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Detection of Epialleles by Measuring Differential DNA Methylation Levels Using Plant-Specific DNA Demethylase

식물 특이적인 DNA 탈메틸화 효소를 이용하여 DNA 메틸화 수준을 파악함으로써 후성대립유전자를 탐지

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ABSTRACT

DNA methylation is one of the crucial epigenetic factors to control gene expression in plants and mammals. Aberrant changes in DNA methylation often lead to the formation of epialleles in plants, and the epimutants that harbor such epialleles sometimes display stable transgenerational inheritance. Therefore, DNA methylation analysis is required to detect epialleles. However, conventional DNA methylation analyses sometimes are not sufficient to detect epialleles. Here we report a novel DNA methylation analysis method combining both 5-methylcytosine (5mC) excision activity of plant-specific DNA demethylase DEMETER (DME) protein and a quantitative real-time PCR (qRT-PCR) technique. Because DME induces a nick in a sequence non-specific manner at the position where 5mC is present, heavily methylated targets cannot be PCR-amplified due to a number of DNA strand breaks, whereas unmethylated or less methylated regions can be easily

amplified. We demonstrate the feasibility of DME-qPCR by successfully distinguishing between wild type and two epialleles, *fwa* and *Cnr*, derived from *Arabidopsis* and tomato, respectively. This novel method has versatility over other enzyme-based tools, and at the same time, may overcome several problems that current DNA methylation analysis principles have – such as pretreatment and intrinsic technical bias.

Key Words**:** Epiallele, DNA methylation, Analysis of DNA methylation, DEMETER

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LIST OF ABBREVIATIONS

5mC	5-methylcytosine
CNR	Colorless, non-ripening
SBP	SQUAMOSA PROMOTER BINDING PROTEIN
FWA	FLOWERING WAGENINGEN
TE	Transposable element
DDM 1	DECREASE IN DNA METHYLATION 1
SINE	Short Interspersed Nuclear Element
ChIP	Chromatin immunoprecipitation
DME	DEMETER
ROS1	REPRESSOR OF SILENCING 1
DML2	DEMETER-LIKE 2
DML3	DEMETER-LIKE 3
qPCR	Quantitative PCR
siRNA	Small interfering RNA
RdDM	RNA-directed DNA Methylation
MBD	Methyl-binding domain
BS-seq	Bisulfite sequencing
TEI	Transgenerational epigenetic inheritance

bns	Bonsai
LINE	Long Interspersed Nuclear Element
BER	Base excision repair
TET	Ten eleven translocate
HhH	Helix-hairpin-helix
GDP	Glycine/proline-rich loop with a conserved aspartate
CAPS	Cleaved amplified polymorphic sequences
ASA1	ATP SULFURYLASE ARABIDOPSIS 1
ATP1	ATP SYNTHASE SUBUNIT 1
DMR	Differential methylated region
GSTP1	GLUTATHIONE S-TRANSFERASE PI
EpiRILs	Epigenetic recombinant inbred lines
AFLP	Amplified fragment length polymorphism

INTRODUCTION

DNA methylation and histone modifications are major epigenetic mechanisms that regulate chromatin structure and the interactions between DNA and proteins, thereby modulating gene expression. Especially, DNA methylation, which refers to the conversion of cytosine to 5-methylcytosine (5mC), plays a crucial role for developmental processes such as transcription regulation, gene imprinting and silencing of transposable elements. DNA methylation of promoter regions is regarded as a strong repressive mark of gene expression. Therefore, alteration of DNA methylation may affect gene expression associated with phenotypic variation. Such variations derived from aberrant DNA methylation, which are transmitted to the next generation, are called epimutations (Chan et al., 2005; Law and Jacobsen, 2010; Schmitz and Ecker, 2012; Zhang and Hsiesh, 2013).

Naturally, spontaneous epigenetic variations might occur by incomplete maintenance of DNA methylation and be transmitted over generations, which would result in the formation of pure epialleles (Schmitz and Ecker, 2012). One of the naturally occurring epimutants is a tomato *colorless non-ripening* (*Cnr*) mutant that has a colorless fruit with the mealy pericarp during ripening. In the wild type, the expression of *CNR*, encoding an SBP-box transcription factor, increases during ripening. By contrast, in the *Cnr* mutant, *CNR* is hardly expressed due to heavy DNA methylation at the promoter (Manning et al., 2006).

However, most of well-studied epialleles are induced by genetic mutations or TE insertions (Schmitz and Ecker, 2012; Heard and Martienssen, 2014). In *Arabidopsis*, a late-flowering epimutant, *fwa*, was induced in the background of

DNA methylation-defective mutation, resulting in genome-wide DNA hypomethylation (Kakutani et al., 1997). In the *fwa* mutant, ectopic expression of *FWA* results from DNA hypomethylation even in the absence of a causative *ddm1* mutation. Also, the repression of *FWA* allele in vegetative tissues in wild type is related with hypermethylation of SINE (Short interspersed nuclear element) around the *FWA* translation start site (Kinoshita et al., 2007). Formation of epialleles is spontaneously induced or by genetic mutation-induced epigenetic variation, and therefore DNA methylation analysis is required for detection of epialleles (Schmitz and Ecker, 2012; Zhang and Hsiesh, 2013).

DNA methylation cannot be detected by conventional PCR-based sequencing technologies. Pretreatments are based on different principles such as enzyme digestion, chromatin-immunoprecipitation (ChIP) and sodium bisulfite conversion. Enzyme digestion relies on DNA methylation-sensitive restriction enzymes. ChIP allows the enrichment of methylated fragments. Sodium bisulfite treatments convert only unmethylated cytosine to uracil. Each pretreatment has strengths and weaknesses, and therefore a proper principle must be carefully chosen (Laird, 2010).

For selecting epimutants in populations, ChIP and sodium bisulfite might not be appropriate, because they are too complicated to analyze a number of individuals at once. The method using the restriction enzymes might be insufficient to analyze DNA methylation at all sequence contexts, because most of the enzymes recognize specific DNA sequences, not DNA methylation per se (Laird, 2010). Therefore, an enzyme capable of removing 5mC directly would be more proper to detect epimutants by analyzing DNA methylation patterns of many individuals in a large scale.

To remove 5mC, 5mC-specific DNA glycosylases can be used, which are involved in active DNA demethylation in *Arabidopsis*: DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2) and DML3. A well-studied enzyme, DME recognizes 5mC and makes a single strand break by β- and δ-elimination on three sequence contexts: CG, CHG and CHH (H=A, T or C) (Gehring et al., 2006; Law and Jacobsen, 2010). The properties of DME can be applied to DNA methylation analysis because DNA strand break represents the presence of 5mC, thereby providing unique information on DNA methylation.

In this study, DME treatment is combined with a quantitative PCR (qPCR) technique to develop DME-qPCR. DME was shown to make a DNA strand break both on CG and CCGG methylated plasmids. DNA cleavage by DME was measured by quantitative PCR. Subsequently, the level of DNA methylation was estimated, which is inversely proportional to the degree of PCR amplification on DME-treated DNA. Finally, I showed that *Cnr* and *fwa* epiallleles can be distinguished from wild type alleles by DME-qPCR. This study demonstrates that DME-qPCR is a promising tool for convenient DNA methylation analysis.

LITERATURE REVIEWS

1. Plant epigenome

The epigenome refers to the description of epigenetic modifications across the whole genome (Bernstein et al., 2007). In practice, "DNA methylome" (a genome-wide DNA methylation pattern) is investigated first to delineate the epigeneome structure of an organism, mainly because DNA methylation is a universal epigenetic modification that is relatively easy to analyze using a high-throughput technology. In addition, several studies propose that there is a strong correlation for gene expression between DNA methylation and other epigenetic factors such as histone modifications and the abundance of small RNAs (Zhan et al., 2006, 2007; Lister et al., 2008). Therefore, profiling DNA methylome would be the first step to build a draft of epigenome, and thereby a general relationship between the global epigenome structure and gene expression patterns can be quickly speculated. As described above, pericentromeric regions are heterochromatic and enriched with repeat sequences such as transposable elements (TEs). These TEs are found to be heavily methylated along with a high abundance of small RNAs, suggesting the primary role of siRNAs triggering DNA methylation by the RNA-directed DNA methylation (RdDM) pathway (Chan et al., 2005; Henderson and Jacobsen, 2007; Zhang et al., 2006). However, not all siRNAs are associated with DNA methylation, implying that rather an RdDM-independent mechanism is engaged.

2. Principles of DNA methylation analysis

Based on the reference genome sequences, a global epigenetic landscape of the whole genome can be investigated by analyzing diverse epigenetic features such as DNA methylation, histone modifications and small RNA distributions. The genome-wide pattern of DNA methylation can be addressed by three major principles: (1) enzymatic digestion using methylation-sensitive and –insensitive restriction enzymes, (2) affinity purification or immunoprecipitation, and (3) sodium bisulfite treatment. These methods are often combined with DNA microarray or high-throughput sequencing technologies to provide genome-wide DNA methylome information.

DNA methylation-sensitive restriction endonucleases such as *Hpa*II and *Sma*I are inhibited by 5-methylcytosine, and therefore, have been widely used to differentiate between methylated and unmethylated DNA. In early studies of DNA methylome profiling, methylation-sensitive enzymatic digestion was usually combined with microarrays or genome-wide tiling arrarys (Tompa et al., 2002; Tran et al., 2005; Zhang et al., 2008). Another class of restriction enzyme, such as McrBC, specifically digests methylated DNA. McrBC-digested DNA can be used as probes for microarray hybridization (Lippman et al., 2005), or directly sequenced by the NGS technology (He et al., 2010). The resolution of restriction endonuclease-based techniques is intrinsically limited to recognition sequences, and therefore, 5-methylcytosine outside the restriction sites cannot be detected by the principle.

Recently, methylated DNA can be enriched by affinity purification using methyl-binding domain (MBD) proteins or by immunoprecipitation with an antibody against 5-methylcytosine. Affinity-purified DNA fragments are combined

with tiling arrays (Penterman et al., 2007; Yan et al., 2010; Zilberman et al., 2007) or high-throughput sequencing to profile the genome-wide DNA methylation patterns (Gehring et al., 2009) .

Sodium bisulfite treatment of denatured DNA induces the conversion of unmethylated cytosines into uracils, but leaves methylated cytosines unaffected. Subsequent PCR amplification converts uracils into thymines, generating C-to-T and G-to-A (on the opposite strand) transitions. The comparison of bisulfite-converted sequences with a reference genome allows the identification of methylated cytosine at the single nucleotide level. Therefore, DNA methylation patterns can be revealed for any sequence contexts, securing the highest resolution compared to other principles. The 'classic' small-scale bisulfite sequencing method is currently combined with a high-throughput sequencing technique, leading to the development of genome-wide bisulfite sequencing (BS-Seq) that is regarded as a gold standard method for DNA methylome analysis in diverse organisms.

3. Epialleles and epimutants

Epigenetic alterations of genes may lead to changes in their expression, and in some cases, both altered epigenetic states and expression patterns are mitotically/meiotically stable and inherited to the offspring. An individual with altered epigenetic modifications may give rise to the offspring with different phenotypes, despite the same DNA sequence at the corresponding gene. The mutant that is not caused by DNA mutation, but rather by epigenetic alteration is called an 'epimutant', and the corresponding epigenetic allele that is responsible for the altered phenotype is called an 'epiallele'. The transgenerational epigenetic inheritance (TEI) involves the formation of epialleles and their consistent

propagation in next generation. Notably, most of naturally occurring or artificially induced epialleles are caused by changes in DNA methylation (Zhang and Hsieh, 2013).

Some of the first epimutants reported in *Arabidopsis* were induced in mutant background defective in DNA methylation. *MET1* and *DECREASE IN DNA METHYLYATION* (*DDM1*) encode maintenance DNA methyltransferase and SWI/SNF-like ATP-dependent chromatin remodeling factor, respectively (Jeddeloh et al., 1999; Vongs et al., 1993). The *met1* or *ddm1* mutants have a genome-wide decrease in DNA methylation level, displaying severe developmental defects and abrupt TE activation (kato et al., 2003; Lippman, 2004). At the same time, epimutations are induced at a high frequency in these mutant backgrounds due probably to abrupt changes in global DNA methylation pattern. Interestingly, some phenotypic changes in these mutants can be inherited to the offspring even without an original causative mutation. For example, the late flowering phenotype of *fwa* mutant was caused by a *ddm1* mutation, which induced a release of gene silencing by decreasing DNA methylation. After outcross to a wild type, and ectopically expressed *fwa* epiallele was consistently transmitted as a dominant allele following Mendelian segregation (Koornneef *et al*., 1991; Kakutani, 1997). A later study verified that a loss of DNA methylation in the repeat regions upstream of the gene causes transcriptional activation of *FWA* leading to late flowering (Soppe et al., 2000). An epigenetically silenced version of *SUPERMAN* (*SUP*) is another example of the formation of an epiallele caused by global DNA methylation changes in *Arabidopsis* (Jacobsen and Meyerowitz, 1997).

In addition to the artificially induced epimutations in DNA methylation-defective mutants, several studies report epimutants and epialleles that

spontaneously occur in nature. The first naturally occurring epiallele was found in *Linaria vulgaris*, in which an asymmetric flower is transformed into a radially symmetric (peloric) flower (Cubas et al., 1999). The abnormal flower structure is caused by DNA hypermethylation and silencing of the *Lcyc* gene, which encodes a *CYCLOIDEA* gene homolog mutant was originally described more than 250 years ago by Linnaeus, suggesting that an epiallele can be stable enough for the maintenance of TEI over a number of generations. Several epimutations associated with agronomically important traits are also found in garden plants. In tomato (*Solanum lycopersicum*), fruit ripening is inhibited in *Colorless non-ripening* (*Cnr*) mutants (Manning et al., 2006). The *Cnr* gene encodes SBP-box transcription factor and its promoter in *Cnr* mutants was found to be hypermethylated leading to gene silencing (Manning et al., 2006). The follow-up study revealed that DNA methylation at the *Cn*r promoter region gradually decreases during fruit ripening in wild type, indicating that DNA methylation in also developmentally regulated (Zhong et al., 2013).

In many cases, epimutations are associated with TE insertions. For example, an insertion of a hAT transposon near the *CmWIP1* gene in melon (Cucumis melo) induces the spreading of DNA methylation to the promoter leading to gene silencing (Martin et al., 2009). Expression of *CmWIP1* promotes carpel abortion and the development of male flowers, whereas its silencing suppresses the anther development, producing mostly female flowers. Therefore, *CmWIP1* acts as a sex determination switch in melon flower development. A recent study shows that expression of tomato VTE3(1) gene, encoding 2-methyl-6-phytylquinol methyltransferase that catalyzes the final steps of ν- and δ-tocopherol (vitamin E) biosynthesis, is modulated by differential DNA methylation of a SINE element

located in the promoter region (Quadrana et al., 2014). DNA methylation of the promoter of VTE3(1) is spontaneously reverted, generating different epialleles with varying expression levels. This indicates that naturally occurring epialleles are also responsible for the regulation of metabolite contents. Actually, repeat-induced DNA methylation and gene silencing is quite prevalent in plant epimutants. As described above, the wild type *Arabidopsis FWA* gene is silenced due to DNA methylation of SINE elements. Silencing of *Arabidopsis bonsai* (*bns*) epiallele is also associated with hypermethylation of LINE elements (Saze and Kakutani, 2007).

4. Plant-specific DNA demethylase

DNA demethylation is a process to reverse DNA methylation, which is often observed during reproductive development in plants and mammals. DNA demethylation occurs passively by DNA replication without maintenance of DNA methylation or actively by removal of 5-methylcytosine. Differences of DNA demethylation processes exist between plants and mammals. In mammals, DNA demethylation occurs via the base excision repair (BER) pathway after oxidation of 5-methylcytosine to other forms of bases by TET family proteins. However, active DNA demethylation in plants is initiated by DNA glycoslyases by directly excising 5-methylcytosine, followed by the BER pathway. In *Arabidopsis*, 5mC-specific DNA glycosylases, referred to as DNA demethylases, include DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2) and DML3 (Law and Jacobsen, 2010; Wu and Zhang, 2010; Bhutani et al., 2011).

DME establishes maternal imprinting of MEDEA by DNA demethylation during female gametogenesis and seems to trigger small RNA production in pollen

vegetative cells for genome stability of reproductive cells (Gehring et al., 2006; Schoft et al., 2011; Law and Jacobsen, 2010). ROS1 represses transcriptional gene silencing triggered by small RNAs (Gong et al., 2002). DML2 and DML3 are reported to prevent improper DNA methylation (Penterman et al., 2007; Ortega-Galisteo et al., 2008). They possess DNA glycosylase domain, which contains a helix-hairpin-helix (HhH) motif and glycine/proline-rich loop (GDP) with a conserved aspartic acid, with iron-sulfur [4Fe-4S] cluster. They are bifunctional DNA glycosylase/AP-lyase that excises 5mC directly from double stranded DNA and breaks an N-glycosidic bond, leading to a single nucleotide gap by β- and δ-elimination processes (Gehing et al., 2006; Mok et al., 2010). The remaining gap is likely filled by BER pathway (Lee et al., 2014).

Among them, the function and structure of DME is well-documented. DME excises 5mC regardless of DNA sequence context. Hemi-methylated or fully-methylated DNA is a substrate of DME (Gehring et al., 2006). DME also excises 5-hydroxylmethylcytosine, an intermediate of oxidation of 5mC during active DNA demethylation in mammals (Jang et al., 2014). The DME family proteins have three essential domains; Domain A at the N-terminus, DNA glycosylase domain and domain B at the C-terminus. A mixed charge cluster at the N-terminal domain A is required for DNA binding (Mok et al., 2010). An iron sulfur [4Fe-4S] cluster in the DNA glycosylase domain is important for DME activity. Though the function is not known, the C-terminus is thought to be required for DME structure and function. Recently, engineered DME without poorly conserved inter-domain regions is also reported to normally excise 5mC (Mok et al., 2010).

MATERIALS AND METHODS

Purification of DNA demethylase, DEMETER (DME)

A recombinant plasmid expressing MBP-DMEΔ677ΔIDR1::lnk (Mok et al., 2010; Jang et al., 2014; Lee et al., 2014) was transformed into *E.coli* Rosetta2 (DE3) strain (Novagen). Cells were grown at 28 °C in four liters of LB containing 50 μg/m_of kanamycin and 50 μg/ml_of chloramphenicol until the OD_{600} reached 0.4. Expression of MBP-DMEΔ677 ΔIDR1::lnk was induced with 0.1 mM IPTG at 18 °C for 16 hr. Cells were harvested by centrifugation at 7,500 rpm for 20 min at 4 °C and resuspended in 30 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol (DTT), 0.1 mM PMSF). Cells were lysed by sonication for 5 min (0.5x duty cycle; Branson Sonifer 250) on ice. Cell extracts were centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatant was filtered through nylon membranes with 0.8 μm and 0.45 μm pore (Advantec), sequentially. The lysate was sequentially purified by three different types of columns: affinity column (HisTrap FF 5 ml, GE Healthcare) with a gradient of imidazole, ion exchange column (Heparin HP 5 ml, GE Healthcare) with a gradient of NaCl and size exclusion (HiLoad 16/60 Superdex 200-pg, GE Healthcare). The final eluted fractions were concentrated and aliquoted with 50% glycerol and stored in a storage buffer (20 mM Tris-HCl, pH 7.4, 40 mM NaCl, 4% glycerol, 20 μM DTT) at -80 °C until use.

Preparation of *in vitro*-methylated plasmids

Unmethylated pUC19 plasmids in methylation-deficient JM110 (dam-,

dcm-) cells were purified using the Bioneer Miniprep Kit. To modify DNA methylation on CG site or internal cytosine of CCGG site, 1 μg of plasmid was methylated with 10 U of CpG methyltransferase (M.*Sss* I) and 8 U of HpaII (CCGG) methytranferase (NEB) according to the manufacturer's instruction, respectively. After *in vitro* methylation, plasmids were purified using Bioneer PCR Purification Kit. To confirm DNA methylation of pUC19 plasmids, plasmids were digested with 10 U of *Msp* I and *Hpa* II. Unmethylated plasmid was prepared in parallel with the same procedure and used as a control.

Plant materials

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (*Col*-0) and ecotype Landsberg erecta (*Ler*) were used in this study. The *fwa-1* was adopted from the previous report (Koornneef *et al*., 1991). Seeds were sterilized and stratified at 4 °C for 2 days in the dark, and sown and grown on Murashige-Skoog (MS) media agar plates in a growth chamber with 16 hours of fluorescent light at 20±5 μmol m^{-2} sec^{-1}, 22 °C, and 70% relative humidity. F_2 *Col*-0 × *fwa*-1 were made by crossing *Col*-0 and *fwa*-1 and self-crossing. To measure flowering time, the number of days from sowing and rosette leaf number to appearance of visible bolt of each plant were counted.

Wild type tomato (*Solanum lycopersicum* cultivar Ailsa Craig) and the corresponding *Cnr* were used. Seeds were sterilized and grown in a growth chamber for 1 month. They were transported and grown in green house conditions (12 hr supplemental lighting at 25 °C and 12 hr at 20 °C) with regular additions of N, P, K fertilizer (Hyponex).

Cleaved Amplified Polymorphic Sequences (CAPS) Analysis

The CAPS marker was used to identify the cross between Col-0 and *fwa-1* and genotypes of F_2 progenies. Genomic DNA was amplified with DG660 and DG661. The PCR products were digested with 20 U of *Taq*$^{\alpha}$I restriction enzyme for 4 hours at 65 °C. Gel electrophoresis was conducted on a 2.5% agarose gel at 25 V for 70 min.

Bisulfite Sequencing

Genomic DNA was treated with EpiTect Bisulfite kit (QIAGEN). The bisulfite treatment steps were performed following the manufacture's protocols. Unmethylated regions were amplified to confirm completed bisulfite conversion. Primers DG662 and DG663 for the *ATP Sulfurylase Arabidopsis 1* (*ASA1*) gene in *Arabidopsis* and primers DG1678 and DG1679 for *ATP Synthase Subunit 1* (*ATP1*) gene in tomato were used as a control (Jeddeloh et al., 1998; Wang et al., 2011). After conversion of cytosine to thymine at unmethylated control genes was confirmed, the 5'-upstream region of *FWA* of *Arabidopsis* was amplified with the primers DG344 and DG345 (Schoft et al., 2011) and the *CNR* promoter of *t*omato was amplified with the primers DG1680 and DG1682 (Manning et al., 2006). The cytosine methylation in the CG, CHG and CHH contexts was analyzed and displayed using CyMATE (Schoft et al., 2011).

Preparation of quantitative real-time PCR templates

Twenty ng of *in vitro*-methylated pUC19 was incubated with 400 ng of DMEΔN677ΔIDR1::lnk in the glycosylase reaction buffer (10 mM Tris-HCl, pH

7.4, 50 mM NaCl, 0.5 mM DTT) and with 20 U of McrBC (NEB) in NEBuffer 2 (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) with 200 ng/μl of BSA and 1 mM GTP, respectively at 37 °C for 1 hr. The reactions were inhibited at 65 °C for 15 min. The reaction products with no enzymes were prepared in parallel with the same procedure and used as a control. The reaction products were diluted 1:2000.

Total gDNA was extracted from leaves using the Cetyl-trimethylammonium bromide (CTAB) extraction method. 500 ng of gDNA was incubated with 600 ng of DMEΔN677ΔIDR1::lnk in the glycosylase reaction buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM DTT) at 37 °C for 2 hr. The reaction products with no enzymes were prepared in parallel with the same procedure and used as a control.

Analysis of DNA methylation using quantitative real-time PCR

Quantitative real-time PCR was performed using Roter-Gene Q (Qiagen) with SYBR green Q-master mix (Genet Bio). The PCR reaction (10 μl) contained 5 ul of 2 × Q master mix, 2 ul of diluted products (1:2000) or 2ul of DME treated gDNA (approximately 50ng) and 200 nM each of forward and reverse primers. To analyze DNA methylation of pUC19 plasmids, DG1764 and DG1882 were used to amplify unmethylated region. CG methylated region was amplified with DG1883 and DG1884. CCGG methylated region was amplified with DG1915 and DG1916. To analyze DNA methylation of plant genomic DNA, DG1287, DG1288 and DG1801, DG1802 were used to amplify unmethylated regions, *ASA1* and *ATP1* in *Arabidopsis* and tomato, respectively. 5'-*FWA*, 216 bp region upstream from the

start codon of *FWA* was amplified with DG1289 and DG1290. 5'-*CNR*, 150 bp region upstream from the start codon of *CNR* was amplified with DG1803 and DG1804. After an initial denaturation of 95 °C for 10 min, thermal cycling condition was as follows: 40 cycles of 95 °C for 10 sec, annealing at 60 °C for 15 sec and extension at 72 °C for 35 sec. Two technical replicates were run.

Relative amplification levels were analyzed using delta-delta Ct method. To calculate $2^{-\Delta Ct}$, Ct values of DME or McrBC treated samples were normalized by Ct values of untreated samples. Then, to obtain values of $2^{-\Delta\Delta Ct}$, $2^{-\Delta Ct}$ of the regions of interest was normalized by $2^{-\Delta Ct}$ of the unmethylated regions. Finally, relative amplification values of the methylated regions were obtained when the relative value of the unmethylated region was 1. Standard deviations were calculated after 2^{-Ct} transformation (Schmittgen and Livak, 2008).

Table 1. List of primers for PCR amplification

Primer name	sequence (5'→3')	use
DG660	CCAGCCAAATATCAGATCTTGCGCC	CAPS marker
DG661	CCTTAAACAACCAAATAGCASTCCGACCAATG	CAPS marker
DG662	TAAGAGAATTAAAATGYAAAYTTTGYAAATA	Bisulfite sequencing
DG663	CCTAACAATRCAATCTRTTTCRAAAATRAC	Bisulfite sequencing
DG1678	TGAAYGAGATTYAAGYTGGGGAAATGGT	Bisulfite sequencing
DG1679	CCCCCTTCCATCAATARRTACTCCCA	Bisulfite sequencing
DG344	GGTTYTATAYTAATATYAAAGAGTTATGGGYYGAAG	Bisulfite sequencing
DG345	CAAARTACTTTACACATAARCRAAAAACARACAAATC	Bisulfite sequencing
DG1680	TTYTAAGYTGYTAYAGAGATATTGGAAGAG	Bisulfite sequencing
DG1682	CARATTAAAACACTTCATCAATTRAAACAA	Bisulfite sequencing
DG1764	TAAGGGATTTTGGTCATGAGATTATC	DME-qPCR
DG1882	GGTAACTGTCAGACCAAGTTTACTC	DME-qPCR
DG1883	CACATTAATTGCGTTGCGCTCAC	DME-qPCR
DG1884	GGATAACCGTATTACCGCCTTTGAG	DME-qPCR
DG1915	GATACGGGAGGGCTTACCATC	DME-qPCR
DG1916	ACTGGCGAACTACTTACTCTAGCTTC	DME-qPCR
DG1287	GAACCCTTTTAAAGGACTAGCCCA	DME-qPCR
DG1288	CGAAAATGACTGTTAAGAGCTCGTTG	DME-qPCR
DG1801	GAATGAGAATGTAGGGATTGTTGTC	DME-qPCR
DG1802	CCCTTCCATCAATAGGTACTCCC	DME-qPCR
DG1289	CATACGAGCACCGCTTTACGG	DME-qPCR
DG1290	GAACCAAAATCATTCTCTAAACAAAGTGT	DME-qPCR
DG1803	CAACAGCTATCATTTATTATGTTACCTC	DME-qPCR
DG1804	AAATCATGTAATATAGCTAGGCAGAGG	DME-qPCR

RESULTS

CCGG methylation was detected by using DME, not McrBC

To verify an efficiency to detect 5mC, DME was compared with McrBC by using *in vitro*-methylated pUC19 plasmids, because both DME and McrBC recognize 5mC and make a cleavage on a methylated DNA strand. Two kinds of *in vitro*-methylated pUC19 plasmids were prepared: CG-methylated pUC19 plasmids and CCGG-methylated pUC19 plasmids (Figure 1A). *In vitro* methylation was confirmed by digestion with *Hpa*II (Figure 1B). CG-methylated pUC19 plasmids were cleaved by McrBC and the methylated region was less-amplified than unmethylated region by qPCR after the McrBC treatment (Figure 2A). Likewise, the methylated region of CG-methylated plasmids was hardly amplified after DME treatment (Figure 2A). After both the McrBC and DME treatments, CG-methylated region was rarely amplified. It showed that both McrBC and DME were available to detect CG methylation.

However, PCR-amplified patterns of CCGG-methylated region were distinct from those of CG-methylated region. CCGG-methylated region was easily amplified after the McrBC treatment whereas it was rarely amplified after the DME treatment (Figure 2B). It indicated that the McrBC treatment did not make a difference between non-methylation and CCGG methylation. McrBC did not digest CCGG methylated pUC19 plasmids because it recognizes only 5mC following adenines or guanines. DME recognizes 5mC and directly excises 5mC, and therefore CG- and CCGG-methylated plasmids were fragmented by the DME treatment. It showed that DME was more efficient to detect 5mC in a sequence

non-specific manner than McrBC and qPCR was appropriate to detect DME-damaged DNA.

Proportion of CG-methylated pUC19 plasmids in total plasmid templates was measured by quantitative PCR after DME treatment

As methylated plasmids were detected by DME, it was tested whether qPCR using DME can measure the proportion of DNA methylation by using mixtures with unmethylated and methylated pUC19 plasmids. Templates were prepared by mixing unmethylated pUC19 and methylated pUC19 with different ratios and confirmed by digestion with *Hpa*II (Figure 3A, B). As CG-methylated pUC19 plasmids were fragmented by DME (Figure 4A), the CG-methylated region of unmethylated pUC19 plasmids in the templates can be amplified by qPCR using DME. So, the degree of PCR amplification was inversely proportional to the ratio of CG-methylated plasmids in templates (Figure 4B). Due to the negative relation, relative degree of methylated fragments in the total DNA fragments would be predicted by qPCR using DME. This technique that combines DME-treatment with quantitative PCR is called 'DME-qPCR' hereafter.

DME-qPCR distinguished epialleles from wild type alleles

It was tested that DME-qPCR had a feasibility to detect epialleles as well as *in vitro*-methylated fragments. To confirm the feasibility, a well-studied epillale, *Cnr* allele of *Cnr* mutants in tomato was used. It is noted that DNA methylation at the promoter of a *CNR* gene represses its expression, inhibiting normal fruit ripening (Manning et al., 2006). To verify *Cnr* epimutant, non-ripening phenotype

was observed and bisulfite analysis of the *CNR* promoter was conducted (Figure 5A, B). Optimization of DME reaction condition was required to prevent incomplete digestion by DME. 500 ng of tomato DNA from leaves was incubated with varying amount of DME. To measure the efficiency of DME-digestion, PCR-amplified levels of the *CNR* promoter between the wild type and *Cnr* mutant were compared. It was shown that 600 ng of purified DME protein was sufficient to digest fully 500ng of genomic DNA (Figure 6). By using DME-qPCR under the condition, hypermethylated *Cnr* allele of the *CNR* mutant was distinct from the hypomethylated wild type allele at the *CNR* locus or an unmethylated gene, *ATP1* as a positive control (Figure 5C).

Also, the adequacy of DME-qPCR was confirmed using *Arabidopsis* genomic DNA. It was reported that a late-flowering *fwa* mutant of *Arbidopsis* was caused by loss of DNA methylation of SINE elements at a 5-regulatory region of *FWA* gene (Kinoshita et al 2007). Hypomethylation of the 5-regulatory region in *fwa* mutants was verified by sodium bisulfite analysis (Figure 7A, B). Likewise, 600 ng of purified DME protein was sufficient to prevent incomplete digestion using 500 ng of *Arabidopsis* DNA from leaves (Figure 8). A relative amplification of the 5-regulatory region was normalized by using an unmethylated gene, *ASA1*. In the *fwa* mutant, a relative amplification of the region was as high as the *ASA1* (Figure 7C). In brief, DNA methylation level of a particular locus could be efficiently detected by DME-qPCR.

DME-qPCR showed difference between hypermethylated wild type allele and hypomethylated *fwa* allele in F_2 population from *Arabidopsis*

Finally, DME-qPCR was applied to estimate the number of hypomethylated *fwa* epialelle at the *FWA* locus. To produce individuals with heterozygous *fwa* epialleles at the *FWA* locus, F_2 population was generated by a cross between the wild type (*Col*-0) and the *fwa-1* mutant (*Ler*). Segregation of *FWA* alleles was confirmed by genotyping with a CAPS (Cleaved Amplification Polymorphic Sequences) marker using a SNP (single nucleotide polymorphism) at the 5'-regulatory region of *FWA* (Figure 9). Genotypes perfectly reflected flowering time. The *fwa* mutant was dominant showing flowering phenotype (Figure 10). Hypomethylated *fwa* allele in total of F_2 individuals were detected by DME-qPCR under the reaction condition described above.

The 5'-regulatory region of *FWA* was hardly amplified in the individuals with homozygous wild type alleles at the *FWA* locus like the wild type, whereas the region was easily amplified in the individuals with homozygous of *fwa* epialleles like the *fwa* mutant. In heterozygous individuals with the wild type and the *fwa*-mutant alleles, the region was amplified, but not as much as the *fwa* mutant (Figure 10). The amplified levels of DME-qPCR were determined by the number of *fwa* eipalleles because hypomethylated *fwa* epiallele was not damaged by DME and easily amplified by qPCR. In summary, hypomethylated *fwa* allele could be amplified by DME–qPCR, and therefore, high relative amplification represented the number of hypomethylated *fwa* alleles at the *FWA* locus.

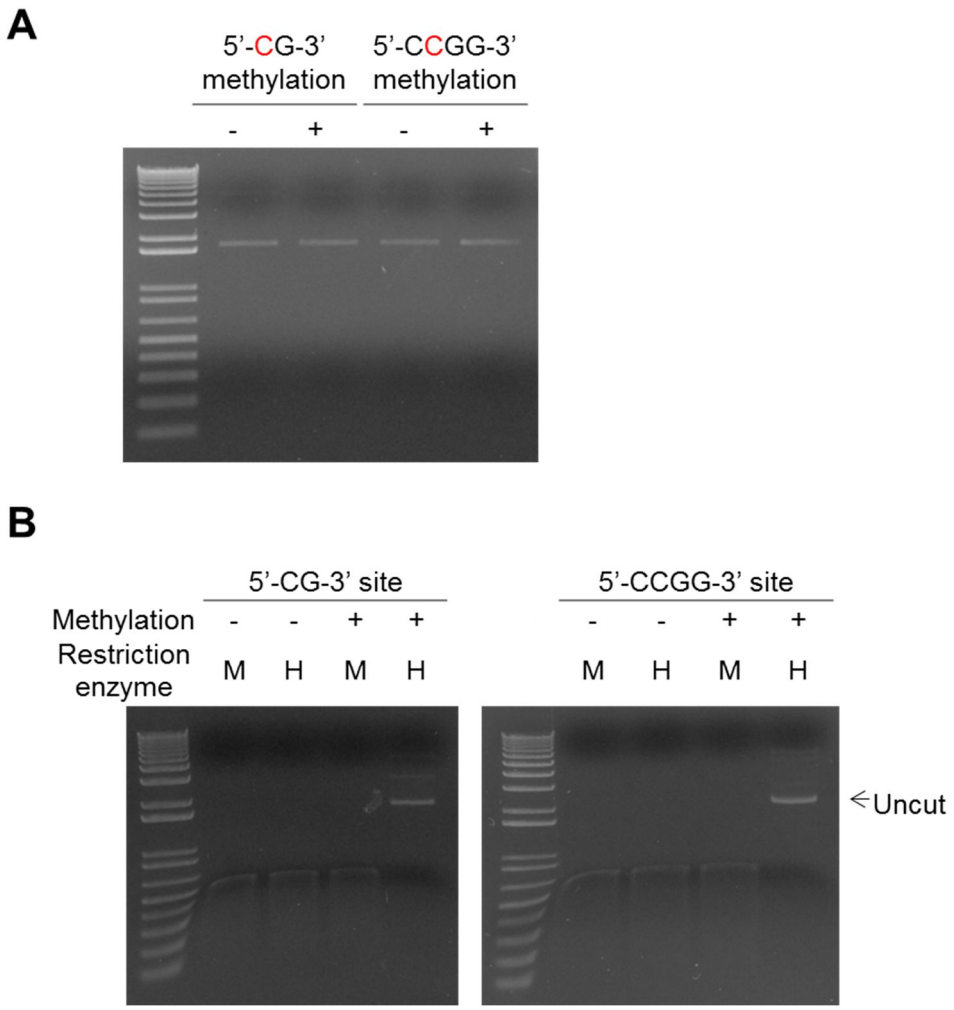

Figure 1. *In vitro* CG and CCGG methylation on pUC19 plasmids.

(A) Preparation of methylated pCU19 plasmids. *M.Sss*I methylates every CG site of pUC19 (lane 2). *Hpa*II methyltransferase methylates internal cytosine at CCGG site of pU19 (lane 4). Red-colored C indicates 5-methylcytosine. **(B)** Verification of *in vitro*-methylation using *Msp*I and *Hpa*II. Unmethylated plasmids were fully digested by *Msp*I (M) and *Hpa*II (H), whereas methylated plasmids were only digested by *Msp*I, not by *Hpa*II.

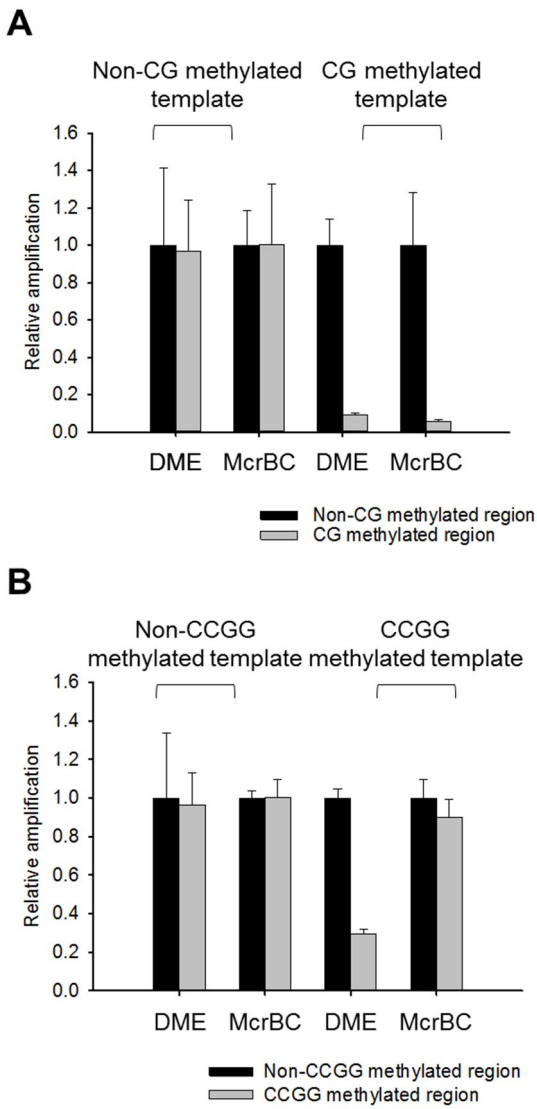

Figure 2. Comparison of DME and McrBC treatments on *in vitro*-methylated pUC19 plasmids.

(A, B) Relative PCR amplification levels after the DME and McrBC treatments on pUC19 plasmids with CG methylation by *M.Sss* I (A) or with CCGG methylation by *Hpa*II methyltransferase (B). Amplification levels of methylated regions were normalized relative to unmethylated regions.

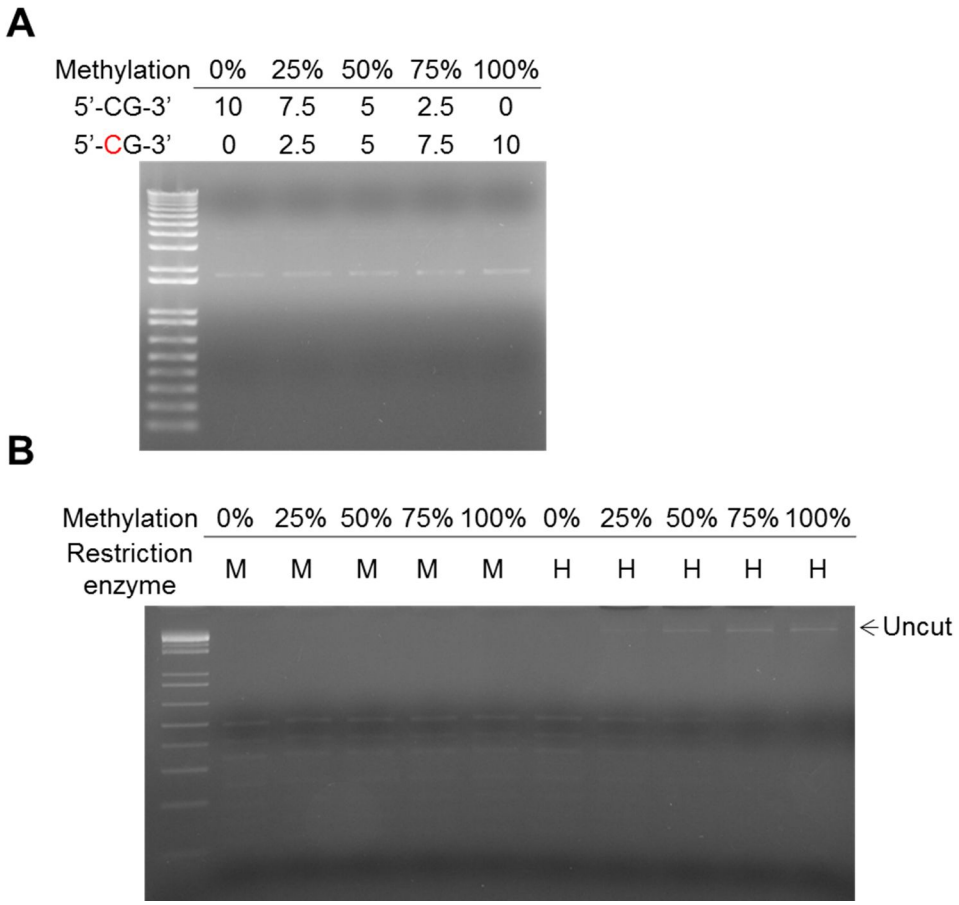

Figure 3. Preparation of pUC19 plasmids with differential DNA methylation.

(A) Preparation of DNA templates with different ratios of unmethylated and CG-methylated pUC19 plasmids. Red-colored C indicates 5-methylcytosine. **(B)** Verification of differential ratio with DNA methylation. Samples with only CG methylated plasmids were not digested by *Hpa*II. 'M' and 'H' indicate *Msp*I and *Hpa*II digestion, respectively. 'Uncut' indicates no digestion with *Hpa*II.

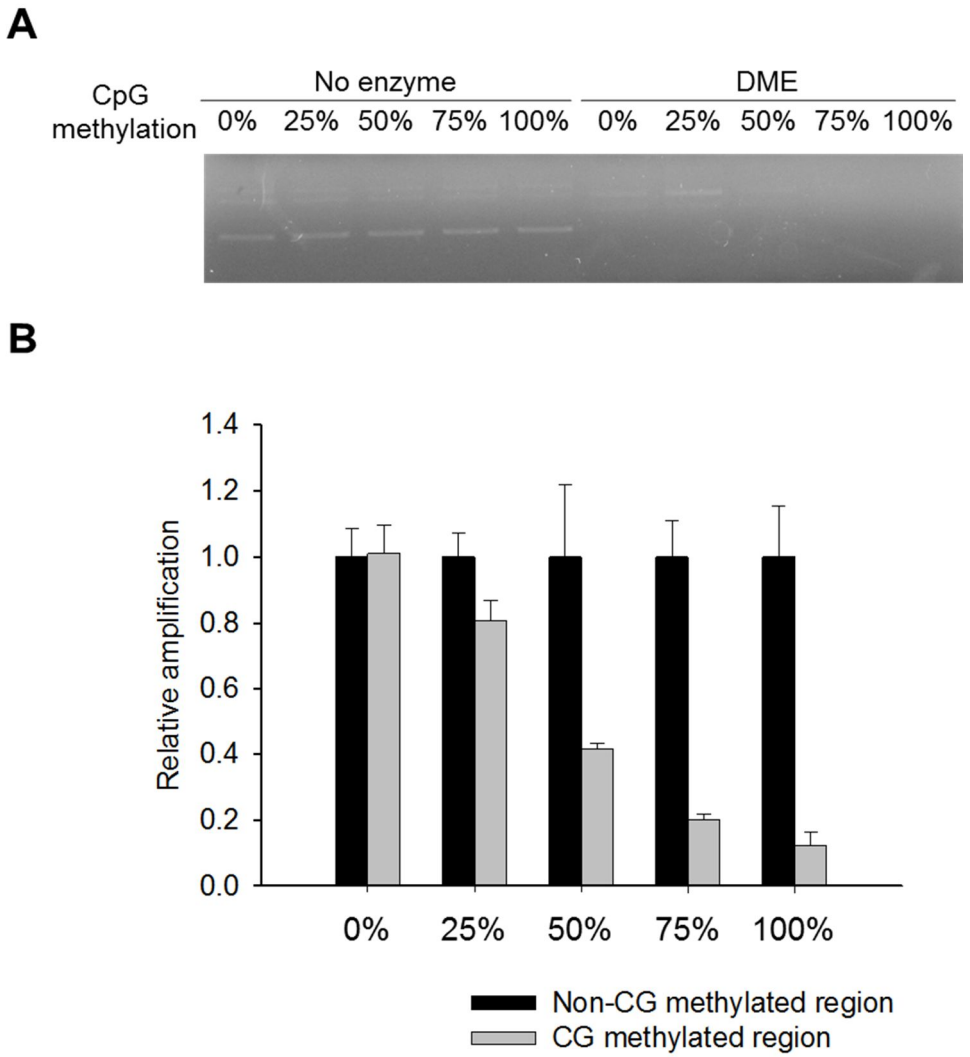

Figure 4. Detection of differential levels of DNA methylation by PCR amplification on DME-treated DNA templates.

(A) DME treatment on plasmid templates with different ratios of unmethylated and methylated pUC19 plasmids. **(B)** Relative PCR amplification levels using templates from (A). Amplification levels of methylated regions were normalized relative to unmethylated regions.

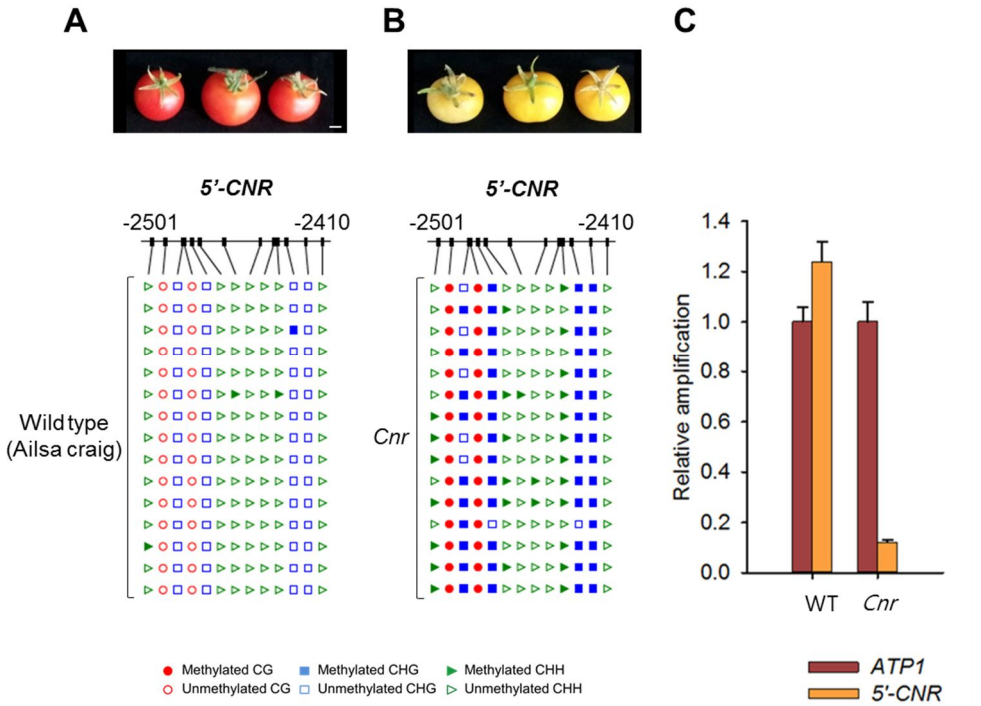

Figure 5. DME-qPCR distinguishes between hypomethylated wild type and hypermethylated *Cnr* alleles in tomato (*Solanum lycopersicum*). (A-B) Mature fruits of wild type (Ailsa Craig) (A) and near-isogenic line of *Cnr* mutant tomato (B). Their DNA methylation levels at the promoter region 150 bp upstream of translation start site were analyzed by bisulfite sequencing. (C) DME-qPCR on genomic DNA from the leaves of the wild type and *Cnr* mutant. *ATP1* was used as an unmethylated control. The PCR amplification level is relative to unmethylated *ATP1* gene. Scale bars=10 mm.

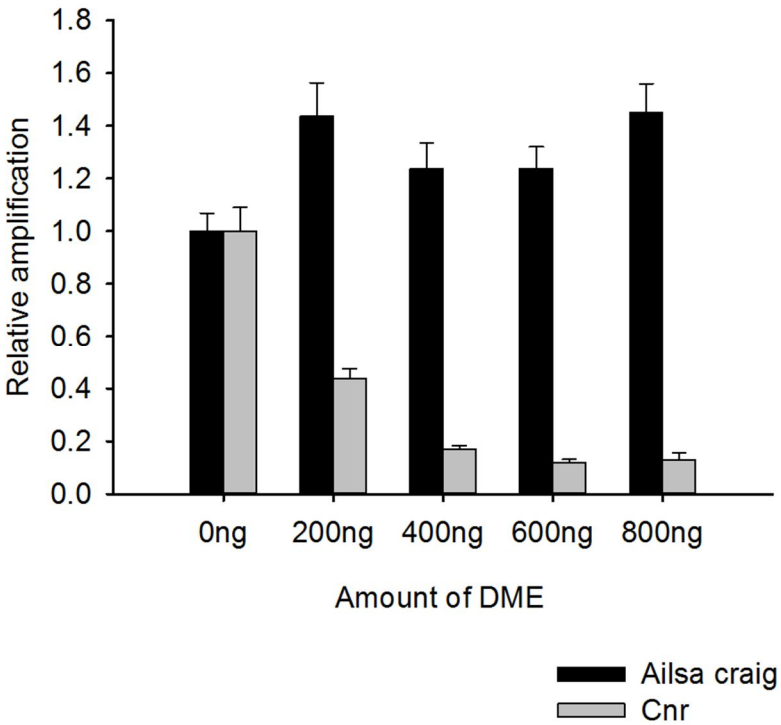

Figure 6. Optimization of DME-reaction condition using tomato genomic DNA.

To find the proper amount of DME for a complete reaction, 500 ng of tomato DNA damage was incubated with varying amount of DME for 2hr at 37℃. Fragmentation of DNA by DME was quantified by amplifying the *CNR* promoter.

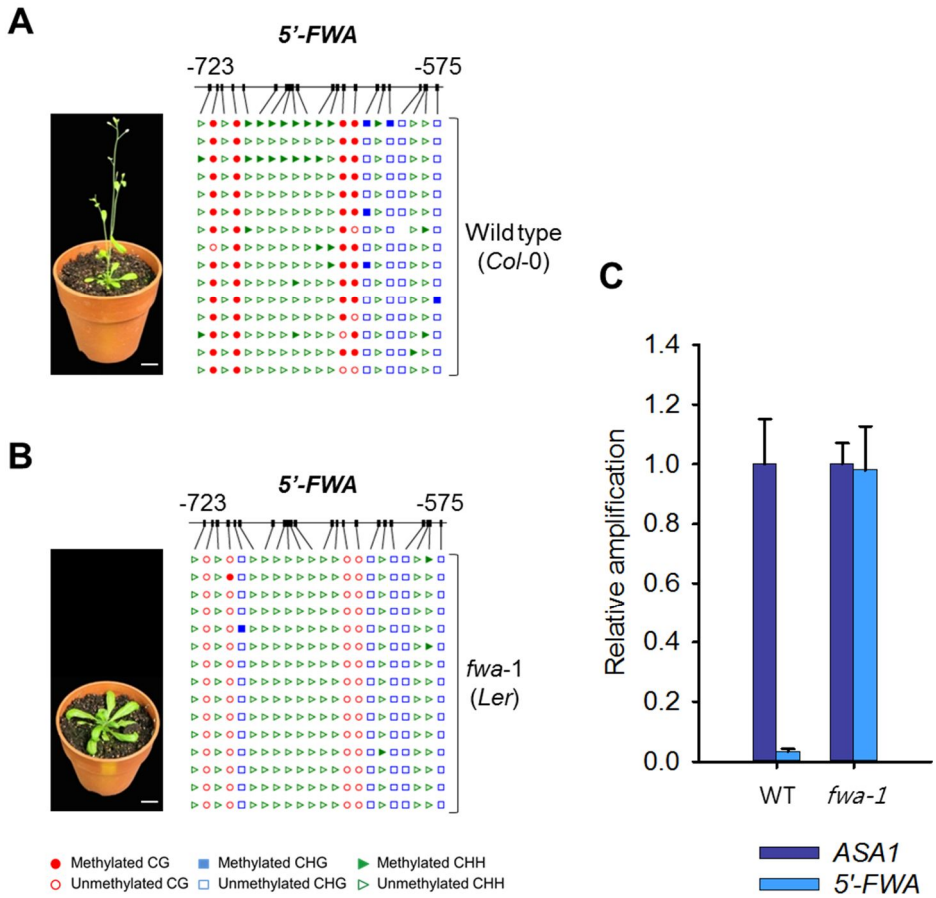

Figure 7. DME-qPCR distinguishes between hypermethylated wild type *FWA* and hypomethylated *fwa* alleles in *Arabidopsis*. (A-B) Flowering phenotypes at 30 days after sowing and bisulfite sequencing results of wild type (A) and *fwa-1* mutant (B). Their DNA methylation levels at the region 216 bp upstream of translation start site were analyzed. (C) DME-qPCR showed hypermethylation on the region 216 bp upstream of translation start site of wild type, compared to *fwa-1*. *ASA1* was used as an unmethylated control. The PCR amplification level is relative to unmethylated *ASA1* gene. Scale bars=10 mm.

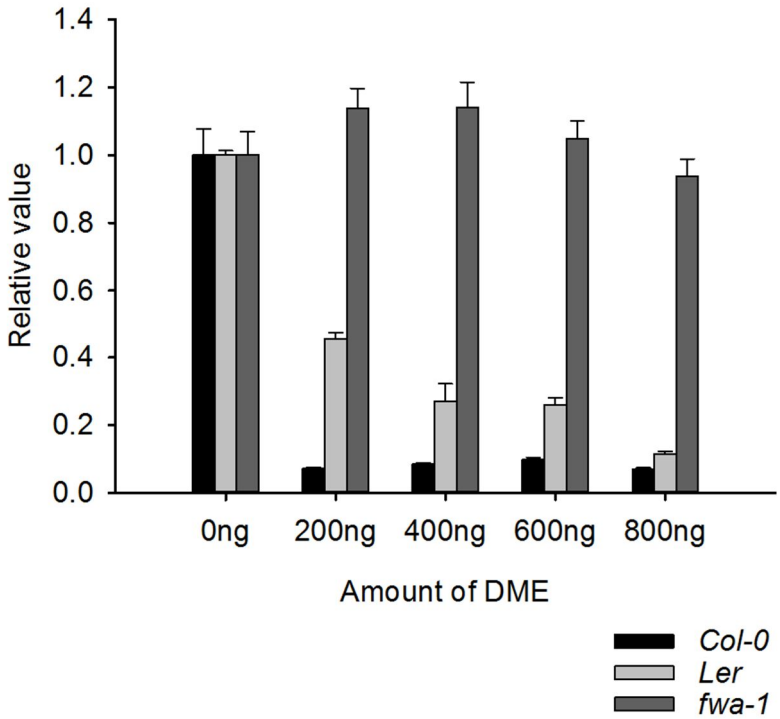

Figure 8. Optimization of DME-reaction condition using *Arabidopsis* genomic DNA.

To find the proper amount of DME for a complete reaction, 500 ng of *Arabidopsis* DNA damage was incubated with varying amount of DME for 2hr at 37℃. Fragmentation of DNA by DME was quantified by amplifying the 5'-regulatory region of the *FWA* gene.

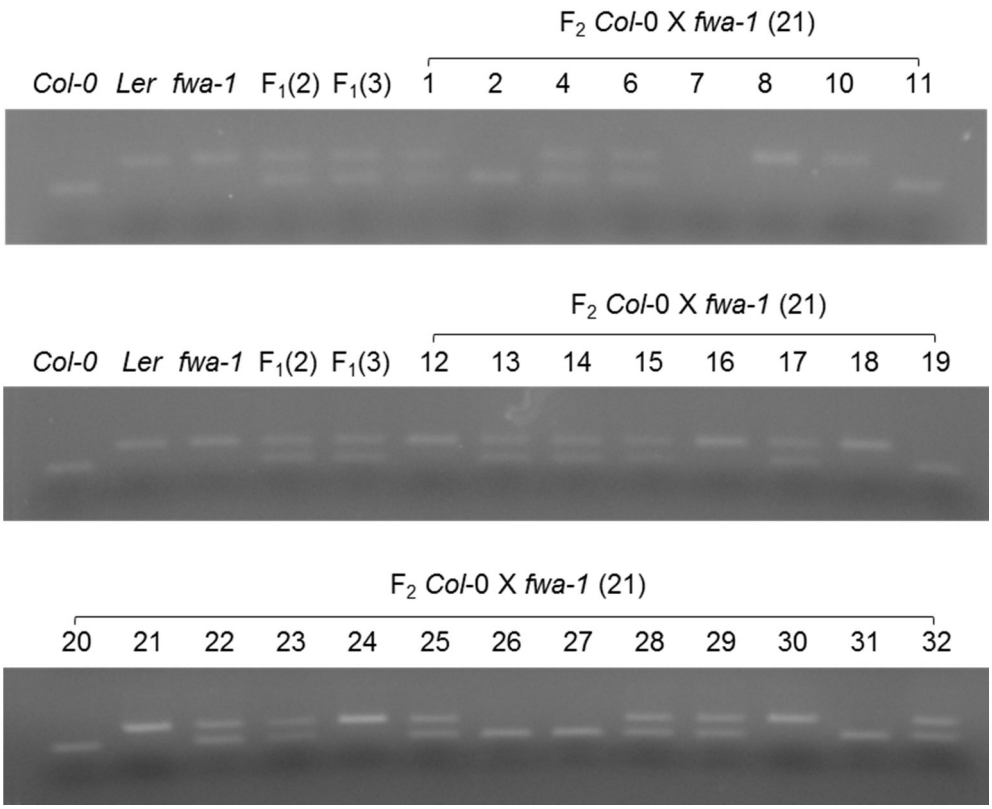

Figure 9. Genotyping the F_2 population from a cross between early-flowering wild type and late-flowering *fwa-1* mutant in *Arabidopsis*.

A CAPS (Cleaved Amplified Polymorphic Sequences) marker is based on SNPs of *FWA* alleles between *Col-0* and *fwa-1* in background *Ler*. PCR product from Col-0 has *Taq*$^{\alpha}$I cleavage site but *Ler* does not. Lower and upper bands display the *FWA* alleles from *Col-0* and *Ler*, respectively. By using this marker, the heterozygosity of the *FWA* allele could be distinguished.

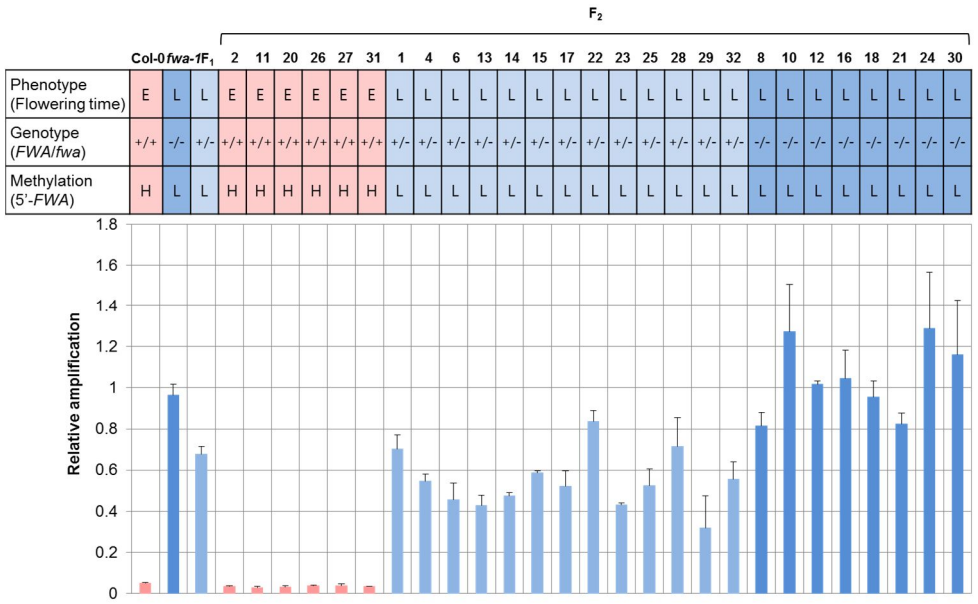

Figure 10. Correlation of DNA methylation levels of the F_2 population in *Arabiodpsis* with genotypes and phenotypes.

F_2 Population was generated by selfing an F_1 hybrid between wild type (*Col*-0) and *fwa-1* (*Ler*). 'E' and 'L' indicate early- and late-flowering phenotypes, respectively. Late-flowering is a dominant phenotype in F_2. A CAPS (Cleaved Amplified Polymorphic Sequences) marker was used for genotyping. '+' indicates wild type *FWA* allele derived from *Col-0*, whereas '–' indicates *fwa* allele from *Ler*. Methylation levels were designated as 'H' for hypermethylation and 'L' for hypomethylation. Red bars of bar graph indicate the plants with homozygous, hypermethylated wild type alleles. Light blue bars indicate heterozygous *fwa* alleles. Blue bars indicate homozygous *fwa* alleles.

DISCUSSION

In this study, a DNA methylation analysis based on the direct removal of 5mC was devised, which was proven useful to detect epialleles induced by aberrant DNA methylation patterns. To remove 5mC in a sequence non-specific manner, a plant-specific DNA demethylase DME was used, which catalyzes 5mC excision accompanied with the cleavage of sugar-phosphate backbone (Gehring et al., 2006; Mok et al., 2010). The level of DNA methylation was measured by quantitative PCR after DME treatment because it is proportional with the degree of DME-damaged DNA. The quantitative PCR combined with the DME treatment, DME-qPCR was tested using *in vitro*-methylated pUC19 plasmids and applied to detect hypermethylated *Cnr* allele of the *Cnr* tomato mutant and hypomethylated *fwa* allele in the *fwa Arabidopsis* mutant.

As McrBC recognizes 5mC and digests DNA unlike DNA methylation-sensitive enzymes such as *Hpa*II, the efficiency of DME treatment was compared with the McrBC treatment. qPCR after the DME treatment can detect both CG and CCGG methylation by measuring DNA fragmentation by DME because DME recognizes 5mC directly and makes a single nucleotide gap (Figure 2; Gehring et al., 2006). However, qPCR after the McrBC treatment did not detect CCGG methylation because McrBC recognizes DNA sequence contexts as well as 5mC (Figure 2B; Raleigh, 1992). Due to the properties of DME, qPCR using DME is more suitable than McrBC to evaluate whether DNA methylation exists or not.

After then, the sensitivity of DME-qPCR was tested on unmethylated or CG-methylated templates. DME-qPCR successfully tells the proportion of CG-

methylated plasmids in the mixed templates because the level of DNA methylation has a negative correlation with PCR amplification after the DME treatment (Figure 4). The sensitivity of DME-qPCR was sufficient to figure out the portion of methylated DNA in total DNA templates. It indicates that DME-qPCR is useful to detect the difference of DNA methylation levels between differentially methylated alleles.

Finally, DME-qPCR was applied to distinguish the epialleles from wild type alleles. Hypomethylated wild type allele of the *CNR* gene in tomato and the *fwa* allele in *Arabidopsis* were easily amplified by DME-qPCR. Only hypomethylated alleles were detected by DME-qPCR because hypermethylated alleles were digested by DME (Figures 5 and 7). Furthermore, DME-qPCR showed the difference between homozygous *fwa* alleles and heterozygous *fwa* alleles in F_2 population (Figure 10). Based on the principles of the method, DME-qPCR should estimate the number of methylated gene copies in an organism which has multiple copies of a gene. In addition, DNA methylation patterns at numerous loci might be analyzed at once using many pairs of primers, implying that DME-qPCR might be employed for genome-wide DNA methylation analysis.

As the dynamics of DNA methylation is associated with many biological processes, epigenetic molecular markers can be developed by DME-qPCR. Recently, the relationship between DNA methylation and plant development was reported in tomato (Manning et al., 2006; Zhong et al., 2013). During tomato fruit ripening, DNA methylation decreased at the two differentially methylated regions (DMRs) which are located downstream from the *Cnr* epiallele (Zhong et al., 2013). In human, the relationship with diseases and aberrant DNA methylation was

reported (Ballestar and Esteller, 2008). Especially, prostate cancer is closely associated with DNA hypermethylation of *GSTP1*, encoding the glutathione S-transferase (Cairns et al., 2001). As it has been revealed that several biological changes are accompanied with the alterations of DNA methylation, DME-qPCR should be adequate to check developmental stages or to diagnose some diseases.

DME treatment is more sensitive than *Hpa*II or McrBC treatment and more straightforward than ChIP or sodium bisulfite treatment to detect 5mC. qPCR combined with the DME treatment should analyze DNA methylation of numerous loci at once as well as a specific locus. Due to the properties of the DME treatment and qPCR, DME-qPCR should be an alternative method especially to screen epialleles or epimutants in populations such as epigenetic recombinant inbred lines (EpiRILs). Furthermore, DME-qPCR might be applicable to analyze DNA methylation of diverse organisms such as mammals. In addition to qPCR, other technologies such as AFLP (Amplified Fragment Length Polymorphism) or microarray can be combined with the DME treatment for genome-wide DNA methylation analysis.

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초 록

식물과 동물에서 DNA 메틸화는 히스톤 변형과 더불어 염색질 구조를 변화시켜 유전자 발현을 조절한다고 알려져 있다. 특히, 프로모터 주위에 위치한 DNA 메틸화는 유전자 발현을 억제하는 기능을 한다. 따라서 DNA 메틸화의 변화는 유전자 발현을 변화시켜 결국 표현형에 영향을 미친다. 이렇게 DNA 메틸화의 변화로 인해 발생하는 유전형질을 후성대립유전자라고 한다. 후성대립유전자를 파악하기 위해서는 DNA 메틸화 분석이 수행되어야 한다. 현재 다양한 원리를 기반으로 한 DNA 메틸화 분석법이 개발되어 있으며, 각기 다른 특징을 가지기 때문에 분석 목적에 맞게 선택되어 이용되고 있다. 본 연구에서는 5-메틸시토신을 직접 제거하여 DNA 메틸화를 분석할 수 있는 새로운 분석법을 고안하였다. 그 과정에서 식물에만 특이적으로 존재하는 DNA 탈메틸화 효소 (DME)를 이용하였다. 이 DNA 탈메틸화 효소는 여타의 DNA 절단효소와는 다르게 DNA 서열과는 상관없이 메틸화된 DNA를 인지하고 직접 제거하여 DNA 단가닥 절단을 유도한다. 따라서 이 탈메틸화효소에 의해 메틸화 정도가 높은 부분은 단가닥 절단이 많이 일어나게 되고 그로 인해 해당 부분의 DNA 증폭이 잘 일어나지 못한다. 이와 같이 탈메틸화 효소처리와 PCR 기법을 이용하여 증폭량과 반비례하여 DNA 메틸화 수준을 파악해볼 수 있다. 본 연구에서는 토마토와 애기장대의 후성대립유전자를 탐지해봄으로써

DNA 탈메틸화 효소를 이용하는 방법이 DNA 메틸화 수준을 파악하는데 유용하다는 것을 확인하였다.

주요어: 후성대립유전자, DNA 메틸화, DNA 메틸화 분석, DNA 탈메틸화 효소

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