Possible Involvement of Locus-Specific Methylation on Expression Regulation of LEAFY Homologous Gene (CiLFY) during Precocious Trifoliate Orange Phase Change Process

Jin-Zhi Zhang*, Li Mei**, Rong Liu, Muhammad Rehman Gul Khan, Chun-Gen Hu*

Key Laboratory of Horticultural Plant Biology (Ministry of Education), College of Horticulture and Forestry Science, Huazhong Agricultural University, Wuhan, Hubei Province, China

Abstract

DNA methylation plays an essential role in regulating plant development. Here, we described an early flowering trifoliate orange (precocious trifoliate orange, Poncirus trifoliata L. Raf) was treated with 5-azacytidine and displayed a number of phenotypic and developmental abnormalities. These observations suggested that DNA methylation might play an important role in regulating many developmental pathways including early flowering trait, and then the expression level of five key or integrated citrus flowering genes were analyzed. Our results showed that FLOWERING LOCUS T (CiFT) relative expression level was increased with the increasing concentrations of 5-AzaC. However, LEAFY (CiLY), APETELA1 (CiAP1), TERMINAL FLOWER1 (CiTF1), and FLOWERING LOCUS C (CiFLC) showed highest relative expression levels at 250 μM treatment, while decreased sharply at higher concentrations. In order to further confirm DNA methylation affects the expression of these genes, their full-length sequences were isolated by genome-walker method, and then was analyzed by using bioinformatics tools. However, only one locus-specific methylation site was observed in CiLFY sequence. Therefore, DNA methylation level of the CiLFY was investigated both at juvenile and adult stages of precocious trifoliate orange by bisulfate sequencing PCR; it has been shown that the level of DNA methylation was altered during phase change. In addition, spatial and temporal expression patterns of CiLFY promoter and a series of 5’ deletions were investigated by driving the expression of a β-glucuronidase reporter gene in Arabidopsis. Exogenous GA3 treatment on transgenic Arabidopsis revealed that GA3 might be involved in the developmental regulation of CiLFY during flowering process of precocious trifoliate orange. These results provided insights into the molecular regulation of CiLFY gene expression, which would be helpful for studying citrus flowering.

Citation: Zhang J-Z, Mei L, Liu R, Khan MRG, Hu C-G (2014) Possible Involvement of Locus-Specific Methylation on Expression Regulation of LEAFY Homologous Gene (CiLFY) during Precocious Trifoliate Orange Phase Change Process. PLoS ONE 9(2): e88558. doi:10.1371/journal.pone.0088558

Editor: Meng-xiang Sun, Wuhan University, China

Copyright: © 2014 Zhang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: chungen@mail.hzau.edu.cn
** Current address: Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang Agricultural and Forestry University, LinAn, Zhejiang Province, China

These authors contributed equally to this work.

Introduction

A large proportion of many eukaryote genomes are variably methylated during the lifetime of the organism [1]. Previously, it has been reported that DNA methylation involved in X-chromosome inactivation [2], the silencing of transposons [3], multicopy genes, and genomic imprinting [4]. DNA methylation may be divided into two types. The first, known as maintenance methylation, maintains the original methylation pattern in the new strand of DNA upon replication. In the second, known as de novo methylation, unmethylated cytosines are methylated [5,6]. DNA methylation is an epigenetic mark mediated by the addition of a methyl group to cytosine in DNA, but methyl groups can also be removed from DNA by DNA demethylation [7]. In both plants and animals, methylation involves the addition of a methyl group to a carbon at fifth position (C5) of the pyrimidine ring of cytosine in CpG dinucleotides (CpG islands) [8]. DNA methyltransferases then transfer a methyl group from S-adenosyl-L-methionine to cytosines in CpG dinucleotides [9]. However, high amounts of 5-methylcytosine found in some plant species suggested that methylation is not restricted to the CG sequence context and led to the discovery that cytosine is also methylated in CNG (N = A, T, G, or C) and, less abundantly, in CHH (H = A, T, or G) sequences [6,10]. In plants, DNA methylation is more common in CpG islands, characteristic of transposons, contributing to the cytosine methylation increased levels, especially due to the high presence of these elements in plant genomes. These CpG islands are located in gene regulatory regions, aberrant methylation of CpG islands is characterized by transcriptional inactivation and subsequent loss of function of the gene regulated in this fashion without structural modifications [11,12].
DNA methylation, one of the most abundant epigenetic modifications in higher plants, plays an important role in regulating developmental processes such as homeotic transformations in floral organs and altered flowering time [13,14]. It is reported that FWA, FERTILIZATION-INDEPENDENT SEED 2 (FIS2), and MEDEA (MEA) are controlled by genomic imprinting of DNA methylation [15–17]. FWA is involved in flowering time [18,19] while FIS2 and MEA regulate endosperm development [20]. The FWA promoter contains two pairs of transposon-associated tandem repeats that give rise to siRNAs [21]; these are sufficient to induce DNA methylation and silence FWA expression, which is the default state [22,23]. The induction of flowering by DNA demethylation was first reported for the low-temperature requiring ecotypes mutants of Arabidopsis [17,24]. These data suggest that DNA methylation in plants regulates the expression of genes with important roles in morphogenesis and development, including molecular mechanism of flowering. However, the regulatory mechanism of woody plants by DNA methylation in relation to physiological events remains unclear so far.

The development of plants is characterized by juvenile and adult phases. After a relatively short juvenile phase, annual plants progress to the adult phase, during which flowering occurs. By contrast, perennial plants have a much longer juvenile phase, in some cases persisting for decades, which poses a major obstacle to the genetic improvement. Much less is known about the regulation of flowering developmental process in perennial species with particular growth habits [25–27]. Most information about the process regulation comes from studies in model plants. Therefore, an understanding of the genetic mechanisms underlying the flowering event is important for genetic improvement in perennial plants. Citrus is the most economically important fruit crop in the world. Its flowering has been the subject of ongoing investigation for many years [28–30]. Recently, several citrus homologs genes such as LEAFY (LFY), APETELA1 (API), TERMINAL FLOWER (TFL1), FLOWERING LOCUS T (FT), APETELA3 (APS), WUSCHEL (WUS), and FLOWERING LOCUS C (FLC) can readily be isolated by exploiting the functional and sequence conservation of flowering genes among flowering plants [27,29–33]. Molecular cloning and functional analyses of LFY have indicated that LFY may be a central regulator of the flowering regulatory network because the LFY gene is controlled by the autonomous, thermoensory, and gibberellin pathways in Arabidopsis [34]. The LFY protein is necessary and sufficient for the vital switch from vegetative to reproductive development in flowering plants [35]. Over-expression of LFY in transgenic plants can induce early flowering in annuals and perennials [36,37]. However, to our knowledge, there are no reports of LFY methylation level during the phase transition in annual and woody plants.

Precocious trifoliate orange with a short juvenile phase derived from trifoliate orange (Poncirus trifoliata L. Raf), twenty percent of the seedlings germinated from the seeds flowered first in the next year after germination [38]. Thus, precocious trifoliate orange provides good material for studying the molecular mechanism of flower formation in woody plants. 5-Azacytidine (5-AzaC) is a pyrimidine nucleoside analog of cytidine that undergoes incorporation into DNA and blocks DNA methyltransferase leading to hypomethylation and potentially beneficial re-expression of abnormally silenced genes, reducing the overall level of DNA methylation in chromatin [39]. In annual plants, phenotypic changes induced by 5-AzaC have been reported in Arabidopsis [24]. Flowering time is the most recurrent and studied change; this process is accelerated in plants treated with 5-AzaC, which flowered earlier as compared with the control plants in Linum usitatissimum [40], Perilla frutescens [14], Pharbitis nil [41], and Silene amuria [42]. However, relatively few reports are available about woody plants. Here, we have reported that 5-AzaC applied to precocious trifoliate orange induced flowering genes expression, suggesting the involvement of DNA demethylation in the flowering process of precocious trifoliate orange. In addition, we have reported on CLFY expression feature in the flowering transition stage, cloning, structural and functional analysis, and DNA methylation level during the phase transition of precocious trifoliate orange.

Materials and Methods

Plant material

Adult and juvenile precocious trifoliate orange samples were collected from the experiment fields of the National Citrus Breeding Center (30°28’ N, 114°21’ E, 30 m) at Huazhong Agricultural University. Apex buds and the following five buds (the major node position for flower formation) from spring flushes were sampled every two months in the year after bud swelling. The adult trees were 3- to 5-year-old. The juvenile material was seedlings germinated from the seeds of the adult mother plants. Because the embryo originates from a nucellar cell in trifoliate orange, the seedlings have the same genetic background as the mother plants. The seeds of precocious trifoliate orange were planted in 20-cm pots containing potting mixture of commercial medium and Perlite at a ratio of 3:1; the juvenile trees were watered regularly with nutrient solution. Shoot apical meristems of the juvenile tree were collected in March, June, September, and December. For spatial expression analysis of CLFY gene, several plant organs from juvenile and adult stages (lateral buds, apex buds, stems, leaves, flowers at full bloom, and whole fruits at 30 days after flowering) were sampled and immediately frozen in liquid nitrogen and stored at −80°C until use. All materials were collected from three individual plants for RNA extraction.

5-Azacytidine treatment

5-Azacytidine (Sigma, Switzerland) solution was freshly prepared for each experiment in phosphate-buffered saline (PBS) at a concentration of 2.4 mg/ml (10 mM) and sterile filtered. After the seed coats of precocious trifoliate orange seed were peeled and sterilized, the embryos were imbibed at 23°C on filter paper soaked with fresh 5-AzaC solution (0, 250, 500, and 1000 μM). The seeds were transferred daily to new filter paper containing fresh 5-AzaC solution. After 15 days, the germinated seeds were planted in 20-cm pots containing potting mixture of commercial medium and Perlite at a ratio of 3:1. The seedlings were watered regularly with nutrient solution and grown in test tubes in artificially lit growth cabinets under long days (16 h light and 8 h dark at 23°C) with fluorescent lights at a photosynthetic photon flux density of 200 μmol m-2s-1.

Analysis of flowering related genes expression by Real-time PCR

Total RNA was extracted according to a previous protocol [30]. The expression levels of flowering related genes were investigated by using real-time PCR with SYBR green I chemistry (QIAGEN, Germany). Primers were designed with the Primer Express software and tested to ensure amplification of single discrete bands with no primer-dimers. Total RNA (3 mg) was treated with 5 units of DNase (Promega, USA) and then used in first-strand synthesis with an oligo (dT) primer (20-mer) and reverse transcriptase according to the manufacturer’s instructions. For real-time PCR, an amount of cDNA corresponding to 25 ng of input RNA was used in each reaction. Real-time PCR was
performed on the LightCycler™ 480 System (Roche Applied Science, Mannheim, Germany) using \( \beta \)-actin as endogenous control. Briefly, the primers were diluted in the SYBER GREEN PCR Master Mix and 20 \( \mu \)l of the reaction mix was added to each well. Reactions were performed by an initial incubation at 50°C for 2 min and at 95°C for 1 min, and then cycled at 95°C for 15 s and 60°C for 1 min for 40 cycles. Data were evaluated by calibrator-normalized relative quantification with efficiency correction using the LightCycler™ 480 software version 1.5 (Roche Applied Science, Mannheim, Germany) and normalized to expression of \( \beta \)-actin. Real-time quantitative PCR was performed in four replicates for each sample, and data were indicated as mean \( \pm \) SD (n = 4). Three biologic repeats were assayed for each sample in this study, giving similar trends. Data from one biologic repeat were presented.

The promoter of flowering related genes isolation and bioinformatic analysis

High quality DNA was extracted according to Cheng et al [43]. Four Genome-Walker libraries were constructed by using the Genome-Walker kit (Clontech, USA) according to the manufacturer’s manual. Genomic DNA was digested with the restriction enzymes Dta I, EcoR V, Hpa I and Sca I. Following digestion, each pool of DNA fragments was ligated to the Genome-Walker Adaptor. The upstream genomic region was amplified from each library using two nested adaptor primers and two nested gene-specific primers. The primary PCR amplification was used for the outer adaptor primer (AP1) provided in the kit and the outer gene-specific primer. The primary PCR mixture was then diluted to 50 folds, and 1 \( \mu \)l of the diluted primary PCR mixture was used as a template for the secondary or “nested” PCR amplification by using the nested adaptor primer (AP2) and the nested gene specific primer. The \( CiLFY \) promoter and gene have been deposited in GenBank under Accession no. FJ238533 and AFY338976, respectively.

Bisulfite DNA sequencing

Bisulfite treatment was performed as described with some modifications [44]. Genomic DNA (3 \( \mu \)g) was digested with EcoR I and EcoR V and purified. DNA (0.5 to 2.0 \( \mu \)g) was added to 18 \( \mu \)l water was denatured at 95°C for 5 min. After quenched on ice, 3 \( \mu \)M NaOH (2 \( \mu \)l) was added and incubated at 37°C for 15 min. The bisulfite solution was prepared by dissolving 5.1 g of sodium bisulfite (Sigma, S-9000) in 8 ml of water with slow stirring to avoid precipitation. The pH was adjusted to 5.1 with freshly prepared 10 M NaOH. Then, 330 \( \mu \)l of 20 mM hydroquinone (Sigma, H-9003) was added and the volume was adjusted to 10 ml with water. Prewarmed bisulfite solution was added to the DNA solution, mixed gently, and overlaid with mineral oil. The bisulfite conversion was conducted by using 5 cycles of 95°C for 5 min, 55°C for 3 h. After bisulfite treatment, DNA was desalted with Wizard® DNA cleanup system (Promega, USA) following the manufacturer’s instructions and recovered in 40 \( \mu \)l of water. 3 \( \mu \)M NaOH (4.5 \( \mu \)l) was added to the DNA solution and incubated at 37°C for 15 min. DNA was then recovered by ethanol precipitation with glycogen carriers and 5 \( \mu \)l of 3 M NaOHAc and dissolved in 25 \( \mu \)l of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and 1 \( \mu \)l aliquot of the bisulfite treated DNA was used for each 20 \( \mu \)l volume of touchdown PCR reaction with three specific sets of primers. Reactions were performed by 94°C/0.5 min×1 cycle; 94°C/ 0.5 min, 60°C to 50°C/45 s, 72°C/1.5 min, −2°C/2 cycles; 94°C/0.5 min, 50°C/45 s, 72°C/1.5 min, ×30 cycles; and 72°C for 10 min for total 40 cycles. Products were cloned and seven individual clones from each tissue were sequenced. The process was repeated three times using biologically independent samples; each biologically sample was collected from three individual plants for DNA extraction in this study.

Vector construction and \( Arabidopsis \) transformation

Full-length promoter (Del 0) and a series of 3’-deletions fragments (Del 1-Del 5) were cloned into the pCAMBIA1391z vector, respectively. Then these vectors were transformed into \( Agrobacterium tumefaciens \) strain EH105. \( Arabidopsis \) ecotype Columbia (Col) plants were transformed by the floral dip method [45]. \( T_0 \) generation seeds were sterilized and germinated on the 1/2 MS [46] solid medium plates containing 23 mg/L hygromycin B (Roche, Germany) as a selective agent in the long-day conditions (16 h light and 8 h dark). Seven days later, positive \( T_1 \) seedlings were transplanted to soil to grow at the same photoperiod. One mg/mL GA3 (Sigma, USA) was added to \( T_1 \) seedlings plated on 1/2 MS medium to obtain 100 \( \mu \)M working concentration [47]. Application of exogenous GA3 after transplanting plants on soil growth was achieved by spraying soil-grown plants twice weekly with a solution of 100 \( \mu \)M GA3 and 0.02% Tween-20 (Bio-Rad). Samples were collected the day after spaying.

Histochemical localization and fluorometric measurement of GUS activity

GUS staining was carried out as described by Jefferson with some modifications [40]. Various tissues from transgenic \( Arabidopsis \) were immersed in X-Glc solution (1 mg/mL X-Glac, 100 mM sodium phosphate buffer [pH 7.0], 10 mM EDTA, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 1% Triton-X100, 100 \( \mu \)g/mL chloramphenicol, 20% methanol) and incubated for 16-24 h at 37°C, and then immersed in 70% ethanol for 3 to 4 times. For fluorometric measurement of GUS activity, plants were ground to a fine powder and then suspended in 1 ml GUS extraction buffer (50 mM sodium phosphate, pH 7.0; 0.1% Triton X-100; 10 mM \( \beta \)-mercaptoethanol; 10 mM EDTA and 0.1% sarcosyl (v/v)). The supernatant, after being centrifuged at 12,000 g for 20 minute at 4°C, was assayed for GUS activity with 4-methyl umbelliferyl glucuronide (Sigma) substrate using an F-4500 fluorescence spectrophotometer at the excitation/emission wavelengths of 365/455 nm. The protein concentrations were quantified according to Gallagher and the GUS enzyme activity was expressed as nmols of 4-methylumbellifereone produced per mg protein per minute [49]. In this study, all materials were collected from three individual transgenic lines for GUS staining and measurement of GUS activity.

Results

Characterization of plants by 5-Azacitidine treatment

Different concentrations of 5-AzaC were treated continuously precocious trifoliate orange seeds at 0, 250, 500, and 1000 \( \mu \)M for 15 days under dark conditions (Figure 1). The treated plants were distinguishable from the control plants (treated with distilled water), the increasing concentrations of 5-AzaC significantly retarded the seedling development especially the root development (Figure 1). For further investigations, these treated seedlings were transplanted to the soil. After 20 days of transplantation, it was noticed that the roots of seedlings treated with 250 \( \mu \)M 5-AzaC grew slowly as compared with the control plants (Figure 1). There were several aberrant leaves grown on seedlings. In addition, there was quite less number of vegetative growing buds, which then tends to decrease with the increase in time. However, seeds treated with 500 \( \mu \)M 5-AzaC concentration were showing apparently
inhibited root and stem development of the seedlings (Figure 1). Shoot apices of some plants were died which then prevented the further elongation of the main stem. Most of the leaves exhibited by the seedlings were having aberrant phenotype. Moreover, the growth of 1000 μM 5-AzaC treated seedlings was nearly standstill compared with 20 days before condition (Figure 1). By summarizing, our results revealed that the plant height was reduced proportionally more with increasing 5-AzaC concentrations as compared with the untreated seedlings.

**Analysis of flowering related genes in 5-Azacytidine treated plants by Real-time PCR**

In order to further understand the involvement of DNA methylation in flowering process of precocious trifoliate orange, total RNA was isolated from throughout the aerial part (including stem, leaf and shoot apical meistem) of 35 days after seed germination with different 5-AzaC concentrations for analyzing some flowering related genes (Figure 2). Previous reports have revealed that *TFL1*, *FT*, *API*, *FLC* and *LFY* play a critical role during the early flowering process of model and woody plants. Therefore, the citrus homologous gene of these genes such as *CiTFL1*, *CiLFY*, *CiAPI*, *CiFLC*, *CiFT* were investigated under different 5-AzaC concentrations. Our findings exhibited that the *CiFT* expression level was increased with the increasing concentrations of 5-AzaC (from 250 to 500 μM). However, *CiTFL1*, *CiLFY*, *CiAPI*, and *CiFLC* showed highest relative expression levels in 250 μM treatment and followed by a sharp decrease in the relative expression in the seedlings treated with 500 μM concentration (Figure 2). These results revealed that demethylation treatment directly or indirectly influences the expression of the above genes during early flowering process of precocious trifoliate orange.

**Sequence characterization of flowering related genes promoter**

CpG and CNG islands are the worthy targets for DNA methylation [6,10]. To confirm DNA methylation affects the expression level of these flowering genes, we focused on specific DNA methylation site in these genes. Therefore, the promoter sequence (about 1.5 kb–2.0 Kb) of the *CiTFL1*, *CiLFY*, *CiAPI*, *CiFLC*, and *CiFT* was isolated from precocious trifoliate orange. The 5’ upstream region and full-length gene of the above five genes were analyzed by using NSITE, TSSP, NSITEM and PromoterScan software. The results revealed the presence of common elements such as TATA box and CAAT box, the putative transcriptional start site and different binding motifs (circadian rhythms; light regulation) in these promoters. However, only one methylation site (CpG Island) was observed in *CiLFY* sequence with Methyl Primer Express v1.0 (Figure S1). The CpG island was located at the tail of 5’-UTR region and gene (from +669 to +1255 bp). The bioinformatic analysis of the region results as A%: 25.81%; T%: 21.10%; C%: 21.33%; G%:30.76%; (C+G%): 52.09%; CG%: 4.8; A+T/C+G%: 0.92%, and sequence analysis by CpMATE was CHH% (N=A, T, G, or C): 47.8%; CHG% (H=A, T, or G): 26.7%; CG%: 25.6%. The result indicated that the *CiLFY* may be regulated directly by DNA methylation in precocious trifoliate orange.

In addition, bioinformatic analysis revealed that the putative transcription start site (A) was located at 746 nucleotides upstream to the start codon (ATG) of the *LFT* promoter consistent with 5’-RACE (rapid amplification of cDNA ends) experiment. A putative TATA box and a putative CAAT box, were located at the regions −25 (−)−200 (−), −49 (−)−90 (−), respectively. The two highly conserved motifs that were involved in the interaction with the RNA polymerase and in the regulation of gene transcription efficiency, respectively. Based on the PLACE software analysis, 394 distinct putative cis-regulatory elements were identified within the *CiLFY* promoter. Of the 394 cis-elements, 40 were singlets, and the remaining was assembled into 50 groups with occurrence ranging from 2 to 28 times (Table S2). There were a number of phytohormone responsive motifs such as auxin response factor binding motifs (ASFI1MOTIFCAMV and AUXRETRACTGALIGMGH3), gibberelin response motifs (PYRIMIDINEBOXOSRAMY1A), and ABA response motifs (EBOXBNNAPA and MYCCONSENSUSAT). Various putative elements in relation to light response were also abundant in *CiLFY* promoter, signifying that this promoter was probably subject to light regulation and involved in photoperiod pathway (Table S1). Interestingly, two flowering gene binding sites (AGAMOUS-like 15 and WUSCHEL gene) were also identified in *CiLFY* promoter. These sites were potentially involved in flowering and apical meristem development; suggesting a regulatory relationship with the two genes in the *CiLFY* promoter. In addition, a number of potential regulatory motifs corresponding to known cis-regulatory signals of eukaryotic genes were also found, including low-temperature-responsive elements, circadian control factors, early responsive to dehydration, enhancer elements and so on (Table S2).

**Analysis of the *CiLFY* expression at juvenile and adult stages by real-time PCR**

A major characteristic of precocious trifoliate orange was that its juvenile phase was shortened to 1 to 2 years. The flower buds of precocious trifoliate orange cannot be recognized visibly by shape and size at early stages. For this reason, paraffin section was performed to identify flower development stage during the transition from the vegetative to reproductive stage. From November to January, some vegetative growth points were transformed into flower buds, it becomes broadened and flattened, forming floral apical meristem, finally give rise to flower meristem and flowers [March] [33]. To understand the relationship between phase change and the expression of *CiLFY* in precocious trifoliate orange, the expression level of *CiLFY* was investigated by real-time PCR (Figure 3). As a result, there was a fluctuation in the *CiLFY* expression at different developmental phases.
expression level accompanying with the season shift and morphological change. The level of \textit{CiLFY} was low during summer and autumn, it slowly climbed up after December and peaked in January, and maintain in high level in March, and then the mRNA level decreased rapidly (Figure 3A). In addition, high levels of \textit{CiLFY} gene were reflected in the adult tissues as compared with juvenile tissue (Figure 3A). One possible explanation for this observation is that this gene may play an important role in inducing early flowering in precocious trifoliate orange and may be directly regulated by methylation.

On the other hand, the temporal expression of \textit{CiLFY} was further examined by real-time PCR in juvenile (leaves, roots and stems) and adult (leaves, roots, stems, flowers, fruits, apex bud and lateral bud) different tissues (Figure 3B). Our results revealed that this gene have shown broad expression patterns, with the transcripts detected in all plant organs except adult roots. The expression levels were particularly high in juvenile stems and adult stems, flowers and apex buds (Figure 3B). As the apex buds are the major node position for flower formation, so this results suggested that the expression may be associated with floral development in the early flowering process of precocious trifoliate orange.

Methylation status of \textit{CiLFY} at juvenile and adult stages of precocious trifoliate orange

Locus specific methylation analysis of the \textit{CiLFY} CpG island was performed to uncover the relationship between the phase change of the precocious trifoliate orange and the methylation status (Figure 4). Observations revealed that the overall DNA methylation status of \textit{CiLFY} was 25.7% and 18.1% at juvenile and adult stages, respectively. The proportion of three different methylation cytosine for juvenile stage was CG\%: 52.3%; CHH\%: 14.8%; CHG\%: 9.1% and for adult stage was CG\%:46.2%; CHH\%: 4%; CHG\%: 4%, respectively (Figure S1). These results indicated that the prominent methylation decrease during the transition from juvenile stage to adult stage in precocious trifoliate orange. DNA methylation occurs mainly at CG sites in mammals, but these often have been observed at CNG and CHH sites in plants [6,10]. In this study, CHH and CNG were relative abundant in \textit{CiLFY} 5’UTR. Therefore, DNA methylation level of the \textit{CiLFY} 5’UTR was also analyzed. Interestingly, bisulfite genomic sequencing results indicated that the overall DNA methylation status of \textit{CiLFY} 5’UTR was 6.3% and 6.0% at juvenile and adult stages, respectively (Figure S1). Therefore, the demethylation of \textit{CiLFY} might be the reason for the reflection of gene expression during flower initiation. The results also suggested that there may be an association of \textit{CiLFY} demethylation to the phage transition in precocious trifoliate orange.

Spatial and temporal expression patterns of \textit{CiLFY} promoter in \textit{Arabidopsis}

To examine the tissue specificity of the \textit{CiLFY} promoter, a GUS expression construct driven by full-length \textit{CiLFY} promoter (1,641 bp) was introduced into \textit{Arabidopsis} (Figure 5). Transgenic plants were confirmed for the presence of appropriate expression cassette by PCR. Overall, histochemical analysis have indicated that the GUS enzyme activity was detected in adult roots, young leaves, floral petals and sepals, but was not in the pistil, stamens, or seeds (Figure 3). At juvenile stage, GUS expression was first detected in 7-day-old seedlings, there was stronger GUS staining.
in the first two true leaves, while there was relatively weaker staining in the hypocotyls, but no staining was observed in cotyledon and radicles. Similar GUS activities were maintained in 14-day-old aerial parts (Figure 5). Histochemical assay at adult stage indicated that GUS staining was found in all floral buds (including apex and lateral). Detailed flower study has revealed that the sepals, peripheral petal, and stigmatic papillae exhibited very intense GUS expression (Figure 5), while anther locules and stamen filaments did not exhibit any GUS expression (Figure 5). The other aerial plant parts like stem, bracts and mature leaves did not exhibit any GUS expression. In fruit, GUS expression was only confined to fruit abscission zone. The GUS expression pattern was also corroborated with the pattern observed by real-time PCR (Figure 3B). All of these results indicated that the CiLFY promoter modulated precise transcriptional regulation of specific and developmental expression in transgenic Arabidopsis.

Expression of promoter-GUS response to GA3 treatment in transgenic Arabidopsis

To gain further insight into the functional role of CiLFY promoter regions, a series of expression cassettes with truncated promoter fragments based on the organ/tissue-specific and hormones responsive region and a GUS reporter gene were generated (Figure 6A). In this study, five deletions were constructed and transformed into Arabidopsis. As a result,
histochemical GUS staining on transgenic plant of deletion of promoter fragment have showed the same positional distribution as compared to that of the full-length promoter (Del 0), but with different expression intensity at different development stages. Del 1 showed stronger promoter activity as compared with Del 0 and Del 2 (Figure 6B), which possibly suggest negative regulating element (−153 to −1331 bp) and positive element (−1331 to −1175 bp) in those regions. We have noticed the presence of a “CACGTTGMOTIF” motif in the promoter sequence between −1,433 and −1,439. This element was of our interest because of its behavior as a transcriptional repressor element, which then involved in regulation of transcription (Table S2). Meanwhile, a CCAATBOX1 element was also identified, which is a cis-enhancer regulatory element essential for increasing the promoter activity. Del 3 showed almost the same activity intensity with Del 2, but Del 4 exhibited the strongest promoter activity amongst them (Figure 6B). It means that there may be a very strong negative element in the region (from −860 to −634 bp). Del 5 (from +254 to +768) did not show any activity, which may indicate the basic promoter element region between −634 and +1 as predicted by MatInspector. From the observations described above, it was therefore conceivable that there was almost no organ-specific located at the region from −1533 to −634 bp. However, there was an obvious intensity difference in terms of different constructs because of the presence of some enhancers and the inhibitors in CiLFY promoter region.

The gibberellin class of plant hormones has been implicated in the control of flowering in several species. Previous studies indicated that gibberelin promote flowering of Arabidopsis by activating the LEAFY promoter. In this study, there was one gibberelin inductive motif PYRIMIDINEBOXHVEPB1 (−177) in CiLFY promoter (Table S2). Therefore, the histochemical GUS activity assays were complemented by analyzing the GA3 induced expression of the GUS reporter gene in transgenic Arabidopsis harboring the different CiLFY promoter-GUS fusion constructs (Figure 6D). Quantitative assay exhibited the enhancement of the full-length promoter activity as compared with control at juvenile stage (Figure 6C). GUS staining was all over the seedlings including radicle, which was absent in the un-treatment. At adult stage, plants exhibited the same expression pattern in terms of flowering organs with un-treatment transgenic plants except significantly enhanced the expression of GUS. For progressive 5′ deletions, the expression of GUS have a sharp increase compared with un-treatment plants, while the expression of GUS gene did not show significant differences among different deletions (Figure 6D). These results suggested that gibberellins inductive motifs “PYRIMIDINEBOXHVEPB1” was essential for GA3 induction in citrus. The above results demonstrated that the promoter had a mechanism responding to gibberellin condition in transgenic Arabidopsis plants and this responding site was within the Del 4 region.

**Discussion**

DNA methylation, one of the most abundant epigenetic modifications in higher plants and animals, plays an important role in regulating developmental processes. Recently in plants, epialleles of genes with ecological importance having effect on development, floral morphology, flowering time, seed pigmentation, and pathogens resistance, have been characterized [1,13,14,41]. Although, early flowering induced by 5-AzaC treatment has reported in annual plants, we report here for the first time on the flower-induction by DNA demethylation in woody plants. There were some morphological abnormalities observed in precocious trifoliate orange by 5-AzaC treatment. The abnormal plants have decreased their stature, smaller leaves, leaves with margins curled toward the upper leaf surface, and reduced apical dominance. Therefore, we supposed that the developmental abnormalities were correlated with altered patterns of gene expression. Treatment of plant and animal cells with 5-AzaC resulted in the demethylation of DNA directly by incorporaration of the analogue in place of cytosine during DNA replication [50] and indirectly by inhibiting the action of methyltransferase [9]. Demethylation of DNA by 5-AzaC has been correlated with induction of transcription in a number of gene systems in plants [14,50]. It has been reported that the reduced DNA methylation by 5-AzaC treatment in Arabidopsis was also resulted in abnormal plant development [42,51]. There was no flowers observed after the application of different concentrations of 5-AzaC. There might be two possible explanations: at first, genetic regulation of floral induction in perennial species is much more complex than in annual plants, so demethylation treatment may cause the metabolic disorders in treated plants. There is evidence supporting the view that demethylation resulted in abnormal plant development. Secondly, the juvenile period of precocious trifoliate orange has been greatly reduced to 1–2 years as compared with the other citrus plants. Thus, the flowering induced by different treatments of 5-AzaC in precocious trifoliate orange was accompanied by only suppression of vegetative growth with no obvious changes in flowering time. Therefore, it is necessary to examine the effect of 5-AzaC in long-juvenile citrus plants which have members showing early flowering and late flowering in the future.

Vernalization requiring late-flowering mutants *lea* and *fy* of Arabidopsis have induced early flowering by 5-AzaC treatment [24], 5-AzaC also induced flowering in the vernalization requiring *Thlaspi arvense*, *Perilla frutescens* and potatoes [13,24,52]. These results indicated that the flowering genes may be up-regulated through the decrease DNA methylation in annual plants [53,54]. Recently, it has been reported that the expression of the *FT* was statistically increased in the 5-AzaC-treated early flowering plants with respect to control plants in potato [13]. Previous studies also exhibited the higher transcription levels and involvement of *FT*, *FT*, *FT*, *FT*, *FT*, *FT*, *FT*,
API, TFL1, FLC and LFY homologous genes during flower induction as well as floral induction, inflorescence development and flowering [32,55–58]. These results indicated that these genes might have an important role in inducing early flowering and shortening the juvenile phase. Our results have reflected an increased relative expression level of CiFT with different 5-AzaC treatments. However, CiTFL1, CiLFY, CiAP1, and CiFLC showed highest relative expression levels at 250 μM concentration. In the past investigations, the transcript level of floral inhibitor FLC is down regulated by treatment with 5-AzaC in Arabidopsis [42,52,59], while CiFLC has shown up-regulated in precocious trifoliate orange. One possible reason may be that the regulatory mechanism of CiFLC was different between Arabidopsis and woody plants. In citrus, the expression profile of CiFLC showed up-regulation during the winter, followed by a decrease in the spring and summer. This kind of cycling is contrary to the pattern observed in Arabidopsis [29]. In addition, the relative expression level of the remaining genes was decreased sharply at higher concentrations of 5-AzaC indicating that they may perform a similar mechanism between citrus and Arabidopsis. In Arabidopsis, the extent of advancement of flowering time is dependent on 5-AzaC concentration, 250 μM being optimum. Higher concentrations of 5-AzaC were inhibitory to bolting and flower development was probably due to the nonspecific toxic effects of 5-AzaC [24]. To examine the differences in the DNA methylation status of five flowering related genes during phase change process, the full-length (including promoter) sequence of these genes was isolated. However, only one CpG Island was identified in CiLFY gene by bioinformatics analysis, the result indicated that the CiLFY may be regulated directly by DNA methylation. LFY is a transcription factor that affects not only inflorescence initiation but also floral organ determination in annual and woody plants [25,32,37]. Thus, CiLFY expression was analyzed at juvenile and adult stages. The expression pattern was closely correlated with floral induction, inflorescence development and flowering (Figure 3), suggesting that the gene may play a critical role in the flowering process of precocious trifoliate orange. Because precocious trifoliate orange was a woody perennial plant, it was difficult to observe a spatial gene expression pattern in the whole plant throughout its life cycle. Therefore, to examine the spatial expression patterns of CiLFY, we generated transgenic Arabidopsis with the GUS reporter gene driven by the regulatory sequences of putative promoters of CiLFY. According to our data for stable expression analysis, GUS expression in the transgenic Arabidopsis was consistent with the result of real-time PCR. These results indicated that transgenic Arabidopsis could reliably reflect the temporal expression of CiLFY in precocious trifoliate orange. Deletion analysis of the CiLFY promoter was also performed for
determining the function of the cis-acting elements. The Del 1 and Del 4 had the higher activity among all the fragments in transgenic Arabidopsis because of the presence of inhibitor and enhancer in this promoter, suggesting that these sequences were important for the regulation of CiLFY promoter activity in citrus. Previous studies have reported that GAs affect plant flowering through a pathway that controls LFY transcription. In this study, GUS fluorescence assays has reflected the visible increase in the gene expression with GA3 treatment, but there was no significant difference between the different promoter deletion lines in response to GA3. Our finding explain that the GA3 regulatory sequence for inducting the gene expression was located somewhere between the −634/+1 fragment of the CiLFY promoter. The above fragment consists of only one GA3 induction cis-elements: PYRIMIDINEBOXHVEPB1 in −177/−169 region. Therefore, we speculated that the GA3 inducing cis-elements might be the main reason for CiLFY promoter response GA3. However, further studies were required because this was a preliminary, inconclusive deduction on our part.

We have performed the bisulfite sequencing in CpG island of CiLFY gene to examine whether the level of DNA methylation will alter during the phase change process or not. There was a decrease in level of DNA methylation at adult stage as compared with juvenile stage. In Arabidopsis DDM1 mutants [60] expressing MET1 in antisense orientation [61], the decrease in DNA methylation affected the duration of both juvenile and vegetative phases and induced flowering [62]. Hence, METases must have a role in regulating developmental phase change [62,63]. These findings indicated that METases might regulate CiLFY expression in precocious trifoliate orange. Previous report proclaimed that methylation was not detected in the LFY of Arabidopsis [44]. The difference of methylation and demethylation during regulation of transition development between annual and perennial plants might be correlated with their meristem determination. It has been reported that the molecular difference between perennials and annuals may be rather small, and a change between these life stages might not require major genetic innovations [64]. In terms of floral process, floral evocation implies epigenetic strategies might not require major genetic innovations [64]. In annuals may be rather small, and a change between these life stages might not require major genetic innovations [64]. In annuals may be rather small, and a change between these life stages might not require major genetic innovations [64].

Conclusions

Epigenetic control, and specifically DNA methylation, plays an essential role in regulating the timing of precocious trifoliate orange flowering. Our findings also suggested that instead of all, only certain key genes were regulated directly by the DNA methylation by depending on related phenomena and species. According to the analyses of a series of truncated CiLFY promoter constructs, the present research has also demonstrated the importance of various regions of the CiLFY promoter for the regulation of citrus flowering. The “PYRIMIDINEBOXHVEPB1” motif was a critical element that determines GA3 induction of the CiLFY promoter. The reporter constructs described here will also provide a useful means of further analyses of CiLFY regulation, with a view to making comparisons with other promoters of flowering genes responsive to GA3, and developmental cues. Further studies will be required to clarify the function of CiLFY by analyzing the transgenic trifoliate orange. For example with an RNAi (RNA interference) construct and by investigating their spatial and temporal expression patterns by in situ hybridization in order to develop the technology to control the flowering of trifoliate orange in the juvenile and adult phase.

Supporting Information

Figure S1 Methylation status of CiLFY gene at juvenile and adult stages of precocious trifoliate orange. Probable sites for the three classes of methylation (CGN, CHG, and CHH) as well as actually methylated sites in all the samples were identified by the software and projected symbolically. Blocked symbols represent actual methylation, whereas unblocked ones represent potential sites. A: Line diagram for 5′-UTR and CG island in CiLFY DNA sequence, a and b present region 1–3 in CpG island of CiLFY gene, respectively; d and e present region 1–2 in 5′-UTR of CiLFY gene, respectively. B: CG island of CiLFY methylation analysis, a, b and c presents region 1–3 in CG island, respectively; d and e presents region 1–2 in 5′-UTR sequence, respectively. (TIF)

Table S1 Primers for genome walking, 5′ deletions of CiLFY promoter, real-time PCR and methylation analysis. (XLS)

Table S2 cis-elements in the CiLFY promoter predicted by database analysis. Different motifs and models were identified using PLACE, PlantCARE and Regsite Plant databases and the Genomatix suite software. Positions are relative to the transcriptional initiation site. The orientation of the motifs is indicated (+, forward; −, reverse). Nucleotides are indicated as follows: N for A, C, G or T; Y for C or T. (XLS)

Author Contributions

Conceived and designed the experiments: JZZ, CGH. Performed the experiments: RI, LM. Analyzed the data: RI, LM, JZZ, MRGK. Wrote the paper: JZZ.

References

1. Li C-Q (2013) DNA demethylation pathways: recent insights. Genetics 2013: 43–49.
2. Mohandas T, Sparker RS, Shapiro LJ (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science 211: 393–396.
3. Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H, et al. (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in Arabidopsis. Nature 411: 212–214.
4. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes & development 16: 6–21.
5. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nature Reviews Genetics 3: 662–673.
6. Shibukawa T, Yazawa K, Kikuchi A, Kamada H (2009) Possible involvement of DNA methylation on expression regulation of carrot LEC1 gene in its 5′-upstream region. Gene 437: 22–31.

According to the analyses of a series of truncated CiLFY promoter constructs, the present research has also demonstrated the importance of various regions of the CiLFY promoter for the regulation of citrus flowering. The “PYRIMIDINEBOXHVEPB1” motif was a critical element that determines GA3 induction of the CiLFY promoter. The reporter constructs described here will also provide a useful means of further analyses of CiLFY regulation, with a view to making comparisons with other promoters of flowering genes responsive to GA3, and developmental cues. Further studies will be required to clarify the function of CiLFY by analyzing the transgenic trifoliate orange. For example with an RNAi (RNA interference) construct and by investigating their spatial and temporal expression patterns by in situ hybridization in order to develop the technology to control the flowering of trifoliate orange in the juvenile and adult phase.

Supporting Information

Figure S1 Methylation status of CiLFY gene at juvenile and adult stages of precocious trifoliate orange. Probable sites for the three classes of methylation (CGN, CHG, and CHH) as well as actually methylated sites in all the samples were identified by the software and projected symbolically. Blocked symbols represent actual methylation, whereas unblocked ones represent potential sites. A: Line diagram for 5′-UTR and CG island in CiLFY DNA sequence, a and b present region 1–3 in CpG island of CiLFY gene, respectively; d and e present region 1–2 in 5′-UTR of CiLFY gene, respectively. B: CG island of CiLFY methylation analysis, a, b and c presents region 1–3 in CG island, respectively; d and e presents region 1–2 in 5′-UTR sequence, respectively. (TIF)

Table S1 Primers for genome walking, 5′ deletions of CiLFY promoter, real-time PCR and methylation analysis. (XLS)

Table S2 cis-elements in the CiLFY promoter predicted by database analysis. Different motifs and models were identified using PLACE, PlantCARE and Regsite Plant databases and the Genomatix suite software. Positions are relative to the transcriptional initiation site. The orientation of the motifs is indicated (+, forward; −, reverse). Nucleotides are indicated as follows: N for A, C, G or T; Y for C or T. (XLS)

Author Contributions

Conceived and designed the experiments: JZZ, CGH. Performed the experiments: RI, LM. Analyzed the data: RI, LM, JZZ, MRGK. Wrote the paper: JZZ.
11. Vanyushin BF, Bakeeva LE, Zamyatina VA, Aleksandrovskii NI (2004) Apoptosis in plants: specific features of plant apoptotic cells and effect of various factors and agents. International review of cytology 233: 153-179.

12. Garcia-Manero G, Daniel J, Smith TL, Kornblau SM, Lee M-S, et al. (2002) DNA methylation of multiple promoter-associated CpG islands in adult acute lymphocytic leukemia. Clinical Cancer Research 8: 2217–2224.

13. Marfil CF, Asurmendi S, Masselli RW (2012) Changes in micro RNA expression in a wild tuber-bearing Solanum species induced by 5-Azacytidine treatment. Plant Cell reports 31: 1449–1461.

14. Kondo H, Shiraya T, Wada KC, Takeno K (2010) Induction of flowering by DNA demethylation in Perilla frutescens and Silene armeria: Heritability of 5-azacytidine-induced effects and alteration of the DNA methylation state by photoperiod conditions. Plant Science 179: 128–136.

15. Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, et al. (2006) DEMETER DNA glycosylase establishes MEDEA polycorn gene self-imprinting by allele-specific demethylation. Cell 124: 495–506.

16. Jiang PL, Kinoshita T, Ohad N, Berger F (2006) Maintenance of DNA methylation during the Arabidopsis life cycle is essential for parental imprinting. Plant Cell 18: 1360–1372.

17. Blazquez MA (2000) Flower development pathways. Journal of Cell Science. 113: 363–369.

18. Kakutani T (1997) Genetic characterization of late-flowering traits induced by 5-azacytidine in Citrus. Tree Physiology 17: 391–397.

19. Kinoshita T, Miura A, Choi Y, Kimura A, Cao X, et al. (2004) One-way traffic of FWA imprinting in Arabidopsis endosperm by DNA methylation. Science 303: 521–523.

20. Kondo H, Ozaki H, Itoh K, Kato A, Takeno K (2006) Flowering induced by 5-azacytidine in Perilla frutescens var. crispa. Physiologia Plantarum 127: 130–137.

21. Pillitteri LJ, Lovatt CJ, Walling LL (2004) Isolation and characterization of a TERMINAL FLOWER organ and its correlation with juvenile in citrus. Plant Physiology 135: 1540–1551.

22. El-Onnui M, Coppins CW, Agusti M, Lovatt CJ (2000) Plant growth regulators in flower initiation: wounding effects. Current Research in plant sciences 19: 595–602.

23. Zhang JZ, Li ZM, Yao JL, Hu CG (2009) Identification of flowering-related LEAFY and APETALA1 homologues from Citrus sinensis L. Osbeck‘Wa-family. Plant Journal 49: 38–45.

24. Pillitteri LJ, Lovatt CJ, Walling LL (2004) Isolation and characterization of CiLFY, a LEAFY homolog from Citrus. Plant Physiology 135: 1540–1551.

25. Ronemus MJ, Galbiati M, Ticknor C, Chen J, Dellaporta SL (1996) Increased CiFT abundance in the stem correlates with floral induction by low temperature in Citrus transgenic lines and their control. Plant Science 124: 216–225.

26. Brown JCL, De Becker MM, Fieldes MA (2008) A comparative analysis of developmental profiles for DNA methylation in 5-azacytidine-induced early-flowering lines and their control. Plant Science 175: 217–225.

27. Li W, Zhou Y, Liu X, Yu P, Cohen JD, et al. (2013) LEAFY Controls Auxin Signalling in the Arabidopsis Floral Transition. Plant Cell 25: 2623–2633.

28. Blazquez MA, Cuebas P, Martinez-Zapater JM (2002) Vernalization induces flowering of Arabidopsis by activating the FLC gene. Nature Biotechnology 19: 263–267.

29. Jefferson RA, Kavanagh TA, Bevan MW (1987) Gus Fusions - Beta-Glucuronidase as a Sensitive and Versatile Gene FUSION Marker in Higher Plants. The EMBO Journal 6: 3900–3907.

30. la Farge MA, Peacock JW, Dennis ES (1998) DNA methylation during the Arabidopsis life cycle is essential for parental imprinting. Plant Cell 10: 1773–1778.

31. Brown JCL, De Becker MM, Fieldes MA (2008) A comparative analysis of developmental profiles for DNA methylation in 5-azacytidine-induced early-flowering lines and their control. Plant Science 175: 217–225.