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ORIGINAL PAPER

Characteristics of cytosine methylation status and methyltransferase genes in the early development stage of cauliflower (*Brassica oleracea* L. var. *botrytis*)

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Abstract DNA methylation is one of the most important epigenetic modifications involved in the development and differentiation in plants. Hypocotyl and cotyledon are the two major tissues of cauliflower (*Brassica oleracea* L. var. *botrytis*) seedlings. Both tissues show significantly different tissue specificity and regenerative abilities in vitro. However, the characteristics of DNA methylation modification and its roles in regulating the organ development in cauliflower remain largely unknown. In the present study, the DNA methylation status between the hypocotyl and cotyledon of cauliflower seedlings were analyzed. The results indicated that although the hypocotyl and cotyledon of cauliflower seedlings share the same genome, the genomic DNA methylation levels and patterns at CCGG sites were different. Compared with the cotyledon, the hypocotyl showed higher DNA methylation level, and more loci showing methylation pattern adjustments were also discovered. Twelve loci with changes of DNA methylation patterns were further explored. The quantitative expression analysis indicated that eight out of twelve sequenced fragments showed differential expression between the hypocotyl and cotyledon, of which the expression of six sequences was identified to be negative correlation with their DNA methylation status. In addition, three main DNA methyltransferase genes *MET1*, *CMT3* and *DRM* were first explored in cauliflower. The results indicated that the expression of these three genes was closely associated with the different DNA methylation status in the hypocotyl and cotyledon. These findings provided more information to further explore the roles of DNA methylation modification in tissue differentiation and development of cauliflower.

Keywords DNA methylation · DNA methyltransferase genes · Cauliflower (*Brassica oleracea* L. var. *botrytis*) · Hypocotyl · Cotyledon

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Introduction

DNA methylation is one of the most important epigenetic regulations in plants. It is usually involved in the conversion of cytosine to 5-methylcytosine in CG, CNG and CNN (N: A, T, C or G) sequence contexts, and is mainly controlled by three types of DNA methyltransferases: *METHYLTRANSFERASE* (*MET*), *CHROMOMETHYLASE* (*CMT*) and *DOMAINS REARRANGED METHYLTRANSFERASE* (*DRM*) (Cao et al. 2003; Zubko et al. 2012; Cao and Jacobsen 2002; Finnegan and Kovac 2000; Lindroth et al. 2001). Increasing investigations have identified that DNA methylation modification play important roles in gene expression regulation, genome stability, genomic imprinting and other genomic processes as well as transgene silencing (Bender 2004; Gehring and Henikoff 2007; Henderson and Jacobsen 2007; Grossniklaus et al. 2001; Weinhold et al. 2013). For example, cytosine methylation of the coding parts of *SUPERMAN* and *AGAMOUS* genes decreased the transcription of both genes (Sieburth and Meyerowitz 1997; Ito et al. 2003). Methylating the 5′end of

FWA transcription factor resulted in the specific expression of *FWA* only in the *Arabidopsis* endosperm (Chan et al. 2005; Cao and Jacobsen 2002). Loss of transgene RNA silencing was detected in some methylation-deficient *Arabidopsis* mutations, and lower level of transgene silencing in roots was found to be associated with reduced DNA methylation levels at non-symmetrical sites (Andika et al. 2006). In addition, in *Arabidopsis*, endogenous long terminal repeat (LTR)-type retrotransposons have been found to be reactivated by DNA hypomethylation (Tsukahara et al. 2009), and several other transposable elements were also found to be regulated by DNA methylation modification (Kubis et al. 2003).

Characterizing whole-genome or gene-specific DNA methylation status is crucial for understanding the roles of DNA methylation modification. A large body of research has been conducted to understand the significance of DNA methylation status in diverse plants. For example, in *Arabidopsis*, a general diminishing of DNA methylation was confirmed to be responsible for changes in meristem identity and organ numbers (Ronemus et al. 1996). Loss of genome-wide methylation has been shown to cause aberrant gametophyte development in *Physcomitrella patens* (Malik et al. 2012). Variation of DNA methylation status has also been reported in oil palm tissue culture-derived explants (Matthes et al. 2001). Similarly, alteration of DNA methylation status in distinct cell lineages of the layers of carnations during vegetative propagation was detected (Yoshida et al. 2004). A higher level of DNA methylation was also detected in seedlings than in flag leaves of rice (Xiong et al. 1999), and significant differences in cytosine methylation status were found to exist between seedlings and adult plants (Sha et al. 2005). Low-paclitaxel yield of the Taxus cell line after long-term culture has been proposed to be associated with higher level of DNA methylation of this cell line (Fu et al. 2012). In addition, during introgressive hybridization of rice and *Zizania latifolia* Griseb, changes in patterns and the extent of DNA methylation were found to be significant in the introgression lines compared to their parent lines (Dong et al. 2006). Alteration of DNA methylation status was found to be induced by tissue culture in rice pure-lines, F_1 hybrids and polyploids (Wang et al. 2013a). The dynamic tissue- and cell type-specific changes that determine the developmental phase of *S. sempervirens* shoots were also found to involve DNA methylation variations (Huang et al. 2012). Moreover, adjustments in DNA methylation status have frequently been observed in plants responding to diverse environmental stresses (Alvarez et al. 2010; Yu et al. 2013a). For example, cold treatment of maize seedlings could result in global demethylation of root genomic DNA, particularly in nucleosome core regions (Steward et al. 2002). Vitro culture and thermotherapy of grapevine somaclones also caused changes in DNA methylation (Baránek et al. 2010). Pathogenic infection by bacteria *Pseudomonas syringae* could lead to significant hypomethylation in host genomic loci (Ruiz-Ferrer and Voinnet 2009). Chemically induced demethylation of the rice *R* gene *Xa21G* has been shown to abolish silencing of this gene and provide heritable resistance to *Xanthomonas oryzae* pv. *oryzae* (Akimoto et al. 2007). Extensive DNA methylation changes in calluses of *Arabidopsis* have been detected to be induced by kanamycin as a selective agent (Bardini et al. 2003). Salt stress has been shown to cause changes in DNA methylation patterns in rice (Karan et al. 2012), soybean (Song et al. 2012) and maize (Tan 2010). In asexual dandelions, considerable DNA methylation variations were found to be triggered by various stresses, most notably chemical induction of herbivore and pathogen defenses (Verhoeven et al. 2010). Similarly, multiple abiotic stresses such as warming and nitrogen addition were found to cause alternations in cytosine methylation in natural populations of *Leymus chinensis* (Yu et al. 2013a). All of these findings indicate that different characteristics of DNA methylation status are closely involved in plant growth and development and response to various biotic and abiotic stresses, although how DNA methylation modification functions in these processes remains to be further elucidated.

Cauliflower is one of the most important vegetables in the *Brassica oleracea* species with abundant nutrients, and is eaten widely all over the world. Hypocotyl and cotyledon are the two predominant tissues of cauliflower seedlings, and both originate from the embryo and share the same genomic DNA sequences. However, the fates of these two tissues show significant differences during cauliflower development as well as in the development of other dicotyledons. The cotyledon is the first organ that becomes senescent and ultimately departs from the plant body soon after the true leaves are formed. By contrast, the hypocotyl, which contains the shoot apical meristem (SAM), further develops and eventually gives rise to other organs such as leaf, stem, curd, and floral organ. In addition, tissue culture experiments have indicated that the regenerative abilities of hypocotyl and cotyledon are significantly different in cauliflower. Under the same in vitro conditions, the hypocotyl of cauliflower seedlings can more easily regenerate to form callus and then explant than cotyledon. The hypocotyl and cotyledon of cauliflower seedlings share the same genomic DNA sequences. Hence, it is conceivable that epigenetic regulation, such as DNA methylation, histone modification and micro RNA-mediated gene silencing, none of which affect DNA sequences, is involved in tissue-specific identification and formation of distinct regenerative abilities exhibited by these two tissues. The objective of this work was thus to explore the

characteristics of DNA methylation status and the expression patterns of three major DNA methyltransferase genes in the hypocotyl and cotyledon of cauliflower seedlings, and further elucidate their potential roles in cauliflower development. Based on the methylation-sensitive amplified polymorphism (MSAP) method, the genomic DNA methylation status at CCGG sites were analyzed in the hypocotyl and cotyledon of cauliflower seedlings. Several MSAP polymorphic bands showing different DNA methylation patterns were cloned, and their transcript levels were further quantitative analysis. Following that, the partial sequences of three major DNA methyltransferase genes: *MET1*, *CMT3* and *DRM* were first cloned and identified in cauliflower. Quantitative expression analysis of these three genes was carried out. The relationship between the DNA methylation status and the expression of three DNA methyltransferase genes, and their relationship with the tissue specificity and different regenerative abilities of hypocotyl and cotyledon were also discussed.

Materials and methods

Plant materials

The cauliflower seeds were treated with 75 % ethanol for 5 min and 2 % NaOCl for 10 min, respectively, and then rinsed thrice with sterile distilled water. Subsequently, the seeds were grown in MS solid medium under controlled conditions with a 16/8 h light/dark cycle at 25 and 22 °C, respectively. The hypocotyl and cotyledon of 5-, 10-, and 15-day-old cauliflower seedlings were collected, immediately frozen in liquid nitrogen, and stored at −80 °C for DNA and RNA isolation. To investigate the regenerative abilities of the hypocotyl and cotyledon of cauliflower seedlings in vitro, the hypocotyl and cotyledon of 5-old-seedlings were collected, and cultured in MS solid medium under the controlled conditions (16/8 h light/dark cycle at 25 °C).

DNA and RNA isolation

Genomic DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method with some modifications (Kidwell and Osborn 1992). In brief, 200 mg hypocotyl or cotyledon of 5-, 10- and 15-day-old cauliflower seedlings, respectively, were ground in liquid nitrogen, and the frozen powder was treated with 2 mL lysis buffer containing 100 mM Tris–HCl (pH 8.0), 100 mM EDTA, 1.4 M NaCl, 0.2 % β-mercaptoethanol, 2 % PVP and 1 × CTAB at 56 °C for 20 min. Subsequently, 2 mL phenol:chloroform:isoamyl alcohol (25:24:1, V:V:V) was added. The following steps were as described by our previous study (Wang et al. 2007). To conduct MSAP analysis, equal amount of DNA from the hypocotyl of 5-, 10- and 15-day-old cauliflower seedlings was pooled to construct hypocotyl DNA pool. Similarly, equal amount of DNA from the cotyledon of 5-, 10- and 15-day-old cauliflower seedlings was also pooled to construct cotyledon DNA pool.

Total RNAs were extracted from the hypocotyl and cotyledon of 5-, 10- and 15-day-old cauliflower seedlings, respectively, by using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After the contaminated DNA was digested by RNase-Free - DNAse I (Promega, USA) for 30 min at 37 °C, the quality of RNA was tested by 1.2 % denaturing agarose gels and 2 μg total RNA were reverse transcribed to cDNA using the AMV reverse transcriptase (Promega, USA).

MSAP assay

The protocol used for MSAP analysis was conducted mainly as previously described (Xiong et al. 1999), with modifications. Briefly, 300 ng DNA from the hypocotyl and cotyledon pools, respectively, were digested for 48 h at 37 °C with 2 U *Eco*RI (TaKaRa, Japan) and 2 U *Hpa*II or *Msp*I (Promega, USA) in a final volume of 15 μL. The digested fragments were then ligated to *Hpa*II/*Msp*I adapters and *Eco*RI adapters using the T_4 DNA ligase (TaKaRa, Japan) in a final volume of 20 μL, and incubated at 16 °C for 12 h. Thereafter, the following reactions of pre-amplification and selective amplification using the preselective primers and selective primers (Online resource 1), respectively, were conducted (Wang et al. 2009). The denatured PCR products were separated on a 6 % denaturing polyacrylamide gel at 35 W for 1.5 h, and then the gels were stained with silver (Chalhoub et al. 1997). Only clear, reproducible bands that appeared in three independent experiments were scored, and these bands were scored in a binary character matrix, with "1" indicating the presence and "0" indicating the absence at a particular position. Statistical analysis was performed by the *U* tests (Zhao et al. 2008).

Cloning of MSAP fragments

The bands, which represent the adjustment of DNA methylation patterns in certain loci detected by MSAP analysis, were isolated and re-amplified with the appropriate selective primer combinations. The corresponding PCR products were subsequently ligated into a pEASY-T1 vector (Transgene, China) and transformed into DH5α, and sequenced with an ABI3770 sequencer (Applied Biosystems, USA). The advanced homology analysis of the sequences was performed using the Blast tool (http://www.ncbi.nlm.nih.gov).

DNA gel blot analysis

To confirm the reliability of MSAP method in detecting the DNA methylation status, Southern blot analysis was conducted. In brief, a total of 12 μg of genomic DNA from the hypocotyl and cotyledon DNA pools, were digested with 15 U *Eco*RI/10 U *Hpa*II and 15 U *Eco*RI/10 U *Msp*I, respectively. The two digested products of each sample were loaded next to each other on a 0.8 % agarose gel, and blotted onto a nylon membrane by capillary transfer with alkali solution (0.4 M NaOH). The probes were selected from the sequenced MSAP bands. The following probes preparation, hybridization, and detection were performed using a DIG DNA labeling and detection kit (Roche, Germany) according to the manufacturer's instructions.

Cloning of partial sequences of *MET1*, *CMT3* and *DRM*

Based on the reported DNA sequences of *MET1*, *CMT3* and *DRM* deposited in the GenBank database, the specific primers used to amplify the corresponding genes in cauliflower were designed. The PCR product of each gene was cloned and sequenced with an ABI3770 sequencer (Applied Biosystems, USA). The gene sequences obtained from the cauliflower with the corresponding sequences from other plant species (Online resource 2) were further analyzed by Clustal W software (Thompson et al. 1994), and phylogenetic analysis was conducted using the Neighbor-Joining method (Saitou and Nei 1987) by MEGA 5 (Tamura et al. 2007).

Quantitative real-time RT-PCR analysis

The quantitative analysis of possible genes with the adjustment of DNA patterns and the three DNA methyltransferase genes in the hypocotyl and cotyledon of cauliflower seedlings were performed by qRT-PCR, respectively. In brief, 100 ng the first-strand cDNA of each sample was used for the real-time PCR analysis. Real-time qPCR was performed with iQ SYBR Green Supermix (Roche, Germany) using the iCycler iQ5 system (Bio-Rad, USA) and specific primers (Online resource 3). Three independent biology replicates were analyzed per sample. Expression values were normalized using the house-keeping gene *actin* and relative expression levels of each sample were performed using (Ct) $2^{-\Delta(\Delta Ct)}$ method.

Results

Distinct regenerative abilities showed by the hypocotyl and cotyledon of cauliflower seedlings

Hypocotyl and cotyledon are two major tissues of cauliflower seedlings. However, significant differences were found in the developmental processes of these two tissues. As a terminal differentiation organ, the cotyledon shows wilt and fall off soon after the generation of true leaves. By contrast, the hypocotyl possessing the SAM can continue to grow and form leaves, stem, and floral organs. In addition, tissue culture experiments indicated that the regenerative abilities of the hypocotyl and cotyledon are significantly different in cauliflower. Under the same condition, the hypocotyl of cauliflower seedlings regenerates to form callus and then explants after about 10 and 15 days, respectively. However, the formation of explants appears to be more difficult from the cotyledon of cauliflower seedlings (Fig. 1).

DNA methylation levels in the hypocotyl and cotyledon of cauliflower seedlings

Sixty-four different selective amplification primer pairs were used for MSAP analysis, of which the amplified results of fifty-one primer pairs showed good repeatability and high polymorphism (Online resource 1). Therefore, the MSAP bands amplified by these 51 primer pairs were used for follow-up data analysis. Based on the results of denaturing polyacrylamide gel electrophoresis (PAGE), a total of 1,583 loci were detected in the hypocotyl and cotyledon of cauliflower seedlings, of which more than half did not show methylation. Methylated loci accounted for 43.52 and 41.12 % of the total loci in the hypocotyl and cotyledon, respectively (Table 1). This result showed that compared with the cotyledon, the DNA methylation level at CCGG sites was higher in the hypocotyl of cauliflower seedlings. Further analysis of different types of DNA methylation showed that the semi-methylation ratio of the hypocotyl (12.51 %) was lower than that of the cotyledon (15.22 %). By contrast, the full methylation ratio of the hypocotyl (24.76 %) was higher than that of the cotyledon (20.85 %). Similarly, the ratio of another type of DNA methylation was higher in the hypocotyl than in the cotyledon, although the status of these DNA methylation sites was not clearly distinguished by MSAP analysis (Table 1). Thus, these data indicated that the total DNA methylation level as well as the ratio of different types of DNA methylation at CCGG sites were different in the two major tissues of cauliflower seedlings.

DNA methylation patterns between the hypocotyl and cotyledon of cauliflower seedlings

The characteristics of DNA methylation patterns at the detected 1,583 loci were further analyzed between the hypocotyl and cotyledon of cauliflower seedlings. In total, four types containing 15 subtypes of different DNA methylation patterns were detected (Fig. 2; Table 2). Data analysis

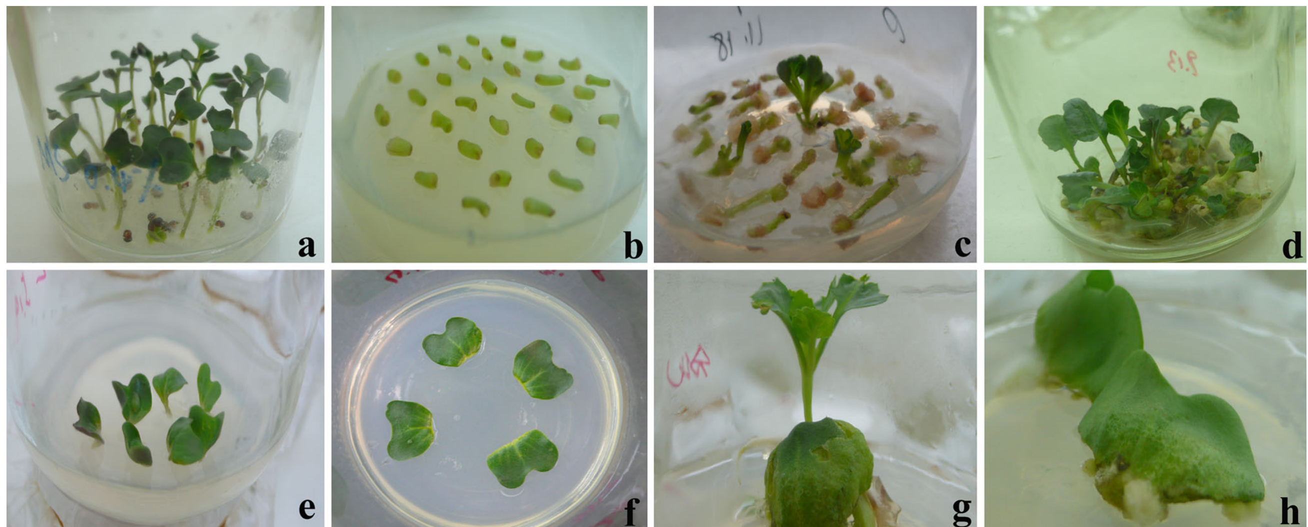

Fig. 1 The regenerative abilities of the hypocotyl and cotyledon of cauliflower seedlings in vitro. **a** The 5-old-cauliflower seedlings, **b** the explant culture of hypocotyl for 5 days, **c** the explant culture of hypocotyl for 15 days, **d** the explant culture of hypocotyl for 25 days, **e** the explant culture of cotyledon with petiole for 5 days, **f** the explant culture of cotyledon without petiole for 5 days, **g** the explant culture of cotyledon with petiole for 35 days, **h** the explant culture of cotyledon without petiole for 35 days

Table 1 DNA methylation levels in the hypocotyl and cotyledon of cauliflower seedlings

Samples	Total sites	Unmethylated sites and ratio	Methylated sites			
			Fully methylated sites and ratio	Hemi-methylated sites and ratio	Other methylated sites and ratio	Total methylation level (%)
Hypocotyl	1,583	894 (56.48 %)	392 (24.76 %)	198 (12.51 %)	99 (6.25 %)	43.52
Cotyledon	1,583	932 (58.88 %)	330 (20.85 %)	241 (15.22 %)	80 (5.05 %)	41.12

indicated that more than 60 % of the 1,583 loci did not show the changes in amplification patterns, of which most of the loci were not methylated (43.15 %), and proportions of semi-methylated and fully methylated loci were 4.23 and 13.27 %, respectively (Table 2). The remaining about 40 % of loci were found to show adjustments in DNA methylation patterns between the hypocotyl and the cotyledon (Table 2). Compared with the cotyledon, 20.66 % of the loci underwent methylation pattern adjustments (type B), and 17.37 % of the loci in the hypocotyl underwent changes in demethylation patterns (type C). These data indicate that the trend of methylation pattern adjustments appears to be more predominant in the hypocotyl. In addition, another type of DNA methylation pattern adjustment, which was undistinguished by the MSAP method (type D), were also detected, and these loci accounted for only 1.33 % of the total detected loci (Table 2).

Cloning and sequence analysis of polymorphic MSAP fragments

Thirteen loci with adjustments of DNA methylation patterns between the hypocotyl and cotyledon were randomly selected. The corresponding polymorphic fragments were isolated, and among them, twelve fragments were successfully cloned (Table 3). Sequence analysis indicated that 4 out of the 12 sequenced fragments showed high sequence homology with reported encoding sequences in other plants (Table 3). For example, E33H25-9-3 and E37H48-3-1 were found to be homologous with the sequences encoding the seed storage and ARM repeat superfamily proteins in *Arabidopsis*, respectively. To further validate the DNA methylation patterns that were revealed by the MSAP analysis, three sequenced fragments (E33H50-16-3, E33H48-13-9 and E33H61-17-1) were randomly selected as probes for a Southern blot analysis. The results identified that the Southern hybridization patterns of E33H50-16-3 and E33H48-13-9 were fully consistent with those detected in MSAP assay. The hybridization pattern of E33H61-17-1 was also coincident with the MSAP assay, except other redundant hybrid signal was showed (Fig. 3). Nevertheless, these results validated that the MSAP method were reliable to explore the DNA methylation status.

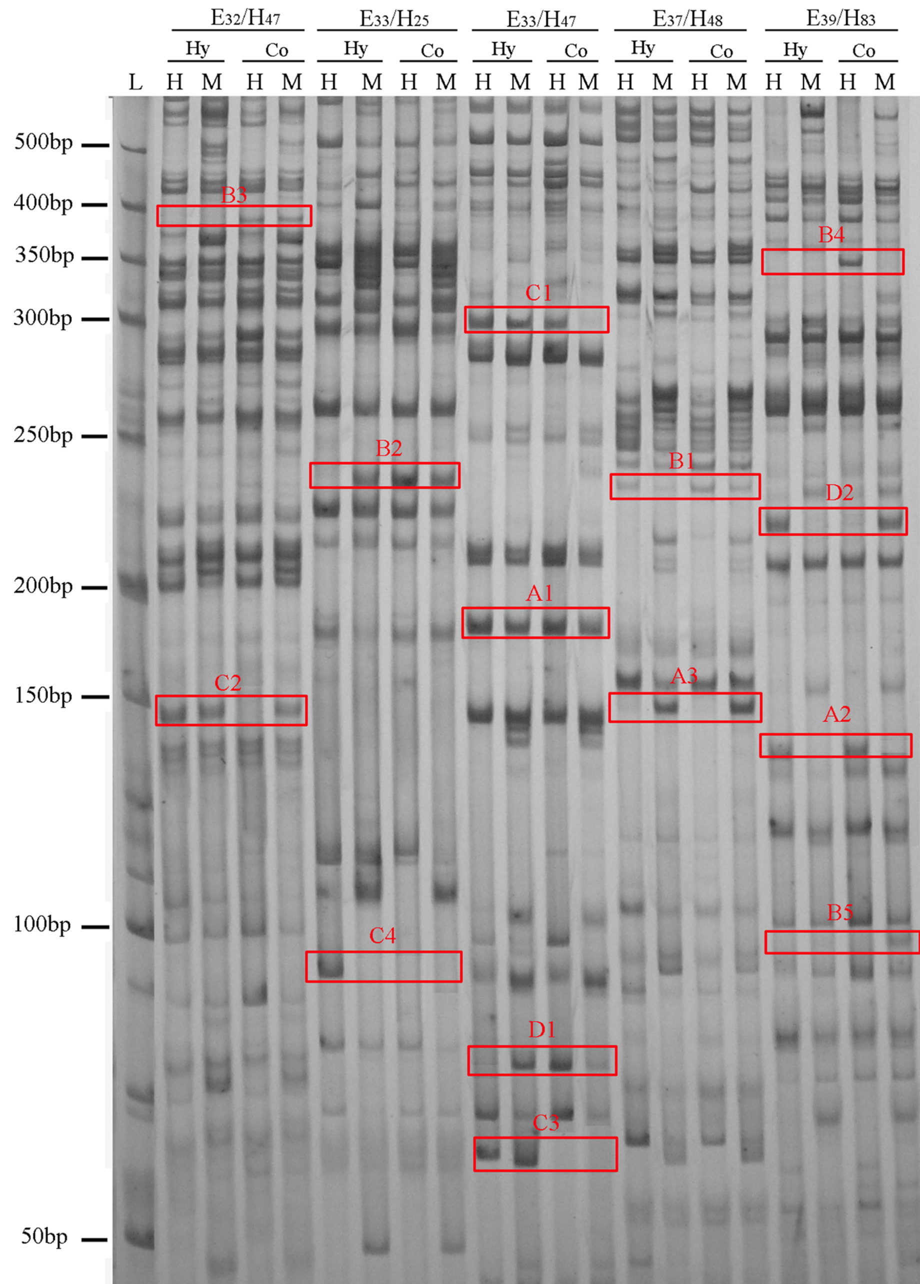

Fig. 2 Representative DNA methylation patterns that were detected in the hypocotyl and cotyledon of cauliflower seedlings by MSAP method. The *red boxes* indicate the different types of DNA methylation patterns in hypocotyl and cotyledon; *H* indicates the selective amplification results used the genomic DNA digested with *Hpa*II and *Eco*RI as templates; *M* indicates the selective amplification results used the genomic DNA digested with *Msp*I and *Eco*RI as templates; *Hy* and *Co* are the abbreviation of hypocotyl and cotyledon, respectively; *L* indicates the 50 bp molecular marker; E_{32}/H_{47}, E_{33}/H_{25}, E_{33}/H_{47}, E_{37}/H_{48} and E_{39}/H_{83} indicate the different MSAP primer combinations, respectively. (Color figure online)

Transcript expression of sequenced possible genes with DNA methylation pattern adjustment

The changes of DNA methylation level or pattern were closely related to the regulation of gene expression. Consequently, qRT-PCR analysis was conducted to explore the transcript expression of twelve sequenced fragments with adjustments of DNA methylation patterns in hypocotyl and cotyledon. The results indicated that except four sequenced fragments (E33H25-8-1, E33H48-13-9, E37H49-4-1 and E39H83-1-2) originating from four different loci, the other eight sequenced fragments all showed differential expression between the hypocotyl and cotyledon. Among them, the expression of most of the fragments (6/8) was showed to be negative correlation with their corresponding DNA methylation status (Fig. 4a–h). These results further confirmed that the characteristics of DNA methylation status could significantly affect the gene transcription expression in early development of cauliflower.

Identification and expression analysis of *MET1*, *CMT3* and *DRM* in cauliflower

Three main types of DNA methyltransferases (CMT, DRM and MET) have been identified in diverse plants. To explore the DNA methyltransferases in cauliflower and

Table 2 DNA methylation patterns between the hypocotyl and cotyledon of cauliflower seedlings based on the MSAP data

Patterns	Hypocotyl		Cotyledon		No. and ratio of patterns		
	H	M	H	M			
A1	—	—	—	—	683 (43.15%)		
A2	—		—		67 (4.23%)		=
A3		—		—	210 (13.27%)		
A					960	60.64%	
B1	—		—	—	101 (6.38%)		
B2		—	—	—	127 (8.02%)		
B3			—	—	21 (1.33%)		↑
B4			—		50 (3.16%)		
B5				—	28 (1.77%)		
B					327	20.66%	
C1	—	—	—		112 (7.08%)		
C2	—	—		—	83 (5.24%)		
C3	—	—			16 (1.01%)		↓
C4	—				21 (1.33%)		
C5		—			43 (2.72%)		
C					275	17.37%	
D1		—	—		12 (0.77%)		
D2	—			—	9 (0.57%)		≠
D					21	1.33%	

"—" indicates the mimetic MSAP bands detected by PAGE gels; " = " indicates the patterns of DNA methylation at CCGG sites were unchanged; " ↑ " indicated the patterns of DNA methylation at CCGG sites were changed due to methylation events in hypocotyl versus cotyledon; " ↓ " indicates the patterns of DNA methylation at CCGG sites were changed due to demethylation events in hypocotyl versus cotyledon; "≠" indicates the alternations of DNA methylation patterns were undistinguished by MSAP method; H indicates the selective amplification results used the genomic DNA as template, which was digested by *Hpa*II and *EcoR*I; M indicates the selective amplification results used the genomic DNA as template, which was digested by *Msp*I and *EcoR*I.

Table 3 Sequence analysis of the MSAP polymorphic bands with different DNA methylation patterns in the hypocotyl and cotyledon of cauliflower seedlings

MSAP fragments	DNA methylation patterns				Length (bp)	Sequence similarity
	H(h)	M(h)	H(c)	M(c)		
E32H47-2-6*			▬		168	unknown
E33H25-8-1	▬				147	unknown
E33H25-9-3*	▬		▬	▬	107	protease inhibitor/seed storage/lipid transfer protein (LTP) family [*Arabidopsis thaliana*]
E33H47-11-7*				▬	106	ATEXO70H4 [*Arabidopsis lyrata subsp. lyrata*]
E33H48-13-9	▬	▬	▬		138	unknown
E33H50-16-3*			▬	▬	326	chloroplast sequence [*Brassica rapa subsp. pekinensis*]
E33H61-17-1*	▬	▬			140	unknown
E37H48-3-1*	▬	▬	▬		169	ARM repeat superfamily protein [*Arabidopsis thaliana*]
E37H49-4-1	▬		▬	▬	179	unknown
E37H49-5-3*	▬		▬	▬	209	unknown
E39H83-1-2			▬	▬	176	unknown
E39H86-2-6*	▬	▬			180	unknown

"▬" indicates the mimetic MSAP bands detected by PAGE gels; H(h) and H(c) indicate the selective amplification results used the genomic DNA digested with *Hpa*II and *Eco*RI as templates in hypocotyl and coteledon, respectively; M(h) and M(c) indicate the selective amplification results used the genomic DNA digested with *Msp*I and *Eco*RI as templates in hypocotyl and coteledon, respectively; * indicates that the corresponding sequenced fragments were anaylzed by qRT-PCR.

elucidate their roles on the DNA methylation status of hypocotyl and cotyledon, partial sequences of three DNA methyltransferase genes, namely, *MET1*, *CMT3* and *DRM* were cloned from cauliflower (accession numbers: KF651133-KF651135). Sequence analysis showed that *MET1*, *CMT3* and *DRM* from cauliflower were all highly homologous with those of the corresponding genes in *Arabidopsis*. Further phylogenetic analysis indicated that *MET1* and *CMT3* were also highly conserved in other plants (Online resource 4). However, homology of cauliflower *DRM* with that of other plants, except *Arabidopsis*, was very low (data not shown). Quantitative analysis of these three DNA methyltransferase genes was conducted. The results indicated that all three of these genes showed differential expression, and the expression levels of *MET1* and *CMT3* in the hypocotyl were significantly higher than those in the cotyledon. By contrast, expression of *DRM* was significantly higher in the cotyledon than that in the hypocotyl (Fig. 4i).

Discussion

DNA methylation is one of the most important epigenetic modifications, and has been identified to be involved in the growth, development and tissue-specific maintenance of plants (Jin et al. 2013; Braszewska-Zalewska et al. 2013; Vining et al. 2013; Iwasaki et al. 2013; Jullien et al. 2012;

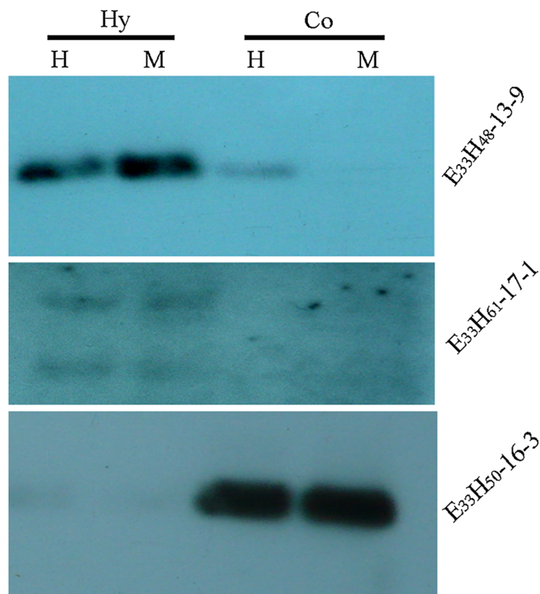

Fig. 3 DNA gel blot analysis using the isolated fragments with changes of DNA methylation patterns as probes. *H* indicates that the DNA samples were digested with *Eco*RI/*Hpa*II; *M* indicates that the DNA samples were digested with *Eco*RI/*Msp*I; *E33H48-13-9*, *E33H61-17-1* and *E33H50-16-3* indicate the different probes, which were detected to show changes of DNA methylation patterns by MSAP analysis; *Hy* and *Co* are the abbreviation of hypocotyl and cotyledon, respectively

Viejo et al. 2012). In these processes, one of the main functions of DNA methylation is to regulate gene expression (Weinhold et al. 2013; Gohlke et al. 2013; Wang et al. 2013b; Kiselev et al. 2013; Li et al. 2012). Generally, if the cytosines of a gene are methylated, especially in the promoter region, expression of the gene is inhibited. By contrast, DNA demethylation is closely associated with the activation of gene expression (Brabbs et al. 2013; Yu et al. 2013b). Therefore, to elucidate the roles of DNA methylation in regulation of gene expression, it is necessary to uncover the whole-genome DNA methylation status or the DNA methylation characteristics of specific genes of interest. Recently, based on analysis of DNA methylomes, a large number of differentially methylated regions have been detected in ripening tomato fruits, and the evidence showed that adjustments in DNA methylation status may ensure the fidelity of this developmental process (Zhong et al. 2013). In fact, variation in DNA methylation status have been discovered in different tissues or different development stages of almost all detected plants, e.g., in the oil palm tissue culture-derived explants (Matthes et al. 2001), the distinct cell lineages of carnations (Yoshida et al. 2004), and the seedlings and flag leaves of rice (Xiong et al. 1999). All of these findings provided crucial clues to further explore the relationship between DNA methylation modification and the growth and development processes in plants. Consistent with the previous reports, different DNA methylation status in the hypocotyl and cotyledon of cauliflower seedlings were also detected in the present study. Further analysis indicated that compared with the cotyledon, the full methylation ratio as well as the ratio of another DNA methylation type which was not distinguished by the MSAP method were obviously higher in the hypocotyl, resulting in higher total DNA methylation level of the hypocotyl. However, the semi-methylation ratio was higher in the cotyledon than in the hypocotyl. Moreover, analysis of the DNA methylation patterns indicated that among the nearly 40 % of detected loci which showed adjustments in their DNA methylation patterns in the hypocotyl and cotyledon, methylation pattern adjustments were identified to be more predominant in the hypocotyl. These results suggested that despite sharing the same genome composition, the hypocotyl of cauliflower seedlings showed higher DNA methylation level, with more loci showing methylation pattern adjustments, compared with the cotyledon. Furthermore, these results implied that expression of more genes in the hypocotyl of cauliflower seedlings may be inhibited. Hypocotyl and cotyledon are two major tissues of cauliflower seedlings, and show significantly different tissue specificity. As a terminal differentiation organ, the main function of the cotyledon is to provide nutrients for seed germination and early development of cauliflower. Therefore, it is conceivable that extensive gene expression and corresponding protein synthesis in the cotyledon are necessary to support the early development of cauliflower. Interestingly, more total RNAs were obtained from the cotyledon despite using the same weights of cotyledon and hypocotyl of cauliflower seedlings (data not shown), which may further imply that gene expression was more active in the cotyledon, even though the hypocotyl shows stronger differentiation and regeneration abilities than the cotyledon in vitro. Accordingly, it can be speculated that the different characteristics of DNA methylation status showed by the hypocotyl and cotyledon of cauliflower seedlings were closely involved in their tissue specificity. Compared with the hypocotyl, lower DNA methylation level and more loci showing demethylation pattern adjustments may be necessary for the cotyledon to activate more genes to support the early development of cauliflower. Nevertheless, further identification of genes that undergo adjustments in DNA methylation patterns and uncovering of their DNA methylation status are required to elucidate the roles of DNA methylation modification in hypocotyl and cotyledon development. In the present study, several loci showing adjustments of DNA methylation patterns were analyzed, and most of them were found to be differentially expressed in the hypocotyl and the cotyledon, implying that the corresponding genes or transcription factors were regulated

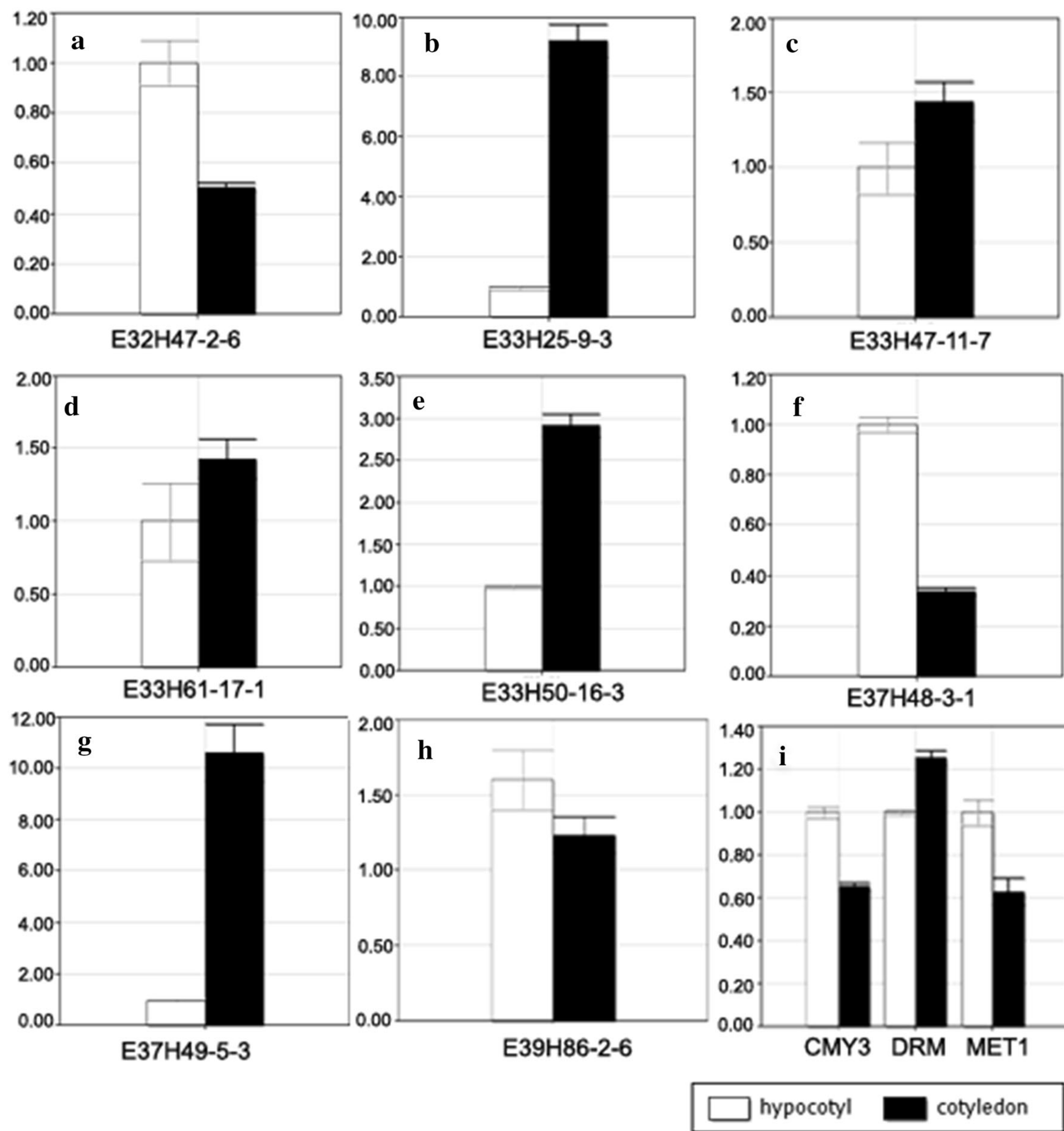

Fig. 4 qRT-PCR analysis of the transcript expression of eight sequenced MSAP polymorphic fragments which may represent possible specific genes (**a**–**h**), and the transcript expression of *MET1*, *CMT3* and *DRM* (**i**). Cauliflower *actin* gene was used as a control

by DNA methylation modification. Thus, these findings provide new insights into the roles of possible genes regulated by DNA methylation in the early development of cauliflower.

Uncovering how genes are regulated by DNA methylation modification is important to elucidate the roles of DNA methylation in plant growth and development or other biological processes. Moreover, understanding how DNA methylation modification is regulated in plants is also crucial to elucidate the functions of DNA methylation. Investigations have indicated that DNA methyltransferases play crucial roles in the cytosine methylation of DNA sequences in diverse plants, and three main DNA methyltransferases genes *MET*, *CMT* and *DRM*, which play different roles in DNA methylation modification, have been identified (Finnegan and Kovac 2000; Steward et al. 2000; Xiao et al. 2006; Cao and Jacobsen 2002). However, the DNA methyltransferases genes in cauliflower and their roles in DNA methylation modification remain to be studied. Consequently, *MET1*, *CMT3* and *DRM* were analyzed for the first time in the present study. Sequence alignment analysis indicated that *MET1* and *CMT3* in cauliflower were highly homologous with the corresponding genes in diverse plants, while *DRM* only showed high

homology with the *Arabidopsis DRM* gene. These results suggested that the functions of *MET1* and *CMT3* are highly conserved in plants. *MET1* is a homologue of the mammalian maintenance methyltransferase *Dnmt1* (Finnegan et al. 1996; Kankel et al. 2003), and has been confirmed to play a role in faithful maintenance of the CG methylation patterns. *CMT3* is specific to the plant kingdom, and has been reported to predominantly control CNG methylation (Lindroth et al. 2001). Transcript expression analysis of *MET1* and *CMT3* indicated that the expression level of both genes were significantly higher in the hypocotyl than in the cotyledon of cauliflower seedlings, which is consistent with the higher full methylation ratio of the hypocotyl. Therefore, this suggests that *MET1* and *CMT3* may be mainly involved in controlling the fully methylated status of CCGG sites during development of cauliflower seedlings. *DRM* is another major class of DNA methyltransferase gene, and is targeted by siRNAs generated by the RNA-directed DNA methylation (RdDM) pathway. It is a homologue of mammalian *Dnmt3* de novo methyltransferases, and is responsible for de novo methylation of cytosines in all sequence contexts (Xie et al. 2004; Cao and Jacobsen 2002). Different from the *MET1* and *CMT3* genes, *DRM* was found to show lower expression level in the hypocotyl than in the cotyledon of cauliflower seedlings, which is consistent with the lower semi-methylated ratio of the hypocotyl. In fact, based on the MSAP analysis, all the detected semi-methylated sites were belonged to asymmetric cytosine methylation. These results suggested that *DRM* should play important roles in controlling the semi-methylated status of CCGG sites during development of cauliflower seedlings. In addition, previous reports have showed that loss-of-function or inhibiting expression of these DNA methyltransferase genes could result in reactivating the expression of some silenced genes in plants (Chen and Pikaard 1997; Chang and Pikaard 2005; Hudson et al. 2011; Pillot et al. 2010). Moreover, the recent report indicated that enhancing expression of these genes also could lead to random hypermethylation in normally unmethylated loci and result in its silencing (Tyunin et al. 2012). These results suggested that the changes of DNA methyltransferase gene expression can significantly affect the expression of other genes, which then affect the plant growth and development. Accordingly, although the roles of DNA methyltransferase genes in cauliflower are still to be elucidated, the detected different expression profiles of these three genes in the present study implied that they should play crucial roles in regulating gene expression in early development of cauliflower.

In summary, findings of the present study suggest that the tissue-specific maintenance and regenerative potential of the hypocotyl and the cotyledon of cauliflower seedlings in vitro are closely associated with their corresponding DNA methylation status. Lower DNA methylation level and more loci showing demethylation changes may be necessary in the cotyledons to activate more genes and thus facilitate synthesis of more proteins to support early development of cauliflower. However, compared with the cotyledon, higher DNA methylation level and more loci showing methylation pattern adjustments did not affect the hypocotyl showed higher differentiation and regeneration abilities. In addition, three main DNA methyltransferases genes were first explored in cauliflower, and the findings indicated that differential expression of these genes may contribute to the different DNA methylation status of the hypocotyl and cotyledon of cauliflower seedlings. Loci showing changes in DNA methylation patterns were also detected, and importantly, differential expression of several such loci in hypocotyl and cotyledon were discovered. Taken together, these findings provided more new insights into the roles of DNA methylation in early development of cauliflower.

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Conflict of interest The authors declare that they have no conflict of interest.

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