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Research article

DNA demethylation during *Chrysanthemum* floral transition following short-day treatment

Zhongai Li [1], Jie Li [1], Yanhua Liu, Zicheng Wang *

Plant Germplasm Resources and Genetic Laboratory, College of Life Science, Henan University, Jinming Road, Kaifeng, Henan 475004, China

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ABSTRACT

Background: Analytical techniques such as methylation-sensitive amplification polymorphism and high-performance liquid chromatography were used to detect variation in DNA methylation of mature *Chrysanthemum* leaves during the floral transition induced by short-day (SD) treatment.
Results: For both early- and late-flowering cultivars, the time from the date of planting to the appearance of the capitulum bud and early blooming were significantly shorter than those of the control. The capitulum development of the early-flowering cultivar was significantly accelerated compared to the control, unlike the late-flowering cultivar. The DNA methylation percentage of leaves was significantly altered during flower development. For the early-flowering cultivar, DNA methylation was 42.2–51.3% before the capitulum bud appeared and 30.5–44.5% after. The respective DNA methylation percentages for the late-flowering cultivar were 43.5–56% and 37.2–44.9%.
Conclusions: The DNA methylation percentage of *Chrysanthemum* leaves decreased significantly during floral development. The decline in DNA methylation was elevated in the early-flowering cultivar compared with the late-flowering cultivar.

1. Introduction

DNA methylation is a common epigenetic phenomenon involving the transfer of a methyl group from S-adenosylmethionine (SAM) to a specific location of the adenine purine ring or cytosine pyrimidine ring of DNA, which is catalyzed by methyltransferase [1]. One of the most important mechanisms is the methylation of the C-5 carbon of cytosine in genomic DNA producing 5-methylcytosine. As an important epigenetic modification, the functional loss of DNA methylation can have an adverse effect on plant growth [2,3], because of the key role of DNA methylation in the growth and development of plants (*e.g.*, influencing flowering time, the complement of embryonic cells, the optical signal, and genetic polymorphisms) [4,5,6,7], genome maintenance, somaclonal variation, foreign gene defense, and endogenous gene expression [8,9,10,11,12].

The transition from vegetative growth to reproductive growth is an important event in plant development. The regulatory pathways include the vernalization, photoperiod, autonomous, and gibberellin pathways. The photoperiod is an important inductive factor influencing flowering time [13]. Many plants exhibit photoperiodism and respond to change in the length of light and dark periods. Depending on their response to night length, plants can be divided into long-day (LD) plants, short-day (SD) plants, and intermediate-day (ID) plants [14]. Mature leaves sense the change in day length and produce a substance that stimulates flowering, ultimately initiating flower bud differentiation and regulating flowering after long-distance transport from the leaves to the shoot tips [15]. Thus, the gene expression of mature leaves is crucial for flowering. The Flowering Locus T (FT) protein is an important component of "florigen," which was first identified in *Arabidopsis* [16,17]. This protein integrates the signals of different developmental pathways, including the photoperiod, vernalization, and autonomous pathways. The signals of these pathways are key to floral development [18].

In recent years, the relationship between DNA methylation and regulation of flowering and that between DNA methylation and photoperiod have been elucidated. Flowering processes induced by photoperiodic changes have been shown to be accompanied by changes in DNA methylation in sample plants such as purple perilla (*Perilla frutescens*) and *Silene armeria* [6]. The DNA methylation rate of individual flowering plants is considerably lower than that of nonflowering plants within the same cluster of *Bambusa multiplex* canes [19]. The use of zebularine, a DNA methylation inhibitor, initiated flowering in the SD plant *Petunia hybrida* without any inductive SD treatment. This indicated that changes in DNA

* Corresponding author.
E-mail address: wzc@henu.edu.cn (Z. Wang).
[1] These authors contributed equally.
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methylation are closely related to the expression of flowering genes during the photoperiod-induced flowering process [20]. The use of 5-azacytidine initiates flowering in the SD plant *Chrysanthemum* 9–16 d before the control [21]. As mature leaves play a key role in flowering, the DNA methylation changes in leaves during flowering *via* SD must be investigated.

Chrysanthemum (*Chrysanthemum* × *morifolium*), one of the most economically important ornamental flowers and a typical SD plant, is often induced to bloom by SD treatment for commercial purposes. In this study, methylation-sensitive amplification polymorphism (MSAP) and high-performance liquid chromatography (HPLC) were used to detect the variation in DNA methylation of mature leaves during SD-induced flowering using two *Chrysanthemum* cultivars with different flowering time as the study materials.

2. Materials and methods

2.1. Plant material and treatment

The early-flowering *Chrysanthemum* cultivar "He Hua Xian Zi" and the late-flowering cultivar "Qiu Shui Chang Liu" were used in this study. The plants were cultivated at the Chrysanthemum Institute of Kaifeng City from 2014 to 2015. Cuttings were taken on 18 May 2014, and the rooted cuttings were planted on 7 July. The plants were grown individually in pots in a medium composed of refuse soil–grass carbon–chicken manure (3:1:1). The plants were watered daily with tap water and given an inorganic nutrient solution once weekly.

SD treatment, consisting of 7-h light and 17-h dark (from 17:00 to 10:00 the following day) periods, was applied from 1 August to 13 September. At the time of treatment, the plants were about 10 cm tall. Plants growing under natural conditions (light time of 13–14 h/d) were chosen as the control group, and 20 pots were used per treatment. The natural day length gradually shortened over the course of the experiment, but it was longer than the treatment duration throughout the experiment. The control group was grown under natural (ambient) climatic conditions. The SD treatment was applied in a shading shed.

2.2. Definition of stages of flowering

The stages of the flowering process were defined as follows: 1. The date of capitulum bud appearance, on which the capitulum bud was first visible to the naked eye; 2. The time for the capitulum bud to appear, that is, the number of days from planting to the date of capitulum bud appearance; 3. The date of early blooming, on which the first whorl of ray florets was first visible; 4. The time to early blooming, that is, the number of days from planting to the date of early blooming; and 5. Capitulum development time, that is, the number of days from capitulum bud appearance to early blooming.

2.3. Analysis of DNA methylation level

Genomic DNA was extracted from mature leaf samples collected every 7 d at 12:00 from 1 August to 18 October. Healthy leaves were chosen for DNA extraction, according to the method proposed by Wang et al. [22].

The DNA methylation level was determined using MSAP and HPLC. MSAP was performed as proposed by Xiong et al. [23] with some modifications. The DNA samples were digested sequentially with EcoRI + MspI and EcoRI + HpaII. The digestion reaction was performed in a volume of 20 μL comprising 300 ng of the DNA template and 10 U of the restriction enzyme. This mixture was then incubated at 37°C for 7 h. The ligation reaction was performed in a volume of 30 μL consisting of 20 μL of enzyme digestion products, 2 U of T4 ligase, 5 pmoL of the EcoRI adapter, and 50 pmol of the HapII/MspI adapter. This mixture was incubated overnight at 16°C. The diluted digestion–ligation (1:10) mixture was amplified using HapII/MspI and EcoRI pre-selective primers with the following protocol: 94°C for 2 min, 26 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. The pre-selective polymerase chain reaction (PCR) products were diluted tenfold and were then amplified using HpaII/MspI and EcoRI selective primers (Table 1). The primers (Table 1) were synthesized by SANGON (Shanghai, China). The selective PCR cycling parameters were as follows: 94°C for 5 min, 94°C for 30 s, 67.5°C for 1 min, and 72°C for 1 min, a decrease in annealing temperature by 0.7°C per cycle for 13 cycles and then 23 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min with a final extension of 10 min at 72°C.

Each PCR reaction was replicated once. Further, two aliquots of each reaction were electrophoresed independently by denaturing polyacrylamide gels (6% (v/v)) for 2 h at 65 W. After silver staining, reproducible and clear bands were scored.

For HPLC digestion of DNA, the method described by Johnston et al. [24] was followed. Each 10-μL (2–5-μg) DNA sample was incubated in an ice bath for 2 min and then immediately placed in a boiling water bath for 5 min. Nuclease P1 (3 μL; 10 U/μL), 4 μL of $ZnSO_4$ (5 mmol/l), and 3 μL of ultrapure water were added. This mixture was then incubated overnight at 37°C. Then, 0.75 μL of alkaline phosphatase and 1.25 μL of Tris–HCl (0.5 moL/l, pH 8.3) were added, and the solution was incubated at 37°C for 2 h. After centrifugation at 1205 ×*g* for 3 min at room temperature, the supernatant was transferred to another centrifugal tube and then filtered through a 0.45-μm microporous membrane. Then it was subject to analysis with a Waters 1515 HPLC Pump (Waters Co., Milford, WA, USA).

The chromatographic conditions used were as follows: a velocity of 0.5 ml/min; a pH of 3.88; a column temperature of 30°C; an ultraviolet (UV) detector; a sample quantity of 10 μL; a wavelength of 280 nm; a mobile phase with a tendency for 7.0 moL/l of heptyl alkyl sulfonate (PIC-B7), 0.2% triethylamine (airport), and 10% methanol; and a C18 column (150 mm × 4.6 mm, 5 μM).

Table 1
Sequences of adapters and primers used in the MSAP analysis.

Primer	Sequence (5′–3′)
Adapter	
E-ad 1	CTC GTA GAC TGC GTA CC
E-ad 2	AAT TGG TAC GCA GTC TAC
HM-ad 1	GAT CAT GAG TCC TGC T
HM-ad 2	CGA GCA GGA CTC ATG A
Pre-selective amplification	
E-00	GAC TGC GTA CCA ATT CA
HM-00	ATC ATG AGT CCT GCT CGG
Selective amplification	
E01	GAC TGC GTA CCA ATT CAA
E02	GAC TGC GTA CCA ATT CAT
E03	GAC TGC GTA CCA ATT CAC
E04	GAC TGC GTA CCA ATT CAG
E05	GAC TGC GTA CCA ATT CTA
E06	GAC TGC GTA CCA ATT CTT
E07	GAC TGC GTA CCA ATT CTC
E08	GAC TGC GTA CCA ATT CTG
E09	GAC TGC GTA CCA ATT CCA
E10	GAC TGC GTA CCA ATT CCT
E11	GAC TGC GTA CCA ATT CCC
E12	GAC TGC GTA CCA ATT CCG
E13	GAC TGC GTA CCA ATT CGA
E14	GAC TGC GTA CCA ATT CGT
E15	GAC TGC GTA CCA ATT CGC
E16	GAC TGC GTA CCAATT CGG
HM1	ATC ATG AGT CCT GCT CGG TCC
HM2	ATC ATG AGT CCT GCT CGG TCA
HM3	ATC ATG AGT CCT GCT CGG TC
HM4	ATC ATG AGT CCT GCT CGG TT
HM5	ATC ATG AGT CCT GCT CGG TA
HM6	ATC ATG AGT CCT GCT CGG TG

2.4. Data analysis

The data were analyzed using SPSS 19 software (IBM Corporation, Armonk, NY, USA) (Table 1).

3. Results and analysis

3.1. Effect of short days on plant height

After SD treatment for 28 and 43 d, the plants heights of the early-flowering cultivar "He Hua Xian Zi" were significantly shorter than those of the control group. For the late-flowering cultivar "Qiu Shui Chang Liu," no significant difference in plant height was noted compared with the control group after SD treatment for 28 d, although significantly shorter plant heights were noted in the SD-treated group for 43 d than in the control (Fig. 1).

3.2. Effect of SD on flowering

After SD treatment, the timings of capitulum bud appearance and early blooming of the early-flowering cultivar "He Hua Xian Zi" in the control group and SD-treated group were 60 and 88 d, and 48 and 67 d after planting, respectively. The corresponding timings for the late-flowering cultivar "Qiu Shui Chang Liu" in the control and SD-treated groups were 77 and 105 d, and 59 and 86 d after planting, respectively. Therefore, after SD treatment, the timing of pre-blooming and early blooming of the two cultivars was significantly advanced by 11 and 12 d, and 18 and 19 d, respectively (Fig. 2). The timings of capitulum bud appearance and early blooming of the SD-treated group were advanced in the early-flowering cultivar compared to the late-flowering cultivar. The period of capitulum bud development was significantly shortened by 9 d in the SD-treated early-flowering cultivar, whereas this period was not significantly affected by SD treatment in the late-flowering cultivar. The timing of capitulum bud development in the control group did not differ between cultivars. However, the period of capitulum bud development for the early-flowering cultivar was 8 d shorter than that for the late-flowering cultivar.

3.3. DNA methylation changes during flowering under SD treatment

Six pairs of MSAP primers were used to detect the variation in DNA methylation during the flowering period. The banding patterns can be divided into four classes: type I bands were present in both profiles, type II bands were present in EcoRI/MspI profiles alone, type III bands were present in EcoR I/HpaII profiles only, and type IV bands were absent in both profiles (Fig. 3).

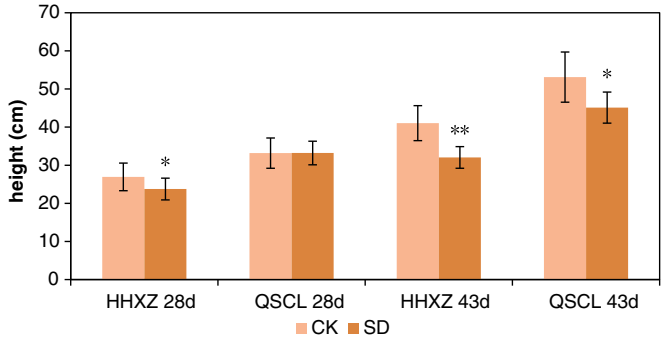

Fig. 1. Effect of short-day (SD) treatment of 28 or 43 d on plant height of the *Chrysanthemum* cultivars "He Hua Xian Zi" (HHXZ) and "Qiu Shui Chang Liu" (QSCL). Notes: Values followed by "*" differ significantly with a p-value of 0.05 and "**" differ significantly with a p-value of 0.01; CK: the control group.

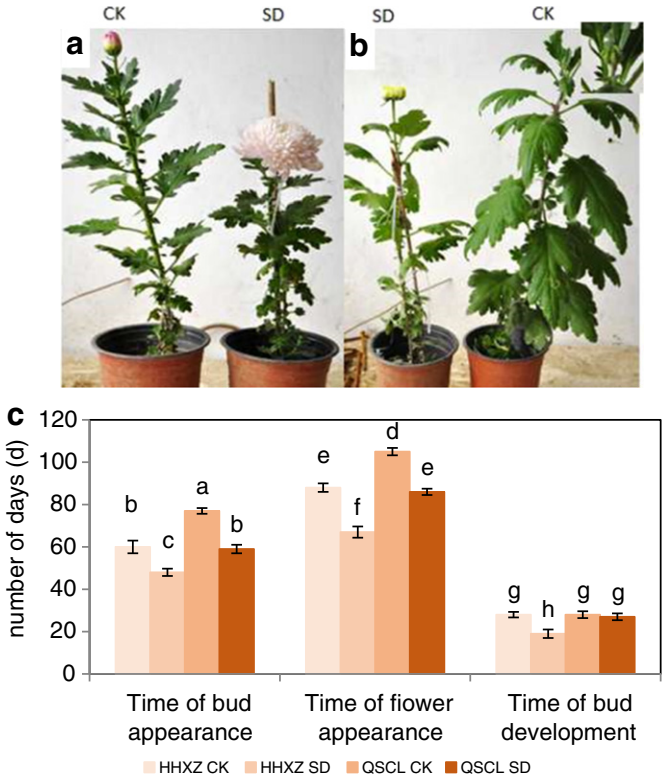

Fig. 2. Effect of short-day (SD) treatment on floral development of *Chrysanthemum*. a: The early-flowering cultivar "He Hua Xian Zi" (HHXZ); b: The late-flowering cultivar "Qiu Shui Chang Liu" (QSCL); c: The time of flowering. Different letters in series are significant at the 5% level.

Reduced DNA methylation was noted in both cultivars during the floral transition process (Fig. 4, Fig. 5) as measured by MSAP. This finding was consistent with the results detected by HPLC analysis (Fig. 6, Fig. 7). The total DNA methylation percentage of the two cultivars as detected by HPLC was slightly higher than that detected by MSAP. With the gradual shortening of the natural day length

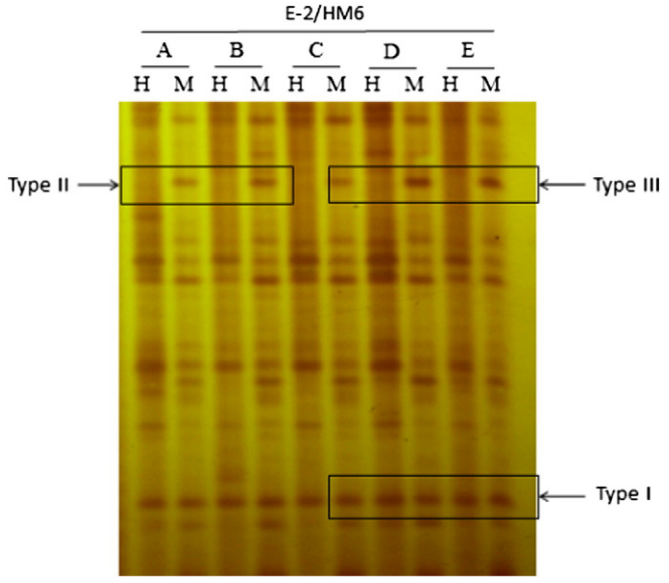

Fig. 3. Part of electrophoretic profiles of MSAP of *Chrysanthemum*. Notes: E-CTG denotes different EcoRI primers; HM-TCA denotes different HpaII/MspI primers; 21, 23, and 25 represent groups of CK; 22 and 26 represent groups of short day; H denotes fragments obtained from digestion by EcoRI/HpaII; and M denotes fragments obtained from digestion by EcoRI/Msp.

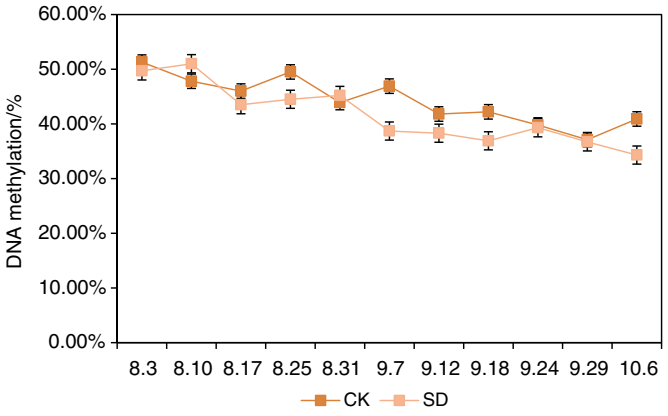

Fig. 4. DNA methylation during floral bud development of the early-flowering *Chrysanthemum* cultivar "He Hua Xian Zi" as measured by MSAP.

during the experiment, the DNA methylation levels of the control group also showed a gradual decline. Over the entire floral transition period, the DNA methylation percentage of the SD-treated group was lower than that of the control group. The range of variation in DNA methylation of the early-flowering cultivar was larger than that of the late-flowering cultivar. For the early-flowering cultivar, the DNA methylation rates were 42.2–51.3% before the capitulum bud appeared and 30.5–44.5% after. For the late-flowering cultivar, the corresponding DNA methylation rates were 43.5–56% and 37.2–44.9%.

3.4. DNA methylation changes at the early flowering stage following SD treatment

The DNA methylation percentage of the two cultivars after SD treatment was significantly lower than that of the control (Table 2). The six primer combinations used generated 149 type I, 72 type II, and 58 type III fragments in the control early-flowering cultivar, and 168, 72, and 58 in the SD-treated early-flowering cultivar, respectively. The equivalent fragments in the control late-flowering cultivar were 145, 74, and 53, and 158, 64, and 48 in the SD-treated late-flowering cultivar, respectively. The mean number of fragments produced by each pair of primers in the control group was similar to that reported by Wang [25]. The DNA methylation rate of the SD-treated early-flowering cultivar decreased by 17.48% compared with the control at the early flowering stage. The DNA methylation rate of the SD-treated late-flowering cultivar decreased by 11.32% compared with

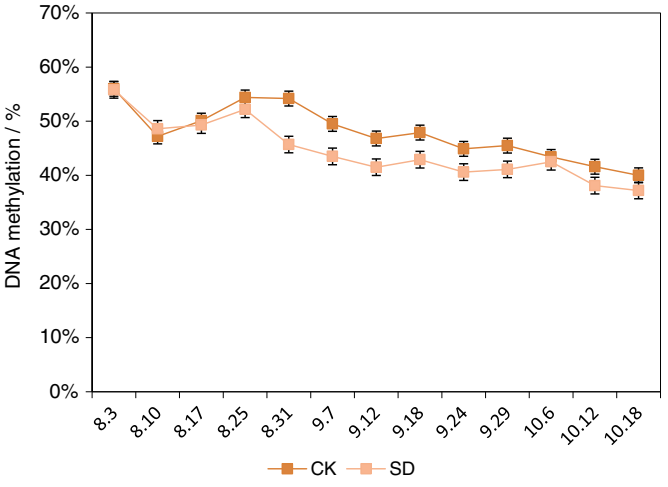

Fig. 5. DNA methylation during floral bud development of the late-flowering *Chrysanthemum* cultivar "Qiu Shui Chang Liu" as measured by MSAP.

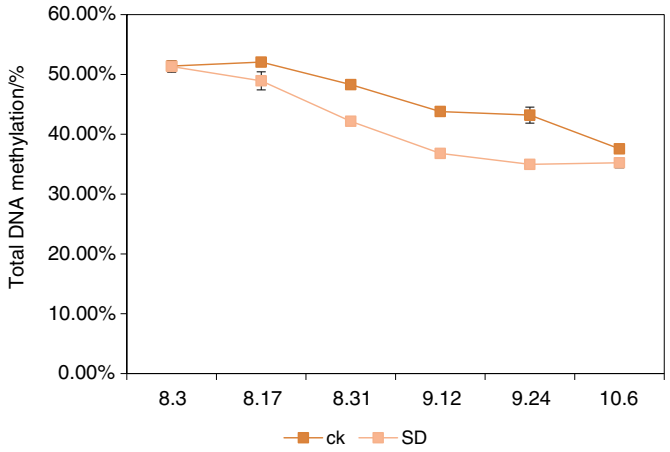

Fig. 6. Total DNA methylation during floral bud development of the early-flowering *Chrysanthemum* cultivar "He Hua Xian Zi" as measured by HPLC.

the control at the early flowering stage. It may be necessary that DNA methylation decrease to a certain critical level for flower induction. DNA methylation decreased to a greater extent in the early-flowering cultivar than in the late-flowering cultivar, which may promote the expression of flowering genes, ultimately resulting in early flowering.

4. Discussion

Some studies have shown that the effective onset of photoperiodic regulation of the floral transition depends on the end of the juvenile stage [26]. Juveniles are unresponsive to the photoperiod and induction of flowering, but plants become responsive once they attain maturity, leading to flower bud differentiation. The process of flower bud differentiation in flowering plants is divided into two general stages: the inflorescence differentiation stage and the floret differentiation stage. These stages can be analyzed *via* nine periods. Generally, cymules can be distinguished in the final stage of floret primordia development. In chamomile, the cymule is distinguishable usually on the 23rd day of SD treatment [18]. In the present study, the capitulum bud appeared on the 23rd day of treatment for the early-flowering cultivar, but on the 33rd day for the late-flowering cultivar. The latter might require a certain SD treatment period to transition from the juvenile to mature stage, and in turn undergo flower bud differentiation. During the flowering process in chamomile, the CIFT gene is increasingly expressed once flower bud differentiation has been initiated [18]. In the present study, the

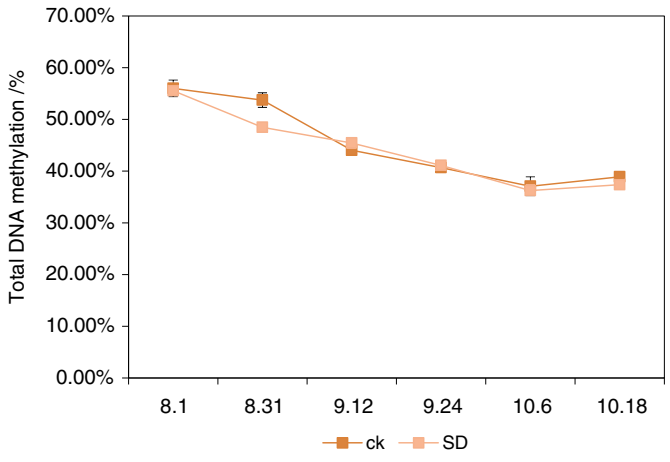

Fig. 7. Total DNA methylation during floral bud development of the late-flowering *Chrysanthemum* cultivar "Qiu Shui Chang Liu" as measured by HPLC.

Table 2
DNA methylation at the early flowering stage after short-day (SD) treatment of the *Chrysanthemum* cultivars "He Hua Xian Zi (early-flowering cultivar) and "Qiu Shui Chang Liu" (late-flowering cultivar).

Cultivar	Total sites	Non-methylated		Methylated	
		Type I	Total	Type II	Type III
HHXZ CK	279	149 (53.4%)	130 (46.6%)	72 (25.8%)	58 (20.8%)
HHXZ SD	275	168 (61.1%)	107 (38.9%)	60 (21.8%)	47 (17.1%)
QSCL CK	273	145 (53.1%)	127 (46.5%)	74 (27.1%)	53 (19.4%)
HHXZ SD	270	158 (58.5%)	112 (41.5%)	64 (23.7%)	48 (17.8%)

significant reduction in DNA methylation percentage begins at the initial flower bud differentiation stage. This implied that the reduction in DNA methylation is associated with floral bud differentiation.

During plant growth and developmental processes, changes in DNA methylation play a key role in blooming, regulation of gene expression for vital functions, genomic defense, cell differentiation, and development [8]. Hypermethylation of the promoter and coding region of a gene can inhibit the binding of transcription factor complexes. This in turn inhibits gene expression, resulting in gene silencing; furthermore, demethylation promotes gene expression. Methylation of the FT promoter causes gene silencing and late flowering in *Arabidopsis* [27]. The present study showed decreased DNA methylation of mature leaves during *Chrysanthemum* flower development induced by SD. This is consistent with previous findings of increased flowering in *Chrysanthemum via* a reduction in the DNA methylation level on applying a DNA methylation inhibitor [21]. DNA demethylation may promote and enhance the expression of the FT gene. The signal is transmitted to the meristem, which may in turn induce the expression of more flowering genes in the meristem. Thus, floral transition is initiated. In our recent study, plants treated with 5-azacydine showed high levels of FT gene expression in leaves (data not shows), which confirms our hypothesis. Further research into this mechanism is needed. The early- and late-flowering cultivars showed different ranges of variation in DNA methylation, which may contribute to different initial flowering times.

The DNA methylation patterns are determined by both DNA methyltransferase and demethyltransferase [28]. It remains to be elucidated whether the reduction in DNA methylation level is due to the decrease in DNA methylase expression level or the increase in demethylase expression level during photoperiod-induced flowering in *Chrysanthemum*. Thus, the expression of DNA methyltransferase and demethyltransferase during this process was investigated in some studies. For instance, in a recent study, Okello et al. [29] showed the significant effect of light on plant cell division, replication, and multiplication. The photoperiod may affect the expression of DNA methyltransferase genes by inducing cyclin expression and a reduction in DNA methylation.

In addition, in the plant genome, the CAG, CTG, and CCG sites are often methylated. However, the MSAP method can only detect the methylation status of CG and part of CCG. It cannot detect double-stranded internal and external cytosine methylation. This accounts for the slightly higher total genomic methylation level detected by HPLC than that measured by MSAP in this study.

Conflict of interest

The authors declare that there are no conflict of interest.

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