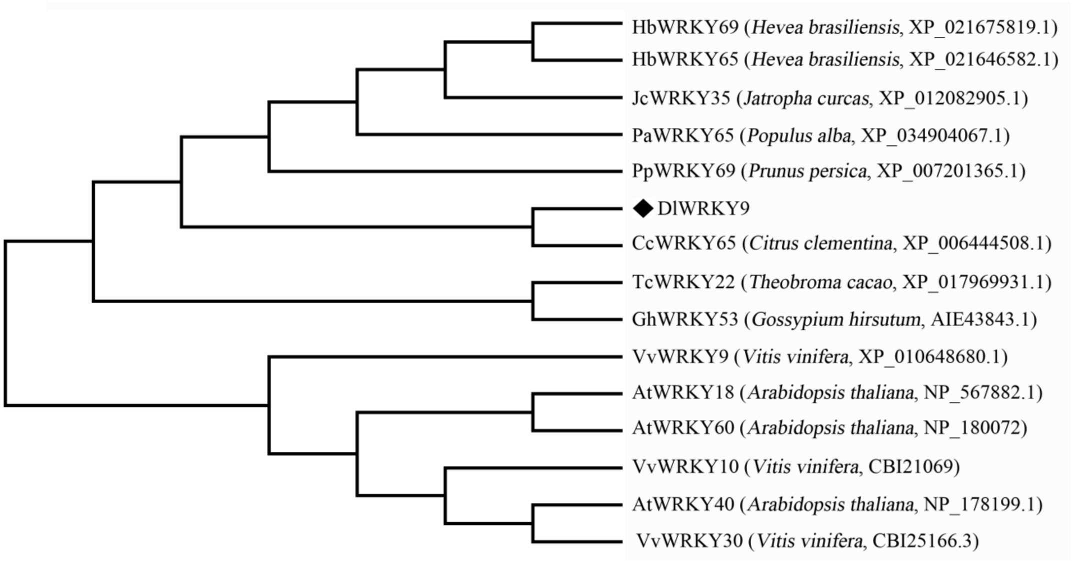
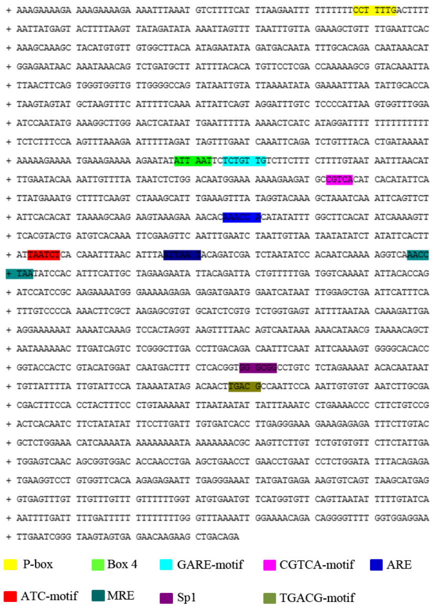


Figure 3. phylogenetic tree analysis between longan DlWRKY9 and similar sequences in GeneBank

Cis-element analysis of DlWRKY9

The Cis-elements in the 2.0-kb upstream sequences of start codon of the *DlWRKY9* gene were analyzed by using PlantCARE. As shown in Figure 4, besides the basic elements, such as W-box, CAAT-box and TATA-box, the promoter of *DlWRKY9* gene also contains several light response elements (such as I-box, ATC-motif, G-box, MRE, Sp1 and Box-4), abscisic acid (ABA) response element (ABRE), gibberellin response element (GARE-motif and P-box), jasmonic acid response element (CGTCA-motif and TGACG-motif) and anaerobic sensing element (ARE). These results suggesting that the expression of *DlWRKY9* gene may regulated by light signal, gibberellin, ABA, jasmonic acid and other signals.



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+ AAAGAAAAGA AAAGAAAAGA AAATTTAAAT GTCTTTTCAT TTAAGAATTT TTTTTTCCT TTTGACTTTT
+ AATTATGAGT ACTTTTAAGT TATAGATATA AAATTAGTTT TAATTTGTTA GAAAGCTGTT TTGAATTCAC
+ AAAGCAAAGC TACATGTGTT GTGGCTTACA ATAGAATATA GATGACAATA TTTGCACAGA CAATAAACAT
+ GGAGAATAAC AAATAAACAG TCTGATGCTT ATTTTACACA TGTTCCTCGA CCAAAAAGCG GTACAAATTA
+ TTAACTTCAG TGGGTGGTTG TTGGGGCCAG TATAATTGTA TTAAAATATA GAAAATTTAA TATTGCACCA
+ TAAGTAGTAT GCTAAGTTTC ATTTTTCAAA ATTATTCAGT AGGATTTGTC TCCCCATTAA GTGGTTTGGA
+ ATCCAATATG AAAGGCTTGG AACTCATAAT TGAATTTTTA AAAACTCATC ATAGGATTTT TTTTTTTTTT
+ TCTCTTTCCA AGTTTAAAGA ATTTTTAGAT TAGTTTGAAT CAAATTCAGA TCTGTTTACA CTGATAAAAT
+ AAAAAGAAAA TGAAAGAAAA AGAATATATT AATTCTCTGT TGTCTTCTTT CTTTTGTAAT AATTTAACAT
+ TTGAATACAA AATTGTTTTA TAATCTCTGG ACAATGGAAA AAAAGAAGAT GCCGTCACAT CACATATTCA
+ TTATGAAATG CTTTTCAAGT CTAAAGCATT TGAAAGTTTA TAGGTACAAA GCTAAATCAA ATTCAGTTCT
+ ATTCACACAT TAAAAGCAAG AAGTAAAGAA AACACAAACC ACATATATTT GGCTTCACAT ATCAAAAGTT
+ TCACGTACTG ATGTCACAAA TTCGAAGTTC AATTTGAATC TAATTGTTAA TAATATATCT ATATTCACTT
+ ATTAATCTCA CAAATTTAAC ATTTAATTAA TACAGATCGA TCTAATATCC ACAATCAAAA AGGTCAAACC
+ TAATATCCAC ATTTCATTGC TAGAAGAATA TTACAGATTA CTGTTTTTGA TGGTCAAAAT ATTACACCAG
+ ATCCATCCGC AAGAAAATGG GAAAAAGAGA GAGATGAATG GAATCATAAT TTGGAGCTGA ATTCATTTCA
+ TTTGTCCCCA AAACTTCGCT AAGAGCGTGT GCATCTCGTG TCTGGTGAGT ATTTTAATAA CAAAGATTGA
+ AGGAAAAAAT AAAATCAAAG TCCACTAGGT AAGTTTTAAC AGTCAATAAA AAACATAACG TAAAACAGCT
+ AATAAAAAAC TTGATCAGTC TCGGGCTTGA CCTTGACAGA CAATTTCAAT ATTCAAAAGT GGGGCACACC
+ GGTACCACTC GTACATGGAT CAATGACTTT CTCACGGTGG GCGGCCTGTC TCTAGAAAAT ACACAATAAT
+ TGTTATTTTA TTGTATTCCA TAAAATATAG ACAACTTGAC GCCAATTCCA AATTGTGTGT AATCTTGCGA
+ CGACTTTCCA CCTACTTTCC CTGTAAAAAT TTAATAATAT TATTTAAATC CTGAAAACCC CTTCTGTCCG
+ ACTCACAATC TTCTATATAT TTCCTTGATT TGTGATCACC TTGAGGGAAA GAAAGAGAGA TTTCTTGTAC
+ GCTCTGGAAA CATCAAAATA AAAAAAAATA AAAAAAACGC AAGTTCTTGT TCTGTGTGTT CTTCTATTGA
+ TGGAGTCAAC AGCGGTGGAC ACCAACCTGA AGCTGAACCT GAACCTGAAT CCTCTGGATA TTTACAGAGA
+ TGAAGGTCCT GTGGTTCACA AGAGAGAATT TGAGGGAAAT TATGATGAGA AAGTGTCAGT TAAGCATGAG
+ GTGAGTTTGT TTGTTTGTTT GTTTTTTGGT ATGTGAATGT TCATGGTGTT CAGTTAATAT TTTTGTATCA
+ AATTTTGATT TTTGATTTTT TTTTTTTTGG GTTTAAAATT GGAAAACAGA CAGGGGTTTT GGTGGAGGAA
+ TTGAATCGGG TAAGTAGTGA GAACAAGAAG CTGACAGA
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🟨 P-box
🟩 Box 4
🟦 GARE-motif
🟪 CGTCA-motif
🟦 ARE
🟥 ATC-motif
🟩 MRE
🟪 Sp1
🟫 TGACG-motif

Figure 4. *DlWRKY9* gene promoter component analysis

Tissue-specific expression of DlWRKY9

By using nine 'SJ' longan tissues, including flower, flower bud, leaf, pericarp, pulp, root, seed, stem, and young fruit, the tissue-specific expression of *DlWRKY9* was analyzed. The result of qRT-PCR shown that the expression of *DlWRKY9* can detected in the nine tissues, with the highest expression displayed in the pericarp, followed by the young fruit and flower, and the lowest expression in the pulp (Figure 5). This result indicates that the expression of *DlWRKY9* gene is tissue-specific and may specifically regulate the development of longan pericarp, young fruit, flower, pulp and other organs.

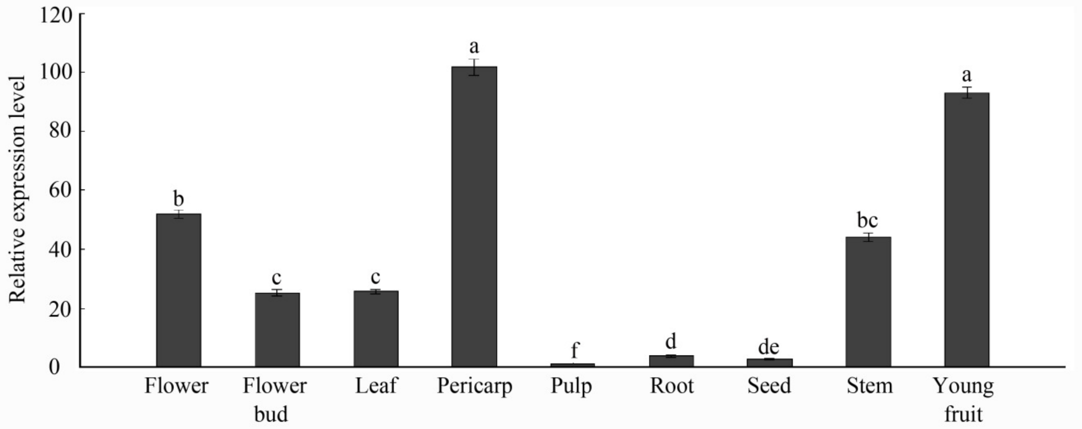

Figure 5. Relative expression levels of *DlWRKY9* in different longan tissues

Subcellular localization of DlWRKY9

In order to determine the subcellular localization of DlWRKY9 protein, recombinant plasmid pBWA (V) HS-DlWRKY9-osGFP was generated and introduced into *Arabidopsis* protoplasts. As shown in Figure 6, the fluorescence signal from the DlWRKY9-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. These results demonstrated that DlWRKY9 is a nuclear protein.

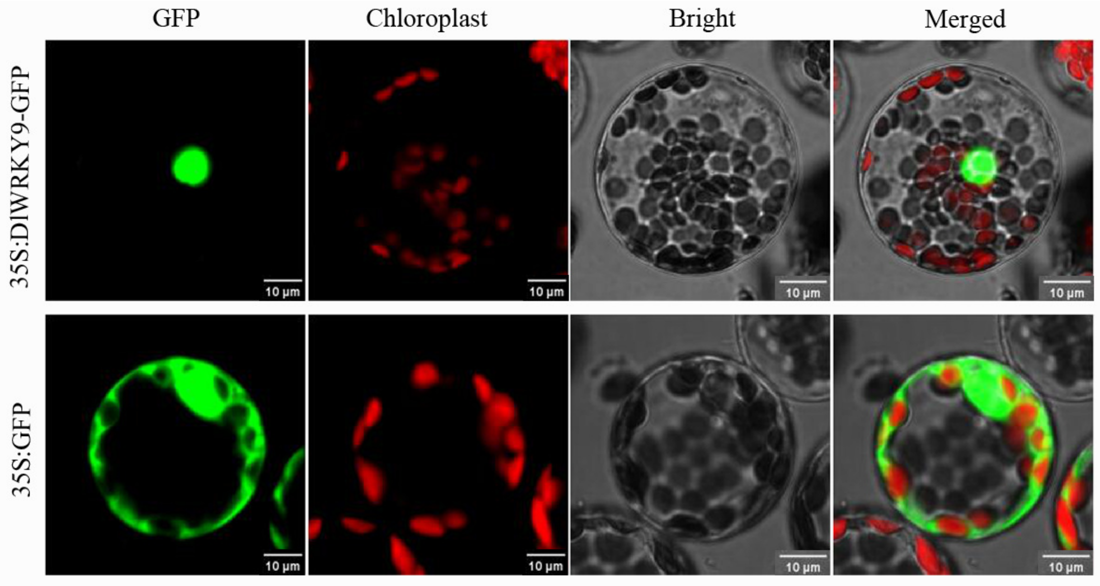

Figure 6 Subcellular localization of the DlWRKY9-GFP fusion protein in Arabidopsis protoplasts
Scale bar= 10 μm.

Overexpression of DlWRKY9 promoting plant flowering

To further investigate the function of *DlWRKY9*, the overexpression vector of *DlWRKY9* 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 7A and 7B). The flowering time ranged from 20 to 22.2 d in the transgenic plants, and from 29.6 to 30.4 d in the control plants (Figure 7A and 7B). The average number of rosette leaves in the WT plants is 8.0 which is lower than transgenic (between 8.4 and 8.9), although the difference is not obvious (Figure 7C).

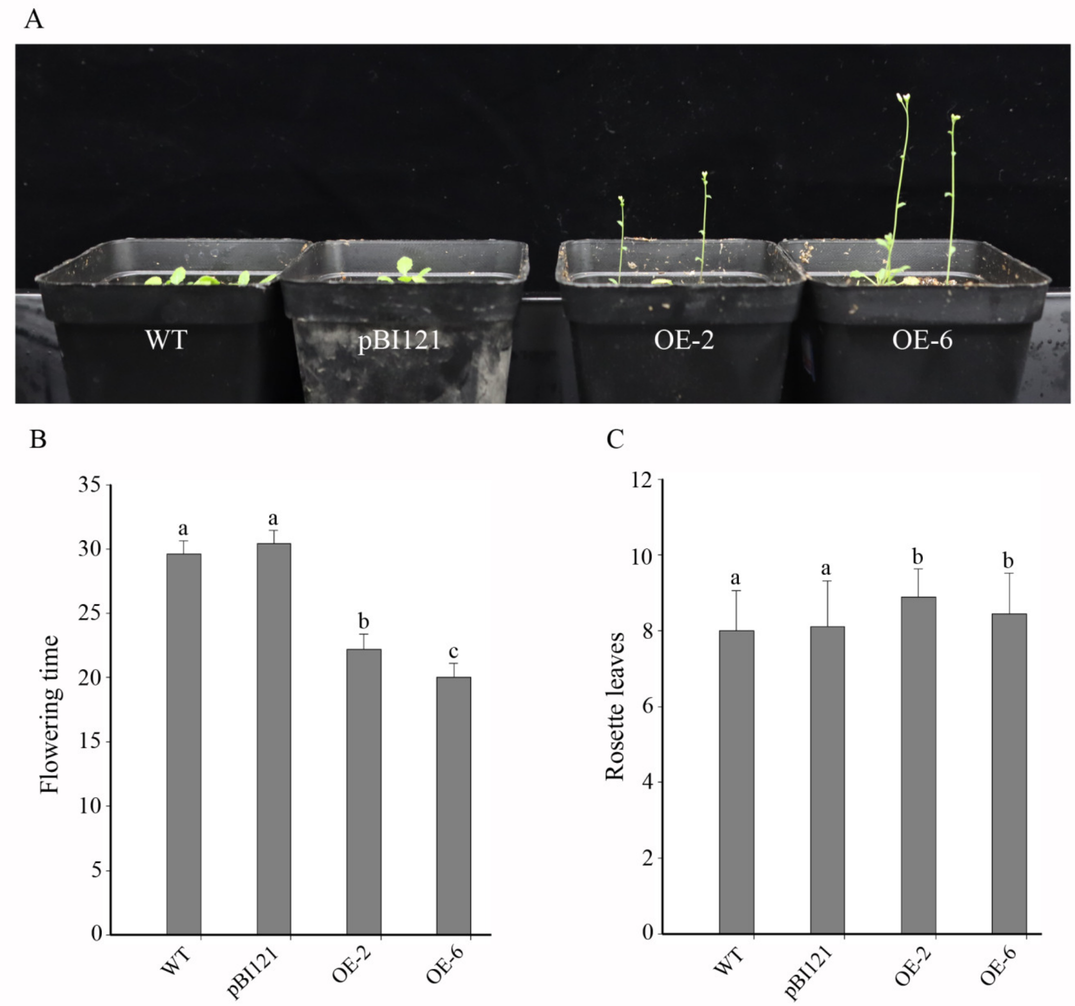

Figure 7. Flowering phenotype of *DlWRKY9* transgenic *Arabidopsis*

(A) Phenotypes of WT *Arabidopsis*, *DlWRKY9*-overexpressing *Arabidopsis* and transgenic pBI121 empty vector *Arabidopsis*; (B) Flowering time analysis; (C) Analysis of rosette leaf number.

Discussion

As one of the largest TFs in plant (Rinerson *et al*., 2015), WRKY proteins have been identified and well-studied in higher plant (Wu *et al*., 2022; Wu *et al*., 2022). In our previous study, 55 *DlWRKY* genes were identified in longan genome (Jue *et al*., 2018). meanwhile, we found some *DlWRKY* genes may be involved in the flower induction of longan. However, due to the long generation time, the function and their regulation mechanism during plant flowering is lagged behind those in other higher plant. In this study, *DlWRKY9* which may be involved in the flower induction of longan was isolated and its potential function was investigated.

WRKYs in plants appear to be deferentially expressed in different tissues and organs (Jue *et al*., 2018). For example, more than half of *VvWRKY* displayed tissue-specific expression (Guo *et al*., 2014). In mulberry, 13 WRKY genes exhibit the highest expression in root tissue (Baranwal *et al*., 2016). consistent with these studies, in this study, the expression level of *DlWRKY9* varied in different tissues with the highest expression in the pericarp, followed by the young fruit and flower, and the lowest expression in the pulp. As the expression level of genes can reflect their role in the development of plant organs and tissues (Rishmawi *et al*., 2014), we speculate that *DlWRKY9* may specifically participate in the development of fruit and flower organs.

Our previous study shown that *DlWRKY9* was down-regulated in the early stage of induction of flowering in 'SJ' longan, which suggesting *DlWRKY9* may involve in regulating flower induction in longan (Jue *et al*., 2018). To better understand the function of *DlWRKY9*, the *DlWRKY9* overexpression *Arabidopsis* lines were conducted. Many studies shown that overexpression different plants WRKY genes resulted early flowering. For example, overexpressed *WRKY12*、*WRKY71*、*GsWRKY20* and *MlWRKY12* gene in *Arabidopsis*, the transgenic lines flowered earlier than WT (Ma *et al*., 2020a). Consistent with these studies, overexpressed *DlWRKY9* in *Arabidopsis*, the transgenic plants flowered earlier ranged from 8 to 10 d than the control plants. This result indicates that *DlWRKY9* gene may be positive regulating plant flowering. The mechanism of WRKY participating in the regulation of plant flowering is relatively complex. WRKY TFs of different subgroups can interact not only with their own promoter regions but also with the promoter regions of other WRKY transcription factors. For example, the promoter region of *PcWRKY1* of celery (*Petroselinum crispum*) has many W-boxes elements. Other WRKY TFs can combine with three W-boxes in the non-coding region of *PcWRKY1*, thus promoting the transcription of *PcWRKY1* gene. However, the interaction of its own promoter region will inhibit the transcription of *PcWRKY1* (Turck *et al*., 2004). WRKY12 and WRKY13 of *Arabidopsis* can participate in the regulation of flowering through gibberellin pathway by forming a complex with DELLA protein. Like *PcWRKY1*, the upstream TFs *SPL10* and its homologous genes can directly bind to the promoter GTAC-motifs of *WRKY12* and *WRKY13*, then positively regulate the expression of *WRKY12* and negatively regulate the expression of *WRKY13*. At the same time, WRKY12 and WRKY13 can directly bind to the W-box of the downstream *GAI*、 *RGL1and FUL* promoter regions to promote or inhibit flower formation (Ma *et al*., 2020b). *AtWRKY71* can affect flowering of plants by directly binding to W-boxes in *FT*, *LFY* and *AP1* promoter regions (Yu *et al*., 2016). In this study, we found that there were also a large number of W-box and gibberellin response elements (GARE-motif and P-box) in the promoter of *DlWRKY9* gene. Subcellular localization analysis showed that DlWRKY9 protein was localized to the nucleus. Therefore, how DlWRKY9, a typical transcription factor, performs the function of promoting plant flowering needs further research.

Conclusions

In the present study, a longan *DlWRKY9* gene was isolated and its gene and protein features were characterized by bioinformatics approaches. This gene encodes a typical Group IIa subgroup of WRKY TFs, and possesses one WRKY conserved domain and one C-$X_{4–5}$-C-X_{23}-HXH zinc finger structure. Expression pattern analysis showed that *DlWRKY9* is preferentially expressed in fruit and flower organs. Meanwhile, overexpressing *DlWRKY9* in *Arabidopsis*, the transgenic lines shown an early flowering phenotype. This study provides useful information for identifying *DlWRKY9* function during flower induction.

Authors' Contributions

Conceptualization: QG; Data curation: XS and YX; Formal analysis: DJ and XS; Funding acquisition: DJ; Investigation: YX and XS; Methodology: DJ and XS; Resources: DJ; Software: DJ and XS; Visualization; Writing-original draft: DJ; Writing-review and editing: DJ, HL and QG. 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.

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