Original article

Dynamic changes of DNA methyltransferase and demethylase gene expression during *Chrysanthemum* × *morifolium* flower induction and development

Kang Dongru, Li Zhongai, Li Mangmang, Li Jie, Liu Yanhua, Ai Penghui, Ding Hongxu, Zhu Yi, Wang Ying and Wang Zicheng

Plant Germplasm Resources and Genetic Laboratory, Kaifeng Key Laboratory of Chrysanthemum Biology, School of Life Sciences, Henan University, Kaifeng, Henan, China

Summary

DNA methylation plays an important role in plant growth and development. The purpose of this study was to observe the expression of DNA methylation related genes in *Chrysanthemum* × *morifolium*. Homologous genes related to DNA methylation in chrysanthemum were cloned, and obtained 1,509-bp for *CmCMT*, 1,470-bp for *CmDRM* and 608-bp for *CmDME*. The *C. morifolium* cultivar ‘Nannonghongxiu’ was exposed to short-day (SD) conditions, and the expression of DNA methyltransferase and demethylase genes in flowering plants was analyzed by quantitative real-time polymerase chain reaction. At flowering stage, *CmFT* expression level exhibited an increasing trend while *CmMET1* and *CmCMT3* showed a decreasing trend; the intensity of decrease was greater in SD-treated plants compared to control plants in the same stage. The expression of *CmDRM2* was up-regulated in the flowering stage. The expression level of *CmDRM2* was lower in control plants as compared to SD-treated plants in the first three growth stages. However, the *CmDRM2* expression level in the control plants was at peak during full bloom stage as well as higher than SD-treated plants. The highest *CmDME* expression level was observed during full bloom stage in both types, however, control plants showed higher level in comparison with the plants exposed to SD conditions. The expression levels of the DNA methyltransferase and demethylase genes undergo dynamic changes during the transition from vegetative growth to reproductive growth (flowering induction stage) and flower development, which seems to influence DNA methylation changes in the leaves.

Keywords

Chrysanthemum × *morifolium*, gene clone, DNA methylation, vegetative growth, reproductive growth

Significance of this study

What is already known on this subject?

- It is well known that DNA methylation plays an important role in the growth and development of plants, but the specific mechanism in the induction of flowering in Chrysanthemums is still unclear.

What are the new findings?

- The expression level of the DNA methyltransferase and demethylase genes undergo dynamic changes during the transition from vegetative growth to reproductive growth.

What is the expected impact on horticulture?

- The study on the expression of DNA methylation-related genes not only provides genetic resources for the subsequent regulation of flowering period in Chrysanthemums, but also provides a basis for the subsequent studies on related mechanisms.

2010). The main regulatory pathways of *A. thaliana* flowering have been characterized, including vernalization, photoperiod, gibberellic pathway, and autonomous pathways. Photoperiod refers to the relative day length, which is a critical factor that influences the timing of flowering (Davis, 2002). Changes in the internal circadian rhythm of plants based on day length are referred to as photoperiodic responses. Depending on their responses to the daily duration of sunlight, plants can be divided into long-day plants, short-day (SD) plants and day-neutral plants (Yeang, 2013).

Epigenetic modifications can control the expression of genes and are playing an important role in the regulation of plant growth and development (Hauser et al., 2011; Paszkowski and Grossniklaus, 2011). These modifications include DNA methylation, which involve the DNA methyltransferase-catalyzed transfer of methyl groups from S-adenosine methionine to CpG and CpXpG sequences to generate 5-methyl cytosine and a small amount of N6-methyl adenine and 7-methyl guanine (Saze et al., 2003; Lindroth et al., 2001). Recent studies revealed that a SD plant, *Perilla frutescens*, can flower even under long-day conditions if its seeds are pre-treated with the DNA methylation inhibitor 5-azaC. The same results were observed when 5-azaC was added to *P. frutescens* stem tips. Thus, *P. frutescens* flowering may be regulated by decreases in the extent of DNA methylation rather than by the photoperiod (Kondo et al., 2007).

Introduction

Flowering, an important event during plant growth and development, is controlled by a complex network of signal transduction pathways that are affected by the internal and external environments (Bratzel and Turck, 2015). Most of the available information regarding flowering pathways came from studies involving *Arabidopsis thaliana* (Amasino,

Chrysanthemum × morifolium species are typical SD plants and exposures to SD condition can induce early flowering. We observed in previous studies that traditional Chinese Chrysanthemum treated with 5-azaC showed difference in flowering time as well as decrease in DNA methylation levels (Li et al., 2017). Treated plants flowered approximately 12 days earlier than wild-type and control plants (Wang et al., 2009). Other Chrysanthemum types such as cut *C. morifolium* species and *C. lavandulifolium* treated with 5-azaC exhibited flowering time difference too (Kang et al., 2019a, b). The DNA methylation level of *C. morifolium* drops gradually during SD treatment, although it is the final consequence of this activity and could either increase or decrease (Wang et al., 2009; Li et al., 2016).

The level and pattern of DNA methylation generally depends on DNA demethylase and DNA methyltransferase activities. In plants, there are three main DNA methyltransferase families, which are represented by cytosine methyltransferase 1 (*MET1*), domains rearranged methyltransferase 2 (*DRM2*), and chromodomain containing methyltransferase 3 (*CMT3*) (Xu et al., 2015; Chang et al., 2009; Chinnusamy and Zhu, 2009; Law and Jacobsen, 2010; Rival et al., 2008). Earlier studies concluded that DNA methyltransferases affect the generation and maintenance of methylated cytosine in *A. thaliana* (Chan et al., 2005; Goll et al., 2005). Additionally, *DRM2* is associated with the *de novo* methylation of the whole genome sequence. This enzyme is involved in RNA-mediated DNA methylations and it is important for the maintenance of asymmetric CHH methylation (Deleris et al., 2010). Furthermore, CG and CHG methylations are mainly catalyzed by *MET1* and *CMT3* (Rival et al., 2008). The methylation of CHG and CHH is controlled by the redundant regulation of *CMT3*, *DRM1*, and *DRM2* (Goll et al., 2005; Li et al., 2019). The four families of DNA demethylases are represented by *DME*, *ROS1*, *DML2*, and *DML3* (Tang et al., 2016; Le et al., 2014; Qi et al., 2015).

DNA methylation is playing an important role in plant growth and development. However, there is a lack of research about the changes of demethylase and DNA methyltransferase expression during the transition from vegetative to reproductive growth in *C. morifolium*.

Materials and methods

Plant materials and treatment

The *C. morifolium* cultivar 'Nannonghongxiu' was used in this study. Plants were cultivated at the Chrysanthemum Institute in Kaifeng. The experimental materials were propagated by cutting. Cuttings were collected on 15 May 2015 and rooted cuttings were planted on 2 July 2015. The plants were watered daily with tap water and given inorganic nutrient solution once a week (Li et al., 2016).

Short-day treatment, consisting of 7-h light and 17-h dark (from 17:00 to 10:00 h the following day) periods. The plants were about 10 cm in height when treated. Plants growing under natural conditions (light time: 13–14 h d⁻¹) were chosen as the control group having 20 pots per treatment. The natural day-length became shorter gradually during the experiments while it was longer than the treatment all the times. The control group was grown under natural climatic conditions and a shading shed was used to apply the short-day treatment (Xu et al., 2015).

Genomic DNA was extracted from mature leaf samples collected every 7 days at 12:00 h from August 1 to 18th of October. Healthy leaves were chosen for DNA extraction, which

followed the method of Wang (Wang et al., 2005). Four development periods of *C. morifolium* were sampled, respectively (1) Vegetative Stage: the period before the induction of SD conditions; (2) Budding Stage: the period when buds are visible to the naked eye; (3) Early Flowering Stage: the first round of petals protruding; (4) Full Bloom Stage: all the petals are in full bloom.

RNA extraction and cDNA synthesis

Total RNA was extracted from *C. morifolium* 'Nannonghongxiu' (at least three plants of equal growth at each stage of development and two or three leaves from the tip of shoots) using the RNA prep Pure Plant Kit (for polysaccharide-rich and polyphenolic-rich plants) (TaKaRa). The first-strand cDNA was synthesized with the Prime Script™ RT Reagent Kit with gDNA Eraser (TaKaRa).

Identification of candidate genes

Sequences for the following DNA methyltransferase and demethylase genes were obtained from the National Center for Biotechnology Information database: *Lycopersicon esculentum* (LOC101252835), *A. thaliana* (DQ335243.1), *Nicotiana tabacum* (LOC104089011), *Vitis vinifera* (LOC100247223), *Citrus sinensis* (LOC102630316), and *Dendrocnide urentissima* (XM_012234578.1). The sequences were aligned with the genes in the *C. indicum* genome (<http://www.icugi.org/chrysanthemum/>) (Tang et al., 2016). The conserved regions of the six gene sequences were identified, and subsequently compared using the DNAMAN program. The conserved sequences were analyzed and used to design gene-specific primers for PCR amplifications. The resulting amplicons were analyzed by agarose gel electrophoresis and sequenced. *DRM* and *CMT* were obtained as described above. The following *DRM2* genes were analyzed: *Cucumis sativus* (XM_004141052.2), *Cucumis melo* (LOC103487106), *Solanum lycopersicum* (LOC101255191), *A. thaliana* (AF240695.1), *V. vinifera* (LOC100254189), and *Elaeis guineensis* (EU117218.1). Additionally, the following *CMT3* genes were included in this study: *C. sativus* (LOC101218956), *S. lycopersicum* (LOC101267211), *Jatropha curcas* (LOC105650961), *A. thaliana* (NM_105645.4), *Nicotiana benthamiana* (JQ957858.1), and *E. guineensis* (EU117217.1).

Acquisition of partial sequences of DNA methyltransferase and demethylase genes

Target fragments were recovered using the MiniBEST Agarose Gel DNA Extraction Kit v. 4.0 (TaKaRa). Primer sequences are provided in Table 1 and the contents of the PCR solution are listed in Table 2. These DNA fragments of methyltransferase and demethyltransferase are highly homologous to other species.

Gene expression analysis

Quantitative real-time (qRT)-PCR was performed using cDNA as the template and the SYBR Premix Ex Taq kit (TaKaRa). The qRT-PCR primers were designed according to the conserved sequence of each gene. The primers specific for *CmMET1* were designed based on the conserved *CmMET1* sequence reported by Wang (Wang et al., 2015). The primers for *CmDRM2*, *CmCMT3*, and *CmDME* were designed according to the conserved sequences determined in this study. *ACTIN* and *SAND* were used as reference genes. The qRT-PCR primer sequences are provided in Table 3. Each PCR was repeated three times.

TABLE 1. Primer sequences.

Primer	Sequence (5'–3')
CMT-F	CCCCACATGAACATTCTCATTCCGA
CMT-R	GCCGAAGGATGAAATGGTGGAAGAT
DRM-F	TCCTGCGAGATTGTTACATGGGCTG
DRM-R	AGGGCAATTAAGACAACGGGGAGG
DME-F	GCAAGATGATGAAGTGTCAACAGCC
DME-R	ACTACCATCCTCAAAGACCAACCGA

TABLE 2. Components of the PCR solution.

Components	Volume (μL)
Reverse transcription product	1.0
Primer 1 (10 μmol L ⁻¹)	1.0
Primer 2 (10 μmol L ⁻¹)	1.0
Taq polymerase (5 U μL ⁻¹)	0.2
dNTPs (2.5 mmol L ⁻¹)	1.6
10°×PCR Buffer	2.0
ddH ₂ O	13.2
Total volume	20.0

TABLE 3. Sequences of quantitative real-time PCR primers.

Primer	Sequence (5'–3')
CmMET1-R	TGAGGCTGAAGAAATGGCTGCAAAG
CmMET1-F	GCAGGGAAGCAAGAGCTAAACGAAA
CmCMT3-R	CCCCACATGAACATTCTCATTCCGA
CmCMT3-F	GCCGAAGGATGAAATGGTGGAAGAT
CmDRM2-R	CTTCAGTTAGGAGACCCCTTCGT
CmDRM2-F	CCAAATGGCAAAGACCGAAG
CmDME-R	ATGATGTGTGTCCGTGCGCTG
CmDME-F	CAAGCTCGGGATCAAACCTCAG
Actin-R	TTACCGTAAAGGTCCTTCCTGA
Actin-F	AGCTTGCATATGTTGCTCTTGA
SAND-F	CGTTGCTCACTACGAGTTCACA
SAND-R	GCAGATGGGTCAACAGGTAAAT

FIGURE 1. Results of agarose gel electrophoresis of the PCR amplicons from *C. morifolium* 'Nannonghongxiu' hypermethylation-related genes. A: *CMT3*; B: *DRM2*; C: *DME*; M: Marker.

Target_fragment	GCAAGATGATGAAGTGTCAACAGCGGCACACATTCCACACTTTTCAGCCCCAAACCCCGGAAACTTAGCAAGTAA	75
Amplified_fragment	GCAAGATGATGAAGTGTCAACAGCGGCACACATTCCACACTTTTCAGCCCCAAACCCCGGAAACTTAGCAAGTAA	75
Consensus	gcaagatgatgaagtgtcaacagcggcacacattccacacttttcagccccaaaccccggaacttagcaagtaa	
Target_fragment	TCCTTTGCTTTGTCTTGGGGAGCATCTCTCAACCAATCGAGGTCAATCGCCCCATGATCTCTCACCAGGCGGTTA	150
Amplified_fragment	TCCTTTGCTTTGTCTTGGGGAGCATCTCTCAACCAATCGAGGTCAATCGCCCCATGATCTCTCACCAGGCGGTTA	150
Consensus	tcctttgctttgtcttggggagcatctctcaaccaatcgaggtcaatcgccccatgatctctcaccaggcggtta	
Target_fragment	AGGAAATCCTTCATTCTATCAGTAGCAGATTGTTTATCCACGTTCCCTTATAGCATCCGAAATTTTCATTGACA	225
Amplified_fragment	AGGAAATCCTTCATTCTATCAGTAGCAGATTGTTTATCCACGTTCCCTTATAGCATCCGAAATTTTCATTGACA	225
Consensus	aggaaatccttcattctatcagtagcagattgtttatccacgttcccttatagcattccgaaattttcattgaca	
Target_fragment	TGGGCACGTCCTCAAGGCTTCATAATCAAGTGAATCCGCTGCGCTTTTGCTTCTTCTTTCTTTTCACTATTAAGT	300
Amplified_fragment	TGGGCACGTCCTCAAGGCTTCATAATCAAGTGAATCCGCTGCGCTTTTGCTTCTTCTTTCTTTTCACTATTAAGT	300
Consensus	tgggcacgctcctcaaggcttcataatcaagtgaatccgctgcgcttttgcttcttcttttcttttcaactattaagt	
Target_fragment	AGTGCCCTCCTTTCTCAAGCTATCCCAAGTCAATGCTCTTCTCTCTTTCTCTCAGCTGTTCCTTTTTCACCTTT	375
Amplified_fragment	AGTGCCCTCCTTTCTCAAGCTATCCCAAGTCAATGCTCTTCTCTCTTTCTCTCAGCTGTTCCTTTTTCACCTTT	375
Consensus	agtgcctccttctcaagctatcccaagtcaatgctcttctctcttctctcctcagctgttctcttttttacccttt	
Target_fragment	GGCATATTCGGACCTGGCCCACTTGGTGGTTCAACGAGAGCCCTTTTGCTCCTTTGAGTTAGCTTCAGTGACTGT	450
Amplified_fragment	GGCATATTCGGACCTGGCCCACTTGGTGGTTCAACGAGAGCCCTTTTGCTCCTTTGAGTTAGCTTCAGTGACTGT	450
Consensus	ggcatattcggacctggcccaacttgggtggttcaacgagagcccttttgctcctttgagtttagcttcagtgactgt	
Target_fragment	TTCTCAAAACAAATGTGTTATTCAACCAAGAGTACTGTGATCAGATTGATTTGTATCCCTTCAAAACATAGCA	525
Amplified_fragment	TTCTCAAAACAAATGTGTTATTCAACCAAGAGTACTGTGATCAGATTGATTTGTATCCCTTCAAAACATAGCA	525
Consensus	ttctcaaaacaatgtgttattcaccacgaagtactgtgacacattgatgtgtatcccttcaaaacatagca	
Target_fragment	GAGTTTGGCTTCGTTTGGTAGATGATGTGTCCGTTGCCCTGCTCATCAAGTACCATGCTCGGTTGGTCTTTGAGG	600
Amplified_fragment	GAGTTTGGCTTCGTTTGGTAGATGATGTGTCCGTTGCCCTGCTCATCAAGTACCATGCTCGGTTGGTCTTTGAGG	600
Consensus	gagtttgccttcggttggtagatgatgtgtccggttgccctgctcatcaagtaccatgctcggttgggtctttgagg	
Target_fragment	ATGGTAG	607
Amplified_fragment	ATGGTAG	607
Consensus	atggtag	

FIGURE 2A. Alignment of partial *DME* (a), *CMT3* (b), and *DRM2* (c) nucleotide sequences from *C. morifolium* 'Nannonghongxiu' with related genes in the *C. indicum* genome.

Target fragment	CCGGAAGGATGAAATGTTGGAAATAGTGTGTTGGGAAGGATCTGTGTCGCCGAAGACGAACGAGGCGAAAC	75
Amplified_frame	CCGGAAGGATGAAATGTTGGGAAGTATGTTTGGGAGGAAATCTGTGTCGCCGAAGACGAACGAGGCGAAAC	75
Consensus	gcccgaagatgaatggttgggaagatagttgttttgggaggaactctgtgtgccggaagacgaagcgaggcgaaac	
Target fragment	TGGCCGCTATCGTTACTTGCCCAAGACGAAAGAGCTTTGGAGAAGCTAGGCACACTGAAACCTTCACGGGGGTGGAG	150
Amplified_frame	TGGCCGCTATCGTTACTTGCCCAAGACGAAAGAGCTTTGGAGAAGCTAGGCACACTGAAACCTTCACGGGGGTGGAG	150
Consensus	tgcccgctatcgttacttgcccaagacgaagagctttggagaagctaggacacttcaacggttgag	
Target fragment	TATATGCAACCTAAGAACATTATACCAAGCACTTATGACGACGCTGTGCACCTTAAGCTTGGTGATGATGGT	225
Amplified_frame	TATATGCAACCTAAGAACATTATACCAAGCACTTATGACGACGCTGTGCACCTTAAGCTTGGTGATGATGGT	225
Consensus	tatatgcaacctaaagacattatatacacaagcacttattgacgacgctgtgcaccttaagcttggatgatggt	
Target fragment	TATGTCAGGCTCTGAGAACGAGGATAGTTATATATGATGATGATTGAAATGTTCAAGGATTCGATTCGATA	300
Amplified_frame	TATGTCAGGCTCTGAGAACGAGGATAGTTATATATGATGATGATTGAAATGTTCAAGGATTCGATTCGATA	300
Consensus	tatgtcaggctctgagaaacgaggaatagttatataatgattgaaatgttcaaggatctgattcgata	
Target fragment	TATATTTTAGGGCTCAATGGTTTATCGGCCCAAGGACAAATATCGAAGCTTTTCAAACTCTCATGATAAT	375
Amplified_frame	TATATTTTAGGGCTCAATGGTTTATCGGCCCAAGGACAAATATCGAAGCTTTTCAAACTCTCATGATAAT	375
Consensus	tatatcttagggctcaatggtttatcggcccaaggacaaatattcgaagcttttcaaaactctcatgataat	
Target fragment	TATATTTTAGGGCTCAATGGTTTATCGGCCCAAGGACAAATATCGAAGCTTTTCAAACTCTCATGATAAT	450
Amplified_frame	TATATTTTAGGGCTCAATGGTTTATCGGCCCAAGGACAAATATCGAAGCTTTTCAAACTCTCATGATAAT	450
Consensus	tatatcttagggctcaatggtttatcggcccaaggacaaatattcgaagcttttcaaaactctcatgataat	
Target fragment	TGCGCTTTGAATGCTGAGGTTGCTGTAAACGAGAAAACTTGGAGCTGGTGACTATTATATATGGCTTAT	525
Amplified_frame	TGCGCTTTGAATGCTGAGGTTGCTGTAAACGAGAAAACTTGGAGCTGGTGACTATTATATATGGCTTAT	525
Consensus	tgccgctttgaatgctgaggttgcgtgtaaacgagaaaaacttggagctgggtgactattatataatggcttat	
Target fragment	TATGACCTTATTCGACATACCAAGCTTGGCCCGGATAACGAGGGGATGAGTAGCTTATTCATCAT	600
Amplified_frame	TATGACCTTATTCGACATACCAAGCTTGGCCCGGATAACGAGGGGATGAGTAGCTTATTCATCAT	600
Consensus	tatgaccttatttcgacataccaagcttggcccgataaacgaggggatgagtagacttattcatcat	
Target fragment	TCGTGCTACTGCTGGTAAATGAAGTCTCAAGTCAAGATAGCAAAAGCTTCAGTTGAAGTCTCGATCTC	675
Amplified_frame	TCGTGCTACTGCTGGTAAATGAAGTCTCAAGTCAAGATAGCAAAAGCTTCAGTTGAAGTCTCGATCTC	675
Consensus	tcgtgctactgctggttaaatgaagtctcaagtcaagatagcaaaaagcttcagttgaagtctcgatctc	
Target fragment	TATTCGGTGTGGAGCTATGCTACGGGGTATGCTTTGGTGCCAAATGCGAGATGTGAATCTTGTACGAGG	750
Amplified_frame	TATTCGGTGTGGAGCTATGCTACGGGGTATGCTTTGGTGCCAAATGCGAGATGTGAATCTTGTACGAGG	750
Consensus	tatttcggtgtggagctatgctacggggtatgctttggtgccaaatgcgagatgtgaatcttgtacgagg	
Target fragment	TGGGCTTAGACTTGAATGAATACGATTCGAGCTTACAACTCAACACCGTGAAGTGAAGGAATGA	825
Amplified_frame	TGGGCTTAGACTTGAATGAATACGATTCGAGCTTACAACTCAACACCGTGAAGTGAAGGAATGA	825
Consensus	tgggcttagacttgaatgaataacgatctcgagcttacaactcaacacccgtgaagtgaaggaaatga	
Target fragment	TACGACAGGATTTTTCAGGTTGTTGAGAGAGTGGCGGCTTTTTCAGAAATTTATGTAGCTGAGAGCGGT	900
Amplified_frame	TACGACAGGATTTTTCAGGTTGTTGAGAGAGTGGCGGCTTTTTCAGAAATTTATGTAGCTGAGAGCGGT	900
Consensus	tacgacaggatttttcagggttgttgagagagtggcggtcttttcagaaatttatgtagctgagagcggt	
Target fragment	GATTTCCGAAGAAATCAAGATGCTGAAATGCTGAAGAGATGATGAAGCTGACGATGGTGATCTGATCTGCA	975
Amplified_frame	GATTTCCGAAGAAATCAAGATGCTGAAATGCTGAAGAGATGATGAAGCTGACGATGGTGATCTGATCTGCA	975
Consensus	gattttccgaagaaatcaagatgctgaaatgctgaagagatgatgaagctgacgatggtgatctgatctgca	
Target fragment	CTGATATAGAGAGGATTTGAGGTTGAAAATTTATGAGTATCTGCTATGCTGATCCTCAAAAGACCAAGGAG	1050
Amplified_frame	CTGATATAGAGAGGATTTGAGGTTGAAAATTTATGAGTATCTGCTATGCTGATCCTCAAAAGACCAAGGAG	1050
Consensus	ctgatataagagaggtttgagggttgaatatgaggtatctgctatgctgattcctcaaaagaccaaggag	
Target fragment	TATGGGCTGATCAAGAGCTTCAATGGGTTTATGGTCTGAGAGACGACATTTGGGCTCATGATGATCATTTG	1125
Amplified_frame	TATGGGCTGATCAAGAGCTTCAATGGGTTTATGGTCTGAGAGACGACATTTGGGCTCATGATGATCATTTG	1125
Consensus	tatgggctgatcaagagcttcaatgggtttatgggtctgagagacgacatttgggctcatgatgatcatttg	
Target fragment	GGGTGATTGTAATTAAGATTAAGAGTTCGTGTTTATGGATCAAGCTTAAGCTTTTGGCTTTGGCTGAGAT	1200
Amplified_frame	GGGTGATTGTAATTAAGATTAAGAGTTCGTGTTTATGGATCAAGCTTAAGCTTTTGGCTTTGGCTGAGAT	1200
Consensus	gggtgattgtaatataagattaaagattcgtgtttatggatcaagcttaagcttttggctttggctgagat	
Target fragment	TGTGATGTCATTGTTGGGCCCCCATGTCAAGGATAAGCGGTTTCAACAGATTTAGGCAAAAGAAACCCG	1275
Amplified_frame	TGTGATGTCATTGTTGGGCCCCCATGTCAAGGATAAGCGGTTTCAACAGATTTAGGCAAAAGAAACCCG	1275
Consensus	tgtgatgtcatttgggtggcccccatgtcaaggataagcggtttcaacagatttaggcaaaagaaacccg	
Target fragment	TTAAAAGACGAAAGAAACAAACAGTAAATGCTACATGGACATCGTTGAATATTGAAGCCAGGTTTGCATAT	1350
Amplified_frame	TTAAAAGACGAAAGAAACAAACAGTAAATGCTACATGGACATCGTTGAATATTGAAGCCAGGTTTGCATAT	1350
Consensus	ttaaaagacgaaagaaacaaacagtaaatgctacatggacatcgttgaatatttgaagccaggtttgcata	
Target fragment	ATGAAATATGTTGATGACATTTGCAAGTTTGCAGAAAGCTTATTTGGTATGATCGCTTTGGGCGCTTATGTC	1425
Amplified_frame	ATGAAATATGTTGATGACATTTGCAAGTTTGCAGAAAGCTTATTTGGTATGATCGCTTTGGGCGCTTATGTC	1425
Consensus	atgaaatatgttgatgacatttgcaagtttgcagaaagcttatttgggtatgatcgctttgggcgcttattgtc	
Target fragment	AATATATATCAAGCCCGTGTGTTTAAATGTTGCGGGGCTCTATGGCTTCCACAGTTCGATGAGAAATGTC	1500
Amplified_frame	AATATATATCAAGCCCGTGTGTTTAAATGTTGCGGGGCTCTATGGCTTCCACAGTTCGATGAGAAATGTC	1500
Consensus	aatatatatcaagcccggtgtgttttaattggttgcggggctctatggcttccacagtttcgatgagaaatgtc	
Target fragment	CATGTGGGG	1509
Amplified_frame	CATGTGGGG	1509
Consensus	catgtgggg	

FIGURE 2B.

Target fragment	TCCTCGGAGATGTTACATGGGCTGCACCAACATCAATCAAGCCACCAACATACATCATATCGCTCAAG	75
Amplified_frame	TCCTCGGAGATGTTACATGGGCTGCACCAACATCAATCAAGCCACCAACATACATCATATCGCTCAAG	75
Consensus	tccctcgagatggttactgggctgcaccaaactcaatcaagccaccaacatacatcatcgctcaag	
Target fragment	TCCTCGGCTGCTTACCTCACTGACATCATCTAGATGAATAAGTTGCTTTTGTGTTGTTTGTCCCAACCA	150
Amplified_frame	TCCTCGGCTGCTTACCTCACTGACATCATCTAGATGAATAAGTTGCTTTTGTGTTGTTTGTCCCAACCA	150
Consensus	tccctcggtcttactcctcactgacatcatctagatgaataagttgcttttgtgtgttgttgtcccaacca	
Target fragment	CTGACAAATGTCAGGTTGCTTACAGAGATCTCAACGATACGATATTTTAAAGGATACCAATCTGATGAA	224
Amplified_frame	CTGACAAATGTCAGGTTGCTTACAGAGATCTCAACGATACGATATTTTAAAGGATACCAATCTGATGAA	225
Consensus	ctgacaaatgtcagggttgcgtttagagatctcaacgatagcatatctttaaaggataccaatctgatgaa	
Target fragment	GAGCTACTTCAAGCACCACCAATCCCGAGAGAGCGCAACATGTTTACAGCTTTGGGATAGATCTTTTACG	299
Amplified_frame	GAGCTACTTCAAGCACCACCAATCCCGAGAGAGCGCAACATGTTTACAGCTTTGGGATAGATCTTTTACG	300
Consensus	gagctacttcaagcaccacccaatcccgagagagcgcaacatgtttacagctttgggatagatcttttacg	
Target fragment	AGAGAGATGATGATCAACGATATCTACCTGAAACGAATGCCAAGTATTATATCTCTGCTCTACTTATG	374
Amplified_frame	AGAGAGATGATGATCAACGATATCTACCTGAAACGAATGCCAAGTATTATATCTCTGCTCTACTTATG	375
Consensus	agagagatgatgatcaacgatattctaccctgaaacgaattgccaaagtatttatctctctgctctacttatg	
Target fragment	CTCTCTCGGCTGATTTCTCGGAAATCCCAACACATTTCACTTCACCGGCTTAAATGCTCCAGTTTGT	449
Amplified_frame	CTCTCTCGGCTGATTTCTCGGAAATCCCAACACATTTCACTTCACCGGCTTAAATGCTCCAGTTTGT	450
Consensus	ctctctcgggtgatgttctcggaatcccaacacatttcaacttcacggtttaaattgtgccaattgt	
Target fragment	TCCTTCCTACACATCAATTCCTTTCGAGCTCATCAACCAATCTTTCGATTCGATTCGATTCGATTCGAT	524
Amplified_frame	TCCTTCCTACACATCAATTCCTTTCGAGCTCATCAACCAATCTTTCGATTCGATTCGATTCGATTCGAT	525
Consensus	tcttctctacacatcaatcttcttctgagctcatcaacacatttctgcatgaaagtgtgtgatcat	
Target fragment	CAACCCATTTTCAGAGCTTTTAAATCTATCACTCACTGACCTCCCAACAGTTTTCAGCAATTC	599
Amplified_frame	CAACCCATTTTCAGAGCTTTTAAATCTATCACTCACTGACCTCCCAACAGTTTTCAGCAATTC	600
Consensus	caacccatttctagagctttttaaacttatctcatctagctcaactagacctcccaaacagttttagcaatctc	
Target fragment	CGTTAGTCTTTTATCCCAATCCGACCACTGTTTGTATGTTAAAGCTTCTTATAGCTGGGAGGTT	674
Amplified_frame	CGTTAGTCTTTTATCCCAATCCGACCACTGTTTGTATGTTAAAGCTTCTTATAGCTGGGAGGTT	675
Consensus	cgttagtcttttatacccaatccgacacctgttgttattggttaagcttcttataagctggggaggt	
Target fragment	GAGGAAGAGAGGAGATCTTGTATGATGAGAGATATGATGATGATCTTTCACCGGACGACAGCAAGT	749
Amplified_frame	GAGGAAGAGAGGAGATCTTGTATGATGAGAGATATGATGATGATCTTTCACCGGACGACAGCAAGT	750
Consensus	gaggaaagagaggagatctgttatgagattggaagatgatgatgatctctttagcggcgagcagcagagat	
Target fragment	ACTTGAATGACAAACTCTGTTTACCTATCACTAAAGAAAGGAGAAATGATCCCAACACCTTAAAGATCA	824
Amplified_frame	ACTTGAATGACAAACTCTGTTTACCTATCACTAAAGAAAGGAGAAATGATCCCAACACCTTAAAGATCA	825
Consensus	acttgaatgacaaactctgtttacactatcaactaaagaaaggaatgattcccaaacacctttagagatca	
Target fragment	AGGCACAATCTTCTAGTAAAGAAATGAGGGGCAATAGCAGCTTACGAGGATTTTACGACTTAAACCATAG	899
Amplified_frame	AGGCACAATCTTCTAGTAAAGAAATGAGGGGCAATAGCAGCTTACGAGGATTTTACGACTTAAACCATAG	900
Consensus	aggcacaatcttctagtaaaagaaatgaggggcaatagcagctttagggagtttctacagagtataaccatag	
Target fragment	GATCAGTAGGGGCTTCAACCCCAATCTTGGCTTGGAGAGCAATCATCTCTTCACTTACGAGGACCT	974
Amplified_frame	GATCAGTAGGGGCTTCAACCCCAATCTTGGCTTGGAGAGCAATCATCTCTTCACTTACGAGGACCT	975
Consensus	gattcagtaggggttcaaccccaatcttggcttggagagcaatcatctcttcaactttaggagacct	
Target fragment	TTCTTTCTTTTTCCTCAATCTTACCTCATCACTCTTCTTCTTAAATGACCCCTTTAGAGAGATATGT	1049
Amplified_frame	TTCTTTCTTTTTCCTCAATCTTACCTCATCACTCTTCTTCTTAAATGACCCCTTTAGAGAGATATGT	1050
Consensus	tcttcttcttcttctcaaatcttaccctcatcaactcttctttaaagctccctttagagagatatgt	
Target fragment	TGGGTTTCTTTCTTCAAAAGATCACTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1124
Amplified_frame	TGGGTTTCTTTCTTCAAAAGATCACTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1125
Consensus	tgggtttcttcttctcaaaagatcacttcttcttcttcttcttcttcttcttcttcttcttcttcttcttct	
Target fragment	TATTCATGCGCGCGGACACATCTGCTATGCTGCGAGAGCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1199
Amplified_frame	TATTCATGCGCGCGGACACATCTGCTATGCTGCGAGAGCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1200
Consensus	tattcatgccccgacacactgtctctatggtcgagaagctctctctatatatacccattatagccaag	
Target fragment	ATAAATAGCTTCTGATCTAGTAAAGAAATGACATCACTGACCTGATCATCTGACCAACTACAGAG	1274
Amplified_frame	ATAAATAGCTTCTGATCTAGTAAAGAAATGACATCACTGACCTGATCATCTGACCAACTACAGAG	1275
Consensus	ataaataagcttctgattctagtaagaaatgacatcactgacctgatcatctgacccaactacagag	
Target fragment	AATCTCGGACACAGAGATCTTGAAGGCTTCTCATCTCTGCGAGACAAATTAACATCATCATCTA	1349
Amplified_frame	AATCTCGGACACAGAGATCTTGAAGGCTTCTCATCTCTGCGAGACAAATTAACATCATCATCTA	1350
Consensus	aactctggacacagagatcttgaaggcttctcatctctgcgagacaaattaaactatcatcatcta	
Target fragment	CACATTTTCTTCTGATTACATGATGATTTCATCAGGAGCTTCTTCAAGATGCGGTATGATGATGATG	1424
Amplified_frame	CACATTTTCTTCTGATTACATGATGATTTCATCAGGAGCTTCTTCAAGATGCGGTATGATGATGATG	1425
Consensus	cacatttcttctgattacatgatttcatcaggagcttcttcaagatgcggtatgattgattgattgattg	
Target fragment	TGAGTATGCGCTTCTTATTTGCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1469
Amplified_frame	TGAGTATGCGCTTCTTATTTGCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1470
Consensus	tgagtatgctgttcttatttggcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttct	

FIGURE 2C.

Data analysis

The data were analyzed using the SPSS 19 program (IBM Corporation, Armonk, NY, USA). A P-value <0.05 was used as the threshold for determining significance. Error bars in graphs were generated using the standard error of the mean values.

Results

Cloning of partial DNA methyltransferase and demethylase gene sequences and nucleotide sequence alignment

The agarose gel electrophoresis results for the PCR-amplified DNA methyltransferase and demethylase genes are presented in Figure 1. The *CmCMT* and *CmDRM* gene fragments were approximately 1,500 bp long, while the *CmDME* fragment was about 600 bp long. The corresponding fragments were recovered, sequenced and compared with sequences in the *C. indicum* genome (<http://www.icugi.org/chrysanthemum/>) (Figure 2). Alignments of these three genes with the original sequences all showed more than 99% sequence conservation.

FIGURE 3. Relative *CmFT* expression levels during various growth stages of *C. morifolium* 'Nannonghongxiu' exposed to short-day conditions ($p=0.01^{**}$). VS: Vegetative Stage; BS: Budding Stage; EFS: Early Flowering Stage; FBS: Full Bloom Stage.

Analysis of FT expression during flower induction and development of *C. morifolium*

As a marker of flowering, *FT* gene plays an important role in flowering induction. The expression of *FT* gene was induced by photoperiod treatment. In the chrysanthemum treatment conducted by us, *CmFT* expression was very low during the vegetative stage, and increased gradually after entering the budding stage and reached the highest level at the full bloom stage. The group treated with SD was higher than the control group in budding stage and early flowering stage, while the group treated with SD was lower than the control group in vegetative stage and full bloom stage (Figure 3).

Analysis of DNA methyltransferase expression during flower induction and development of *C. morifolium*

The results of the methyltransferase analyses during transition from vegetative to reproductive growth of *C. morifolium* are provided in Figures 4–6. The *CmMET1* and *CmCMT3* expression level was decreased gradually during the flowering process and was significantly lower at the budding stage where the capitulum bud arises in the vegetative growth stage. Additionally, the expression levels were lower in plants treated with SD conditions compared to control

FIGURE 4. Relative *CmMET1* expression levels during various growth stages of *C. morifolium* 'Nannonghongxiu' exposed to short-day conditions ($p=0.01^{**}$). VS: Vegetative Stage; BS: Budding Stage; EF: Early Flowering Stage; FBS: Full Bloom Stage.

FIGURE 5. Relative *CmCMT3* expression levels during various growth stages of *C. morifolium* 'Nannonghongxiu' exposed to short-day conditions ($p=0.01^{**}$). VS: Vegetative Stage; BS: Budding Stage; EFS: Early Flowering Stage; FBS: Full Bloom Stage.

FIGURE 6. Relative *CmDRM2* expression levels during various growth stages of *C. morifolium* 'Nannonghongxiu' exposed to short-day conditions ($p=0.01^{**}$). VS: Vegetative Stage; BS: Budding Stage; EFS: Early Flowering Stage; FBS: Full Bloom Stage.

plants at the same stage. The relative *CmMET1* expression level was lowest in the budding stage, while *CmCMT3* expression level was observed the lowest during the full bloom stage. *CmDRM2* expression exhibited an increasing trend during the flowering process of *C. morifolium* while the level was relatively stable in the control plants from vegetative stage to bud stage. In contrast, *CmDRM2* expression in the SD-treated plants was up-regulated initially in the budding stage, then reached its peak during the early flowering stage. Thus, the *CmDRM2* expression level under SD conditions was higher than control conditions from the bud stage to the early flowering stage. However, we observed that the *CmDRM2* expression level in the control plants was highest at the full bloom stage with higher intensity compared to SD-treated plants. Three DNA methyltransferase genes have different pattern of expression as *CmMET1* and *CmCMT3* expressed similarly while *CmDRM2* expression is exactly the opposite.

Analysis of demethylase gene expression during flower induction and development of *C. morifolium*

DME is an important demethylase gene, the *CmDME* expression level undergoes through dynamic changes during flower development in the control and SD-treated plants. The changes in *CmDME* expression of *C. morifolium* plants induced by the SD treatment are presented in Figure 7. We observed that the *CmDME* expression level was lower at the budding stage compared to vegetative growth or early flowering stages. In addition, there was no obvious difference in *CmDME* expression between the SD-treated and control plants. *CmDME* expression was at peak during the full bloom stage while control plants showed higher level in contrast with SD-treated plants (Figure 7).

In addition, it appears that artificial short-day exposure reduces the expression of most DNA methylase-related genes (*CmMET1*, *CmCMT3*, *CmDME*), which may be one of the reasons for the early flowering induced by artificial short-day exposure.

Discussion

DNA methyltransferase gene plays an important role in the flowering process

A previous study revealed that the inhibition of DNA methylation promotes flowering of *C. morifolium* (Li et al., 2016). According to the results of an earlier study, the extent of DNA methylation in plants decreases under SD conditions of *C. morifolium* (Li et al., 2016). However, the underlying mechanism regulating this decrease had not been characterized. The results of this study suggest that the expression of DNA methyltransferase genes (i.e., *CmMET1* and *CmCMT3*) are down-regulated during the flowering process of *C. morifolium*. This is in contrast to the expression of *CmDRM2*, which exhibits a slightly increasing trend. Additionally, the changes in gene expression were larger in the SD-treated plants than in control plants. We also observed that the expression of *CmDME* was relatively stable during the flowering process of *C. morifolium*. These results are consistent with the fact that the overall expression levels of DNA methyltransferase genes decrease during flowering of *C. morifolium*. According to another study, *DRM2* mainly regulates asymmetric CHH methylation, while CG and CHG methylations are mainly catalyzed by *MET1* and *CMT3* (Rival et al., 2008). In flowering plants, the variability in DNA methyltransferase gene expression level is likely related to the various types of DNA methylation catalyzed by different enzymes.

FIGURE 7. Relative *CmDME* expression levels during various growth stages of *C. morifolium* 'Nannonghongxiu' exposed to short-day conditions ($p=0.01^{**}$). VS: Vegetative Stage; BS: Budding Stage; EFS: Early Flowering Stage; FBS: Full Bloom Stage.

We recently confirmed that decrease in the extent of DNA methylation can promote the expression of *CmFT*, thereby affecting the flowering of *C. morifolium* plants exposed to SD conditions (Li et al., 2019). Decreases in DNA methylation rates are mainly caused by down-regulation of DNA methyltransferase gene expression; besides, the expression levels of DNA methyltransferase genes are reportedly correlated with the expression of the cyclin gene. However, Okello indicated that light conditions considerably influence plant cell division, replication, and growth (Okello et al., 2016). We hypothesize the expression of DNA methyltransferase and demethylase genes change during the growth and development, which ultimately increases or decreases the extent of DNA methylation (Sun et al., 2018; Yan et al., 2016).

DNA methylation related genes, both DNA methylase and demethylase genes, showed dynamic changes during flowering induction. We can confirm that DNA methylation plays an important role in the process of flowering induction. However, it is not clear which genes in the flowering induction regulation network are regulated by DNA methylation; further studies are needed.

The reduced DNA methylation level in flowering-promoted plants maybe be mainly from CG and CHG sequences

Among four genes examined in this study, the two DNA methyltransferases, *CmMET1* and *CmCMT3*, showed significant changes when plants went through the transition from vegetative stage to reproductive stage (bud appearance stage). When we assume that mRNA expression level of these genes are correlated with active protein levels. Decreases in DNA methylation rates are mainly caused by the down-regulation of DNA methyltransferase gene expression (Li et al., 2019). This study seems to indicate that the activities of *CmMET1* and *CmCMT3* determine DNA methylation levels of genes that are associated with flowering time regulation unlike *CmDRM2* and *CmCME*. The reduced DNA methylation level in flowering plants promoted may be mainly from CG and CHG sequences. Of course, this needs to be demonstrated by DNA methylation detection in the genome in the future.

Conclusions

The decrease of DNA methylation in *Chrysanthemum* flowers during SD treatment was realized by the combination of up-regulation and down-regulation of different DNA methylase.

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Authors' contributions

ML and YL: wrote the first draft; DK: edited and reviewed the manuscript before submission. JL: performed the experiments; ZL, PA, YW, HD and YZ: analyzed the data. ZW: guided the whole experiment, reviewed and edited the manuscript before submission.

Conflict of interest

The authors have no conflicts of interest to declare.

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Address of authors:

Kang Dongru^a, Li Zhongai^a, Li Mangmang^a, Li Jie, Liu Yanhua, Ai Penghui, Ding Hongxu, Zhu Yi, Wang Ying and Wang Zicheng*

Plant Germplasm Resources and Genetic Laboratory, Kaifeng Key Laboratory of Chrysanthemum Biology, School of Life Sciences, Henan University, Jinming Road, Kaifeng, Henan, 475004, China

* Corresponding author; E-mail: wzc@henu.edu.cn

Tel.: +86 13 849126494

^a These authors contributed equally to this work.