



Effects of the silencing of *CmMET1* by RNA interference in chrysanthemum (*Chrysanthemum morifolium*)

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Abstract

DNA methylation is an epigenetic modification involving many biological processes. It is known that epigenetic mechanisms such as cytosine methylation play a pivotal role in regulating plant development. In this current study, a conservative domain-spliced hairpin RNA (ihpRNA) plant expression vector aimed at gene of DNA METHYLTRANSFERASE1 (*CmMET1*) has been constructed. Transgenic chrysanthemum materials (Zijingling) were obtained by *Agrobacterium*-mediated transformation with expression vectors. Transgenic plants were used as rootstock, grafted onto non-transgenic the scions [Guoqinghong (GQH) and Huanshuijinqiu (HSJQ)], which silencing *CmMET1* gene, and exhibited the early flower phenotypes. A high-performance liquid chromatography analysis indicated that the decrease of *CmMET1* expression decreased the methylation level of genomic DNA. Similarly, a quantitative real-time polymerase chain reaction analysis revealed that *CmMET1* expression levels decreased in transgenic chrysanthemum plants and in the scions grafted onto transgenic plants. This decrease of *CmMET1* expression upregulated the expression of the methyltransferase gene, METHYLTRANSFERASE2 (*CmDRM2*), but downregulated the expression of the demethylating enzyme gene, DEMETER (*CmDME*), while the CHROMOMETHYLASEA (*CmCMT3*) expression level remained low and could be almost undetectable. Among the *CmFT*-likes genes that affect flowering time, *CmFTL1* expression was downregulated, as well as *CmFTL2* and *CmFTL3* expression levels which were upregulated. Our data indicated that silencing *CmMET1* could decrease plant height, change the phenotype of chrysanthemum, and promote earlier flowering. The transgenic plants bloomed 8 days earlier. GQH and HSJQ scions grafted onto transgenic plants were 12 and 9 days earlier than the scions grafted onto non-transgenic plants, respectively. Overall, the results have some meanings for promoting the flowering of chrysanthemum scion varieties using genetic modified rootstock.

Keywords RNA interference · Chrysanthemum grafting · Gene expression · Flowering · DNA methylation

Introduction

Epigenetics is defined as the study of changes in gene function that consisting of the entirety of epigenetic marks in the form of histone modifications, DNA methylation, histone variants, and small RNAs, and that do not entail a change in DNA sequence (Jablonka and Raz 2009; Wu and Morris 2001). Epigenetic modifications occur frequently in plants

(Courtney-Cutterson et al. 1993), and result in changes to color (Zheng et al. 2001), plant type (Aswath et al. 2004), flowering cycle (Yepes et al. 1995), etc. DNA methylation is the best studied and most stable of all epigenetic modifications in most eukaryotic organisms (Feng and Jacobsen 2011; Vanyushin and Ashapkin 2011). In animals, DNA methylation happens in the CG context, while, in plants, it occurs on cytosines in any context (CG, CHH, and asymmetric CHG, H = A, C, or T) with CG being the most commonly methylated dinucleotide (Henderson and Jacobsen 2007; Law and Jacobsen 2010). Methylation in each context is believed to be primarily catalyzed by a specific family of DNA methyltransferases: DNA METHYLTRANSFERASE 1 (MET1) for CG, CHROMOMETHYLASE (CMT) for CHG, and METHYLTRANSFERASE (DRM) for CHH (Law and Jacobsen 2010). In addition to methyltransferases, plants encode enzymes that can remove methylation from DNA (Zhu 2009). One of these,

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DEMETER (DME), is expressed in *Arabidopsis thaliana* male and female gametophytes, primarily in the vegetative and central cells, respectively (Ibarra et al. 2012; Zhang and Zhu 2012). Therefore, characterization of DNA methylation modifiers in chrysanthemum would be of great significance for understanding of epigenetic regulatory mechanisms.

The phenotypic plasticity of plants is thought to involve epigenetic regulation, and DNA methylation may be responsible for reinforcing changes in the patterns of gene expression that occur during plant development (Richards 1997; Finnegan et al. 2000; Tariq and Paszkowski 2004). In some plant species, phenotypic and/or developmental changes have been induced by the DNA demethylating agent 5-azacytidine (azaC), and in a small of these species, the transmission of these changes into subsequent generations has been observed. For example, heritable azaC-induced changes have been reported in *Oryza sativa* (Sano et al. 1990), *Brassica oleracea* (King 1995), and *Nicotiana tabacum* (Vyskot et al. 1995).

Chrysanthemum (*Chrysanthemum x morifolium*) are among the most important and economically valuable ornamental plants worldwide. However, most chrysanthemum cultivars are late-flowering plants. The flowering is regulated by environmental factors, such as night length in photoperiodic flowering, coldness in vernalization, and multiple stress factors in stress-induced flowering. Short-day treatments are often used to promote the flowering of chrysanthemums, which are typical short-day plants. We previously observed a decrease in the DNA methylation levels of chrysanthemum plants exposed to short-day treatments to promote flowering (Li et al. 2016). We also observed that chrysanthemum plants treated with 5-azacytidine, which inhibits DNA methylation, bloom 7–10 days earlier than expected (Nie et al. 2009). According to these studies, a decrease in DNA methylation can promote the early flowering. However, the current methods for artificially controlling DNA methylation levels, including chemical treatments, are often laborious, time-consuming, and potentially harmful to the environment. Developing methods of controlling the expression of DNA methylation-related enzymes could change DNA methylation levels, which may lead to promote flowering of chrysanthemum plants. The purpose of this study is to control chrysanthemum DNA methylation-related gene expression by RNAi technology, thereby reducing DNA methylation and promoting chrysanthemum flowering.

Materials and methods

Plant materials

The chrysanthemum cultivar materials ZJL and early- and late-flowering chrysanthemum cultivars (GQH and HJSQ,

respectively) were preserved in Laboratory of Plant Germplasm Resources and Genetic Engineering, Henan University, and were grown in a growth chamber, set at 14 h light/10 h dark cycles, 24 ± 2 °C.

Plasmid construction

The construction of RNAi interference vectors was provided in Supplementary Material 1.

Chrysanthemum transformation using an *Agrobacterium*-mediated method

The new and green of ZJL leaves were cut into 0.5–1 cm² pieces along their veins. The leaf pieces were incubated in Murashige and Skoog (MS) medium supplemented with 2.0 mg dm⁻³ 2,6-benzylaminopurine (BA) and 1.5 mg dm⁻³ 2,4-D butylate in darkness to induce the production of calli. The generated calli were infected for 10 min with RNAi vector-carrying *Agrobacterium tumefaciens* cells in a 100-ml solution consisting of 10 g sucrose and half-strength MS medium. The calli were subsequently dried using sterile blotting paper and then added to a co-culture medium consisting of MS medium supplemented with 1.0 mg dm⁻³ 6-BA and 0.6 mg dm⁻³ indole-3-butyric acid (NAA). After a 2-day incubation in darkness, the calli were transferred to a screening culture medium that was refreshed every 2 weeks [i.e., MS medium-containing 1.0 mg dm⁻³ 6-BA, 0.6 mg dm⁻³ NAA, 10 mg/l dm⁻³ kanamycin (Kan), and 300 mg/l dm⁻³ sodium cefotaxime (Cef)]. Growing buds with 2 cm tall were transferred to the rooting medium (i.e., MS medium-containing 0.3 mg/l NAA and 100 mg/l Cef) for reproducing.

Analysis of transgenic plants using a polymerase chain reaction

Leaves were collected from the transgenic lines screened by Kan and from wild-type chrysanthemum (control) grown in the greenhouse. Genomic DNA was extracted using DNA extraction kit (Takara, Dalian, China). The silencing with RNAi vector contains a kanamycin resistance gene (Kan). Therefore, we used Kan primers [TG-KanSelect-LP (5'-TGAAGATGAACAAAGCCCTGAA-3') and TG-KanSelect-RP (5'-GCAGAAGGCAATGTCATACCACT-3')] to amplify specific fragments, and gel electrophoresis showed the fragment size that the silencing vector was successfully transferred to chrysanthemum ZJL. In addition, we designed RNAiF (5'-GGAGCATCGTGGAAAAGAAGA-3') and RNAiR primers (5'-CATTAGAATGAACCGAAACCGG-3') in silencing vectors for further identification of transgenic plants. The reactions were carried out in a total volume of 20 µl and contained 2 µl of DNA, 1 µl of each primer to final

concentration 10 μM , and 10 μl of 2 \times ES Taq Mastermix (Dye), up to 20 μl of nuclease-free water. PCR conditions were as follows: 95 $^{\circ}\text{C}$ for 5 min; 35 cycles of 95 $^{\circ}\text{C}$ for 30 s, 56 $^{\circ}\text{C}$ 1 min and 72 $^{\circ}\text{C}$ for 1 min, 72 $^{\circ}\text{C}$ 10 min, and 16 $^{\circ}\text{C}$ 30 min. The amplified products were analyzed by 1% agarose gel electrophoresis using a Tris–acetic acid–ethylenediaminetetraacetic acid buffer. Fragments with expected size were purified using a SanPrep Column DNA Gel Extraction kit (Takara). The purified fragments were connected to pMD18-T vector and sequenced (Huada, Beijing).

SiRNA detection in scions

Total RNA was isolated from the samples using RNAPrep Pure Plant Kit (Tiangen, Beijing, China) reagent according to the manufacturer's instructions. cDNA was synthesized from the isolated RNAs using the PrimerScriptTM RT reagent Kit with Gdna Eraser (Takara, Dalian, China) according to the manufacturer's instructions. We used sense-LB-FADi-XbaI (5'-GCTCTAGAAGGGGTAATGACGGCAGA-3') and antisense-RB-FADi-KpnI (5'-GGGGTACCCAGGCC ATATCTGTTTCG-3') to amplify specific fragments, and gel electrophoresis showed the fragment size that the silencing signal was successfully transferred to the scions.

Grafting procedure

The material plants which were grown individually in pots in a medium consisting of refuse soil: limestone (3:1) by vegetative propagation in growth chamber. The 30 pots for the treatment and control groups were transplanted to the experimental field of Henan University for further cultivation. The plants were grown individually in pots in a medium consisting of refuse soil:grass carbon:chicken manure (3:1:1). The plants were grown in 18 cm \times 18 cm pots in a growth chamber, set at 14 h light/10 h dark cycles, 24 \pm 2 $^{\circ}\text{C}$, watered daily with tap water, and fertilized with an inorganic nutrient solution once 2 weeks. Rootstocks were prepared by removing the shoots above at least two basal leaves and then making a 1–2-cm vertical cut at the center of the stem. Scions (3–5 cm) were prepared by removing leaves and trimming the base of the scion to form a wedge. The scion/rootstock junction was wrapped with Parafilm and a clip. To prevent dehydration, plants were covered with plastic bags for 2 weeks until the graft was completed.

RNA isolation and qPCR analysis

For qPCR analysis, total RNA was isolated from the samples using RNAPrep Pure Plant Kit (Tiangen, Beijing, China) reagent according to the manufacturer's instructions. cDNA was synthesized from the isolated RNAs using the PrimerScriptTM RT reagent Kit with Gdna Eraser (Takara, Dalian,

China) according to the manufacturer's instructions. Real-time qPCR was conducted using the SYBR[®] Premix Ex-TaqTM II kit (TaKaRa). The qRT-PCR cycling conditions were as follows: the initial denaturation at 95 $^{\circ}\text{C}$ for 10 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, and annealing at 60 $^{\circ}\text{C}$ for 1 min, with a final at 95 $^{\circ}\text{C}$ for 30 s; 40 cycles of 95 $^{\circ}\text{C}$ for 5 s and 60 $^{\circ}\text{C}$ for 30 s. The relative gene expression was calculated by the comparative C_t ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen 2001). The reactions and samples were carried out in triplicate, and the expression levels were normalized to that of the actin gene. The housekeeping gene–actin gene was used as endogenous control for normalization and the untreated sample was accepted as reference. All the gene-specific primers designed by Primer Premier 5.0 software were listed in Supplementary Table 1.

DNA isolation and HPLC analysis

Modified CTAB DNA isolation method was used for the isolation of DNA from transgenic plants and the scions (Delaporta et al. 1983; Jobes et al. 1995). For HPLC analysis, first, DNA from each sample (5 μg) was treated with 0.5 μl RNase A and 1 μl RNase T1 at 37 $^{\circ}\text{C}$ for 1 h with the purpose of not leaving any RNA residues. DNA samples subjected to RNase application were purified with chloroform precipitation before proceeding to DNA hydrolysis and were denatured for 3 min at 100 $^{\circ}\text{C}$. DNA hydrolysis was performed with the application of 3 μl Nuclease P1 for 17 h at 37 $^{\circ}\text{C}$ and later with 5 μl alkaline phosphatase for additional 2 h at 37 $^{\circ}\text{C}$. The hydrolyzed DNA samples were purified using 0.45 ml Millipore-Amicon Ultra Centrifugal Filter unit. For analysis of 5-methylcytosine, 50 ml of hydrolysed DNA samples was injected into Waters 1515 (Massachusetts, America) HPLC system. A Zorbax Eclipse XDB-C18 column (150 \times 4.6 mm \times 5 μm , Agilent, USA) was used. A binary gradient of a mixture of acetonitrile and water (ACN: H₂O, 90:10 v/v) (A), and a mixture of acetonitrile and isopropyl alcohol (ACN: ISOPROH, 85:15 v/v) (B) were optimized, leading to the best separation of the derivatives and interferences at a constant flow rate of 0.5 ml min⁻¹. The hydrolyzed DNA samples were purified using 0.5 ml Millipore-Amicon Ultra Centrifugal Filter unit. The chromatographic run time was 20 min and an additional period of 4 min was necessary to reestablish the initial chromatographic conditions. The column temperature was 30 $^{\circ}\text{C}$ and the injection volume was 10 μl . The solvent mixtures were ultrasonically degassed prior to use.

Data analysis

Morphological and physiological experiments, evaluation of HPLC results, and analysis of gene expression were statistically evaluated via the one-way ANOVA Tukey's test and

two-way ANOVA using the GraphPad Prism[®] 5.01 computer program. *P* values < 0.05 were considered significant. Morphological and physiological experiments were performed with five biological repetitions, HPLC, and gene-expression analyses with three biological and two technical repetitions.

Results

Production and identification of transgenic plantlets

Agrobacterium GV3101 cells carrying the RNAi vector were used to infect ZJL calli. The transformation of chrysanthemum ZJL produced several transformed clones. DNA and RNA from young leaves of 5–6-week-old plants were assayed both for the presence and expression of *Kna* and the *CmMET1*, respectively, by PCR and RT-PCR. PCR amplification produced a fragment of the expected size in all of the examined samples (Figs. 1, 2). To investigate the interference effect of *CmMET1* gene, we used RT-PCR for the transcription analysis. A quantitative real-time polymerase chain reaction analysis revealed that *CmMET1* expression levels in transgenic ZJL plants were 52.5% lower than that of non-transgenic plants (Fig. 9a). Compared with the control, the transformed ZJL seedlings contained long petioles, few leaves, and chlorotic tissues, and exhibited delayed growth and dwarfism (Fig. 3).

Chrysanthemum morphology due to the silencing of *CmMET1* by RNA interference

Chrysanthemum plant height and phenotype

The silencing of *CmMET1* resulted in obvious changes in plant height and phenotype. The stem node of transgenic plants was shorter than that of control plants. Compared

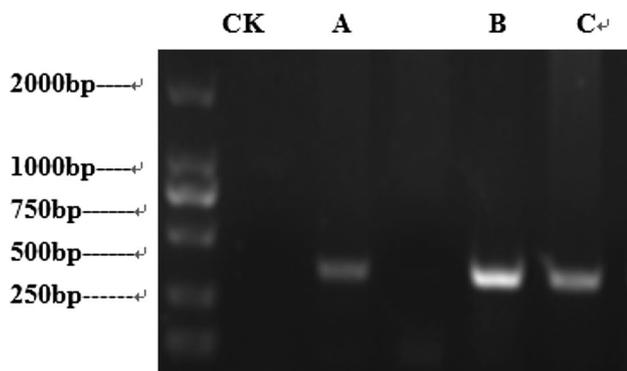


Fig. 1 Kanamycin results of chrysanthemum *CmMET1* gene-transformed buds. A, B, and C: transgenic ZJL

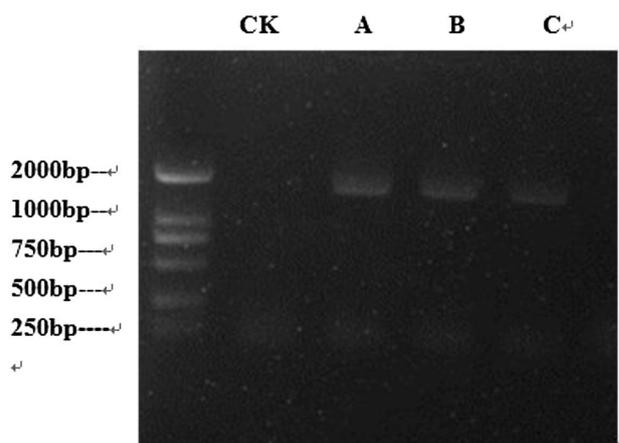


Fig. 2 RNAi primer results of chrysanthemum *CmMET1* gene-transformed buds. A, B, and C: transgenic ZJL

with the control plants, Transgenic ZJL plants show obvious dwarfism during vegetative growth (Fig. 4). In addition, the apical leaves of the treatment group are more yellow or shallower, and they can be recovered by themselves during the flowering stage. The lower leaves were dark in color and with thin stem (Fig. 5). As well, there was no significant change in bud size.

Chrysanthemum flowering time

Growth and development of flower buds were recorded daily since the August. We found that the budding and blooming of the transgenic ZJL and the scion HSJQ grafted onto transgenic rootstock were significantly shorter than those of the control group. The budding and flowering of plants were 10, 8 days and 12, 9 days earlier than control group, respectively (Figs. 6, 7b). In addition, there was no difference in bud development time between the treatment group and the control group. Similarly, GQH scions' treatment group and the control were almost simultaneously budding; however, the bud development time of the treatment group was significantly shortened which made the flowering time 12 days ahead of time (Fig. 7a; Table 1).

The scions' detection

The early flower phenotypes could be achieved in non-transgenic scions grafted on the transgenic rootstocks. After 30 day inoculation, silencing signal of all scions was investigated. RNA from young leaves of 5–6-week-old scions was assayed both for the presence and expression of SiRNA and the *CmMET1*, respectively, by PCR and RT-PCR. PCR amplification produced a fragment of the expected size in all of the examined samples (Fig. 8). Therefore, it is convinced to say that RNAi signal can be transmitted by grafting.

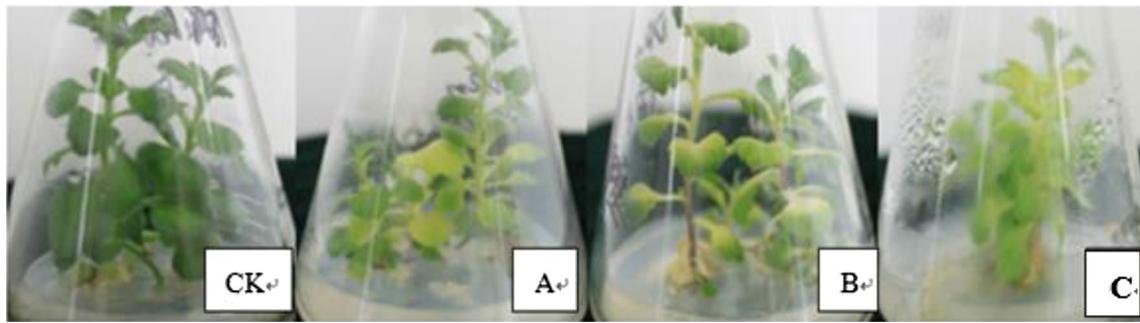


Fig. 3 Representative control and transgenic ZJL seedlings. A, B and C: transgenic ZJL

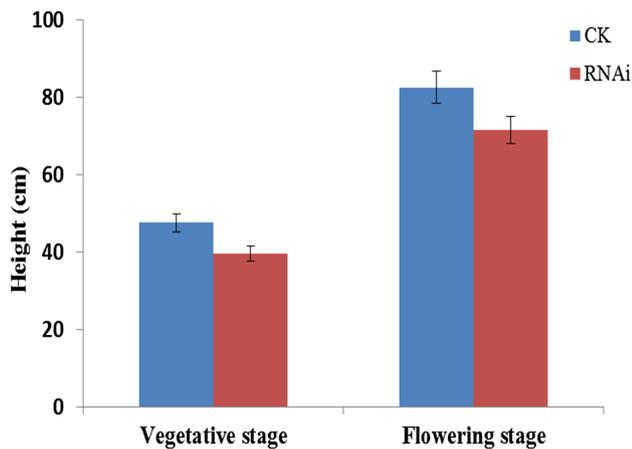


Fig. 4 Effect of the silencing of *CmMET1* by RNA interference on ZJL plant height

High-performance liquid chromatography analysis of DNA methylation in scions and transgenic plants

The methylation changes determined by HPLC analyses are shown in the graphs in Fig. 9. As a result of these analyzes, the rate of DNA methylation in transgenic plants was 14.88% lower than that of non-transgenic plants. In addition, the methylation rates of plants with GQH or HSJQ scions grafted onto transgenic rootstock were 11.2% and 13.84% lower than that of non-grafted transgenic material, respectively. These observations indicated that the decrease of *CmMET1* expression decreased the methylation level of genomic DNA.

RT-PCR analysis

The transgenic ZJL plant, control plants, and the scions were also analyzed for expression levels of the *CmMET1*, *CmDRM2*, *CmCMT3*, *FT-like*s, and *CmDME* genes known to be involved in epigenetic mechanisms. The variation of



Fig. 5 Terminal bud changes of transgenic plants of chrysanthemum. a ZJL non-transgenic, b ZJL transgenic, c HSJQ grafted to the non-transgenic of ZJL, and d HSJQ grafted to the ZJL transgenic

gene expression after qPCR analysis is summarized in the graphs given in Figs. 10, 11, 12, and 13.

In transgenic ZJL plants, the relative *CmMET1* expression levels were 52.5% lower than that non-transgenic plants. Meanwhile, the *CmMET1* expression of GQH and HSJQ scions grafted onto transgenic ZJL rootstock was decreased by 58.67% and 52.5% than the scions grafted onto non-transgenic materials. The relative *CmMET1* expression levels in the transgenic plants were significantly different from that in the control plants for the three analyzed cultivars ($P < 0.01$) (Fig. 10). The results show that the RNA silencing signal appeared to be transmitted from the rootstock to the scions. However, the effects of RNAi differed slightly among the chrysanthemum

Table 1 Effects of the silencing of *CmMET1* by RNA interference on flowering

Varieties	Type	Budding date (m–d)	Budding time (d)	Bloom date (m–d)	Bud development time (d)
ZJL (stock)	Control group	9–26	109	10–27	26
	Treatment group	9–16	99*	10–19	28
HSJQ (scion)	Control group	10–8	121	11–10	33
	Treatment group	9–26	109*	11–1	36
GQH (scion)	Control group	8–24	74	10–10	48
	Treatment group	8–26	76	9–28	34*

*Significant difference ($P < 0.05$)**Fig. 6** Flowering of transgenic and non-transgenic ZJL chrysanthemum plants. Plants 1 and 4: non-transgenic ZJL chrysanthemum; Plants 2 and 3: transgenic ZJL chrysanthemum

cultivars, probably because of the variability in the genome sequences.

The relative *CmDRM2* expression level was 31% higher in transgenic ZJL plants than in non-transgenic plants. In addition, the relative *CmDRM2* expression level in the scions was decreased by 1.29% and 1.21%. The observed differences in the relative *DRM2* expression levels were significant ($P < 0.05$) (Fig. 11). Meanwhile, the relative *CmDME* expression level in transgenic plants was 73.58% of the level in non-transgenic materials. The relative *CmDME* expression levels in the GQH and HSJQ scions grafted onto transgenic ZJL rootstock were 85.01% and 68.75% of the level in the scions grafted onto non-transgenic ZJL rootstock, respectively (Fig. 12). Furthermore, the relative *CmCMT3* expression levels were low or almost undetectable (i.e., relative expression: 0.02–0.06). In plant cells, DNA methylation levels remain relatively stable for a certain period (Kutueva et al. 2016). Down-regulated *CmMET1* expression affected the abundance of

**Fig. 7** Flowering of different chrysanthemum scions. **a** Plant 1: GQH under natural growth conditions; Plant 2: GQH grafted onto a non-transgenic ZJL rootstock; Plant 3: GQH grafted onto a transgenic ZJL

rootstock. **b** Plant 1: HSJQ grafted onto a non-transgenic ZJL rootstock; Plant 2: HSJQ under natural growth conditions; Plant 3: HSJQ grafted onto a transgenic ZJL rootstock

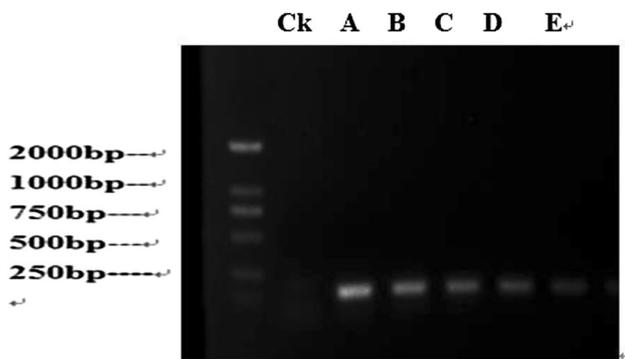


Fig. 8 The detection of silencing signal in the scions. CK: the scions grafted onto non-transgenic ZJL a, b, c, d, e: scions grafted onto transgenic ZJL

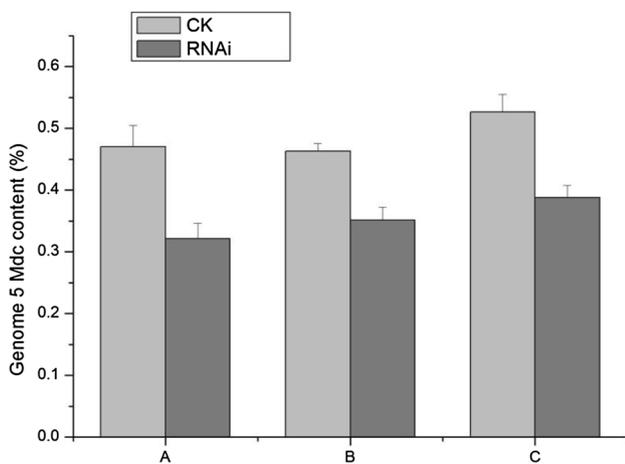


Fig. 9 Methylation level of the chrysanthemum genome as determined by HPLC. A RNAi: transgenic ZJL, CK: non-transgenic ZJL, B RNAi: GQH scions grafted onto transgenic ZJL, CK: GQH scions grafted onto non-transgenic ZJL, C RNAi: HSJQ scions grafted onto transgenic ZJL, and CK: HSJQ scions grafted onto non-transgenic ZJL

the methyltransferases, *CmDRM2* and *CmDME*, which are responsible for maintaining DNA methylation levels to ensure that plants survive and grow normally.

The *FT-like*s genes are involved in the regulation of flowering time. Mutant *Arabidopsis thaliana* and tomato plants overexpressing *FT-like*s genes produce flower buds significantly faster than wild-type plants. We observed that silencing *CmMET1* significantly affected the expression of *FT-like*s genes, with *CmFTL1* expression being downregulated and *CmFTL2* and *CmFTL3* expression being upregulated (Fig. 13).

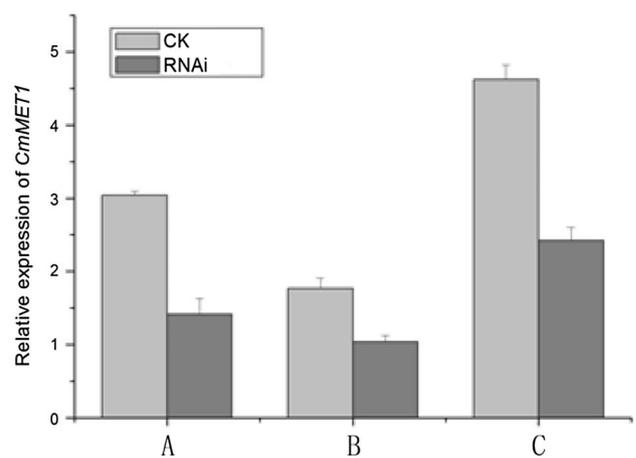


Fig. 10 The *CmMET1* expression in chrysanthemum plants and scions (A, B, and C was the same as the A, B, and C meaning of Fig. 7)

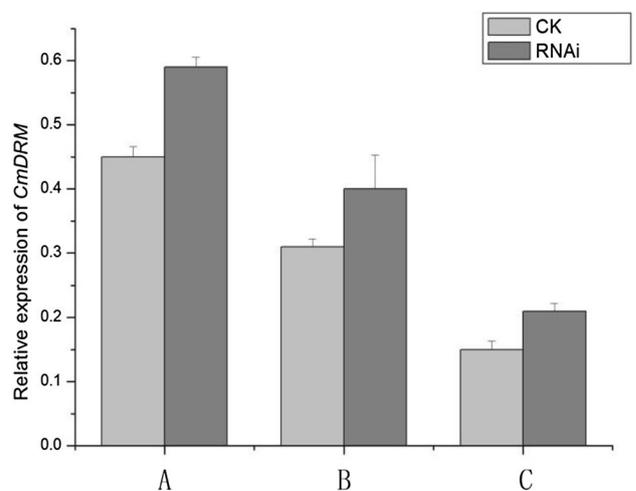


Fig. 11 The *CmDRM* expression in chrysanthemum plants and scions (A, B, and C was the same as the A, B, and C meaning of Fig. 7)

Discussion

The epigenetic regulation, such as DNA methylation, has been shown to control the phenotypic plasticity of many plant species. Numerous studies have revealed that plant development was closely related to DNA methylation. In rice and *Arabidopsis*, *MET1* is the major methyltransferase that maintains DNA methylation levels required for normal growth and development (Yamauchi et al. 2014). A previous study revealed that silencing two rice methyltransferase genes, namely *OsMET1-1* and *OsMET1-2*, by RNAi results in stunted growth, early flowering, leaf curling, and a shortened growth period (Miki and Shimamoto 2008). *Arabidopsis MET1*-antisense transgenic plants obviously showed abnormal developmental phenotypes,

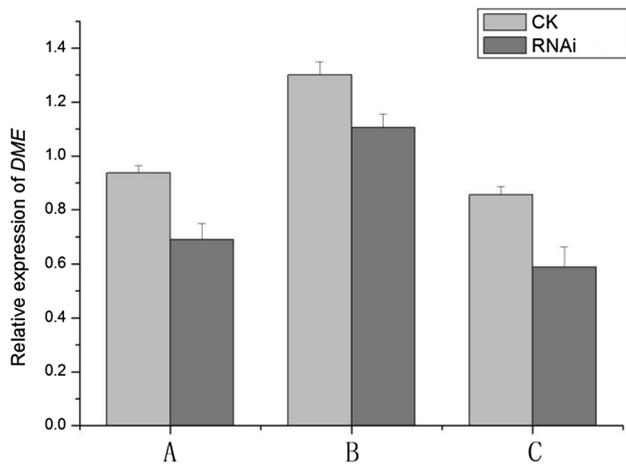


Fig. 12 The *CmDME* expression in chrysanthemum plants and scions (A, B, and C was the same as the A, B, and C meaning of Fig. 7)

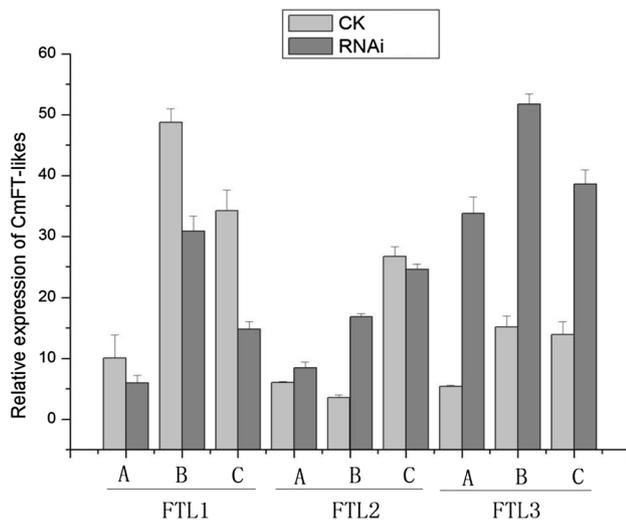


Fig. 13 The expression *FT-like* in chrysanthemum plants and scions (A, B, and C was the same as the A, B, and C meaning of Fig. 7)

such as small plant size, altered leaf size and shape, and altered flowering time (Finnegan et al. 1996). *Arabidopsis drm1drm2cmt3* triple mutants showed retarded growth, reduced plant size, and partial sterility (Cao and Jacobsen 2002). A previous study involving *A. thaliana* concluded that methylating the FT promoter decreased the gene-expression level and delays flowering (Helliwell et al. 2006). In this study, we observed that the relative *CmFTL1* expression levels exhibited a decreasing trend in transgenic plants, which was in contrast to the increasing trend of *CmFTL2* expression. The *CmFTL3* expression level was significantly higher in the transgenic plants than in the controls. That may be due to the difference in the regulation of the expression of the three genes. The joint

action of the three genes ultimately promotes the flowering of chrysanthemum. In addition, the silencing *CmMET1* could decrease plant height, change the phenotype of chrysanthemum, and promote earlier flowering. The transgenic ZJL plants bloomed 8 days earlier. GQH and HSJQ scions grafted onto transgenic ZJL plants were 12 and 9 days earlier than the scions grafted onto non-transgenic ZJL plants, respectively.

DNA methylation is a conserved epigenetic gene regulation mechanism that is utilized by cells. Growing plants exhibit tissue-specific DNA methylation levels, which vary as plants development (Gehring and Henikoff 2007). For example, the DNA methylation level is lower in immature tomato tissues, such as stems, roots, and protoplasts, than in mature leaves, fruits, and seeds (Teyssier et al. 2008). In the current study, we observed that a decrease of *CmMET1* expression resulted in the up-regulation of *CmDRM2* expression levels, while the *CmCMT3* expression levels remained low or almost undetectable. The lack of significant changes to *CmCMT3* expression suggests that the functions of *CmCMT3* were independent from the other chrysanthemum methyltransferases relatively. Our data also revealed that a decrease of the *CmMET1* expression level was associated with the decrease of *CmDME* expression, which is consistent with the results of the previous study involving *A. thaliana* mutants (Xu et al. 2014). These studies indicated that the expression of genes mediating DNA methylation or demethylation is coordinated to maintain a relatively stable DNA methylation level.

RNA interference (RNAi) is a double-stranded RNA (dsRNA)-induced gene-silencing mechanism that is widely conserved among eukaryotes, including animals, plants, and fungi (Md et al. 2013). Because of its high specificity and efficacy, it has been widely used as an efficient tool to analyze gene function. Recent studies have revealed the natural roles of RNAi and RNAi-related phenomena, including suppression of transposon activity, resistance to virus infection, post-transcriptional and post-translational regulation of gene expression, and epigenetic regulation of chromatin structure (Hannon 2002; Grewal and Moazed 2003). A characteristic of mobile signals is that they can spread to neighboring cells through plasmodesmata or systemically to the entire plant through the microtubule system (Mlotshwa and Vance 2002). In plants, RNA silencing can spread between cells through plasmodesmata and over long distances via the phloem (Palauqui et al. 1997; Sonoda and Nishiguchi 2000). Recent advances in analytical techniques show that large amounts of mRNA and siRNA are present in the phloem, and some RNA transport through the sieve tube and play a role where they are needed (Mermigka et al. 2015). In this study, the plants in which a scion was grafted onto transgenic rootstock flowered earlier than plants with a scion grafted onto non-transgenic rootstock. This implies that siRNA can

be transported over long distances in grafted chrysanthemum plants. Consequently, we only need to use a kind of genetically modified material as the rootstock, which can promote the flowering of various scion varieties. In addition, as the rootstock does not have to blossom, it can avoid the problem of transgene escaping through pollination.

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