Analysis of Nucleic Acids Methylation in Plants

Bi-Feng Yuan

Abstract 5-methylcysine (5-mC) and N^6-methyladenosine (m^6A) are important epigenetic marks occurring in nucleic acids of plants with regulatory roles in a broad range of biological processes. Recently, some novel modifications with potential regulatory roles such as 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-foC), and 5-carboxylcytosine (5-caC) have also been discovered in plants. Systematic investigation of the functions of nucleic acid modifications will promote the understanding of the mechanism underlying association of epigenetic modifications with plant development and response to environmental stresses. In this respect, great advances have been made in the development of methods for investigation of the occurrence and localization of these epigenetic modifications in nucleic acids of plants. Here, we focus on the recent methodological advances for the analysis of the global levels of DNA and RNA methylation. In addition, we will discuss the mostly used methods for mapping the genome-wide distribution of DNA and RNA methylation.

Keywords DNA methylation • RNA methylation • 5-methylcysine • 5-hydroxymethylcytosine • 5-formylcytosine • 5-carboxylcytosine • N^6-methyladenosine • global detection • mapping

Contents

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1 General Functions of DNA and RNA Methylation in Plants

Methylation modifications, notably in the forms of 5-methylcytosine (5-mC) and N^6-methyladenosine (m^6A) in both DNA and RNA, perform important regulatory functions in various biological processes (Chen et al. 2016; Shen et al. 2014; Wu and Zhang 2014). DNA and RNA methylation occurs in almost all living organisms, from bacteria to fungi, plants, and mammals (Motorin and Helm 2011; Zemach and Zilberman 2010). The fine-tuning of chromatin structure by DNA and RNA methylation is one of the major hallmarks of gene regulation during cellular development (He 2010; Jones 2012).

1.1 DNA Cytosine Methylation in Plants

Although the levels of 5-mC are relatively low in mammalian genomes (3–8% of total cytosine), 5-mC presents a much abundant level in plant genomes (5–25% of total cytosine) (Rangwala and Richards 2004). Plants have more complicated and sophisticated system of the genome methylation compared to animals. In plants, cytosine can be methylated at CG, CHG, and CHH sites (H is A, T, C) (Matzke et al. 2015). DNA methylation in plants is predominantly controlled by domains rearranged as methyltransferases 2 (DRM2) via the RNA-directed DNA methylation pathway and maintained by DNA methyltransferases 1 (MET1), chromomethylase 3 (CMT3), and DRM2 (Chan et al. 2005). CG methylation is mediated by MET1; CHH methylation is controlled by DRM2, while the plant-specific CMT3 regulates CHG methylation (Chan et al. 2005). DNA methylation in plants is involved in the control of genetic functions including transcription, replication, gene transposition, and cell differentiation (Law and Jacobsen 2010).

1.2 RNA Cytosine Methylation in Plants

In addition to occurring in DNA, 5-mC has also been identified in different RNA species from all kingdoms of life (Motorin and Helm 2011; Motorin et al. 2010). 5-mC residues in tRNAs are known to influence their secondary structural, stabilization, and codon recognition (Helm 2006; Squires and Preiss 2010). 5-mC sites

are also found in rRNA where they play critical roles in recognition of tRNA and translational fidelity (Chow et al. 2007). And internal 5-mC in mRNA was also identified (Edelheit et al. 2013). In plants, the level of 5-mC in total RNA is about 0.88% in *Lepidium sativum*. When plant was exposed to abiotic stress, such as Cd (II) or Se(IV), 5-mC in both DNA and RNA changed (Yanez Barrientos et al. 2013). The advances in the field of epigenetics suggest that RNA cytosine methylation might play a similar role in the modulation of genetic information as DNA cytosine methylation in plants (Mattick et al. 2009).

1.3 RNA Adenine Methylation in Plants

Recent discovery of reversible m^6A modification on mRNA and mapping of m^6A in mammals revealed potential regulatory functions of this RNA adenine modification. In *Arabidopsis thaliana*, m^6A content in mRNA varies across tissues with a high ratio of m^6A/A found in flower buds, and defects in m^6A methyltransferase cause an embryo-lethal phenotype, suggesting a critical role of m^6A in plant development (Zhong et al. 2008). Recently, m^6A mapping analysis showed that m^6A is a highly conserved modification in mRNA of *Arabidopsis thaliana* (Luo et al. 2014). m^6A in mRNA of *Arabidopsis thaliana* is enriched around both the stop codon and the start codon. A positive correlation between m^6A deposition and mRNA levels indicates a regulatory role of m^6A in plant gene expression (Luo et al. 2014).

2 Global Detection of DNA and RNA Methylation in Plants

Established methods for the determination of global DNA and RNA methylation in plants mainly include liquid chromatography (LC), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis (CE), thin layer chromatography (TLC), and immuno-based detection. The global detection of DNA and RNA methylation requires the liberation of DNA/RNA components with enzymatic/chemical treatments followed by determination of the components with various methods.

2.1 Liquid Chromatography

The analysis of global DNA and RNA methylation by LC is based on the chromatographic separation of the components by enzymatic or chemical hydrolysis of DNA/RNA. Therefore, the baseline separation of the DNA/RNA components is necessary to avoid co-elution of analytes.

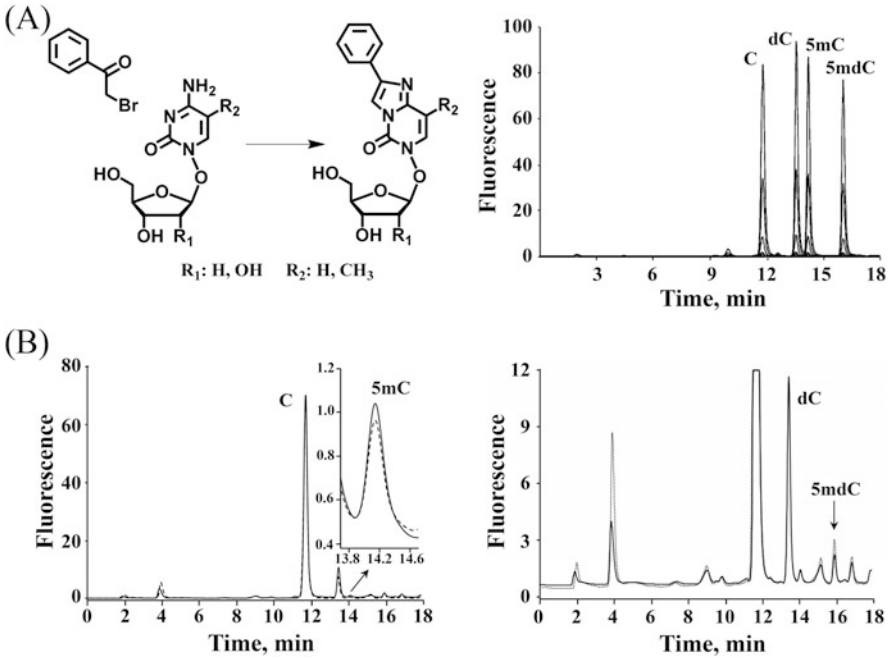

Fig. 1 (**a**) Schematic illustration for selective derivatization of cytosinemoieties with 2-bromoacetophenone for the determination of global DNA methylation by reversed phase liquid chromatography with spectrofluorimetric detection (Reprinted with permission from Torres AL, Barrientos EY, Wrobel K. *Anal Chem*, 2011, 83: 7999–8005). (**b**) LC-FLD chromatograms of control plant (*solid line*) and plant exposed to 1.0 mgL^{-1} Se(IV) (*dashed line*). *Left panel*: Y-scale and insert adjusted to visualize 5-mC and C signals (RNA methylation); *right panel*: Y-scale adjusted to visualize 5-mdC and dC signals (DNA methylation) (Reprinted with permission from Yanez Barrientos E, Wrobel K, Lopez Torres A, Gutierrez Corona F, Wrobel K. *Anal Bioanal Chem*, 2013, 405: 2397–2404)

Johnston et al. (2005) examined the methodological factors in LC analysis of plant DNA methylation using in vitro cultures of *Ribes ciliatum*. The results demonstrated that complete removal of RNA from plant DNA is difficult using RNase digestions and LiCl precipitation, suggesting that nucleobases analysis should be avoided as nucleobases from residual RNA fragments will interfere DNA-derived nucleobases. Nucleoside or nucleotide analysis is therefore recommended as a more suitable option. Liquid chromatographic techniques generally are quantitative, reproducible, but less sensitive. In this respect, a relatively large amount of genomic DNA (~1–50 μg) is normally needed.

To increase the detection sensitivity of 5-mC by LC-based detection, selective derivatization of cytosine moieties with 2-bromoacetophenone followed by reversed phase LC with spectrofluorimetric detection was developed (Torres et al. 2011; Yanez Barrientos et al. 2013) (Fig. 1). The proposed method was capable for the detection of as low as 0.06% of methylation in 80 ng of DNA and can be used

for the evaluation of RNA methylation at the same time. With this method, Barrientos et al. (2013) analyzed the global DNA and total RNA methylation in *Lepidium sativum* and further assessed the effect of Cd(II) and Se(IV) exposure on DNA and RNA methylation of *Lepidium sativum*.

2.2 *Liquid Chromatography-Mass Spectrometry*

Due to the good selectivity and sensitivity, LC-MS has been widely used in the analysis of nucleic acid modifications.

Our group developed various methods for the detection of DNA and RNA methylation by LC-MS (Chen et al. 2013; Huang et al. 2016a, b, 2015; Jiang et al. 2015, 2016; Shen et al. 2015; Tang et al. 2013, 2014, 2015; Wang et al. 2013; Xiong et al. 2015; Yuan 2014; Yuan and Feng 2014; Yuan et al. 2011; Zhang et al. 2016). Specifically, we recently established a chemical derivatization strategy combined with liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method to determine 5-formyl-2′-deoxycytidine (5-fodC) and 5-carboxyl-2′-deoxycytidine (5-cadC) in plants (Tang et al. 2014) (Fig. 2). Derivatization of 5-fodC and 5-cadC by Girard's reagents significantly increased the detection sensitivities of 5-fodC and 5-cadC by 52 to 260folds. Using this method, we demonstrated the widespread existence of 5-fodC and 5-cadC in genomic DNA of various plant tissues, with contents of 5-fodC ranging from 2.1 to 4.7 modifications per 10^6dG and 5-cadC ranging from 0.2 to 3.6 modifications per 10^6dG. Moreover, we found that environmental stresses of drought and salinity can change the contents of 5-fodC and 5-cadC in plant genomes, suggesting the functional roles of 5-fodC and 5-cadC in response to environmental stresses.

In addition, Liu et al. (2013) reported the use of a reversed-phase LC coupled with tandem mass spectrometry method and stable isotope-labeled standards for assessing the levels of the oxidized 5-mC nucleosides in DNA of *Arabidopsis*

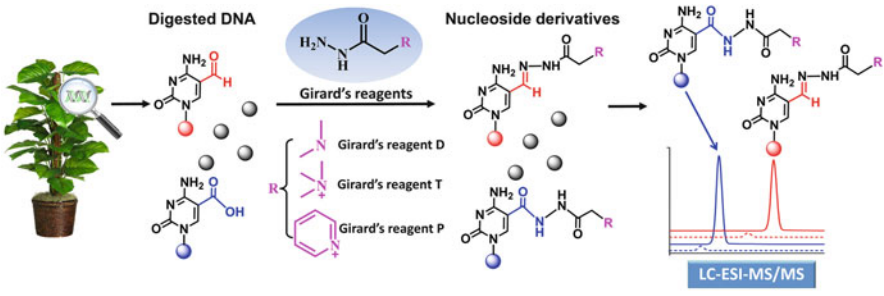

Fig. 2 Schematic diagram for determination of 5-fodC and 5-cadC in genomic DNA of plant samples by chemical derivatization using Girard's reagents coupled with LC/ESI-MS/MS analysis (Reprinted with permission from Tang Y, Xiong J, Jiang HP, Zheng SJ, Feng YQ, Yuan BF. *Anal Chem*, 2014, 86: 7764–7772)

thaliana. The quantitative results showed that the occurrence of 5-hmC, 5-foC, and 5-caC in DNA of *Arabidopsis thaliana* were 0.79, 0.79, and 0.78 modifications per 10^6nucleosides, respectively. This method is involved in the pre-enrichment of these modified nucleosides by LC, which can minimize the interference from other abundant nucleosides during subsequent mass spectrometry analysis but will increase the analytical time.

Recently, Magana et al (2016) investigated the effect of CuO nanoparticles on global DNA and RNA methylation in *Lepidium sativum* by liquid chromatography/ion trap mass spectrometry. Enhanced selectivity toward cytosine-containing nucleosides was achieved using proton-bound dimers formed in positive electrospray ionization as precursor ions for multiple reaction monitoring (MRM) quantification. The quantitative results showed that 13.03% and 0.92% methylated cytosines were found in DNA and RNA, respectively. Upon CuO nanoparticles treatment, DNA hypomethylation was observed, but RNA methylation did not present significant changes.

2.3 Capillary Electrophoresis

Several studies have developed capillary electrophoresis methods for the detection of genomic DNA methylation content in plant tissues. Fraga et al. (2000) used open-tube capillary electrophoresis system to separate acid hydrolyzed genomic DNA (nucleobases) from *Pinus radiate* trees for the evaluation of genomic DNA methylation. The problem is that the contamination of RNA may also contribute to the acid hydrolyzed products of nucleobases. In this respect, later they developed micellar capillary electrophoresis with UV-Vis detection to analyze the nucleosides of enzymatic products of DNA from *Pinus radiate* trees (Fraga et al. 2002). The detection and quantification of nucleosides through enzymatic hydrolyses notably increases the specificity and allows its exploitation in the analysis of poorly purified and/or concentrated DNA samples.

The capillary electrophoresis method offers the advantages of high resolution and cost-effective separations, providing an efficient approach to quantify nucleic acids methylation. The drawback of capillary electrophoresis method is that sample loading volume is limited and separation reproducibility can be affected by slight variations, which requires further improvements.

2.4 Thin Layer Chromatography

TLC-based method normally requires ribo- and deoxyribonucleotides that are generally distinguishable. After isolation of nucleic acids, the digested nucleosides were labeled with radioactive phosphate which enables sensitive determination of the contents of global DNA and RNA methylation. With the TLC method, global DNA methylation was successfully detected and quantified in various plant samples, including *Arabidopsis* and *Cardaminopsis arenosa* (Madlung et al. 2002),

Artemisia annua (Pandey and Pandey-Rai 2015), *Pyruscommunis* (Michalak et al. 2013), and *Quercus robur* (Michalak et al. 2015).

TLC method is cost-effective, and there is no need for sophisticated instrumentation. So TLC method is frequently used in biological lab for discovery and quantification of modified DNA and RNA. However, TLC-based method involves radioactive isotope labeling, and the analytical procedure is relatively tedious.

2.5 Immuno-Based Detection

Immunostaining is a technique widely used to evaluate the presence of DNA and RNA methylation. This technique relies antibodies that can selectively recognize the corresponding modified DNA and RNA inside cells for cell-based visualization.

Zluvova et al. (2001) employed immunostaining technique to examine the global changes of DNA methylation during seed germination and shoot apical meristem development in *Silene latifolia*. The data showed that a rapid decrease in global DNA methylation during seed germination occurred first in endosperm tissue and subsequently in the hypocotyl. To reveal the dynamics of the methylation pattern, correct epitope retrieval sometimes is essential and a deep denaturation step is needed.

In addition to 5-mC in DNA, Yao et al. (2012) found that DNA in leaves and flowers of *Arabidopsis thaliana* contains low level of 5-hmC by immuno-based dot-blot technique (Fig. 3). Using in vitro binding assays, the authors observed that full-length VIM1 protein binds preferentially to hemi-methylated DNA. However, when 5-hmC replaces one or both cytosine residues at CpG site, VIM1 binds with tenfold lower affinity. These results suggest that 5-hmC may contribute to VIM-mediated passive loss of cytosine methylation in vivo during DNA replication in *Arabidopsis thaliana*.

In the past several years, many commercially available kits, such as enzyme linked immunosorbent assay (ELISA) with chemiluminescence detection-based analysis, were also produced and employed for the detection of global 5-mC in *Phelipanche ramosa* (Lechat et al. 2015) and rice (Ferreira et al. 2015) and global 5-mC and 5-hmC in *B. oleracea* and *C. sativus* (Moricova et al. 2013).

3 Location Analysis of DNA and RNA Methylation in Plants

The quantitative distribution information of DNA and RNA methylation is crucial to understand their biological functions. The advance in sequencing technologies accelerates and revolutionizes the genome-wide distribution studies of DNA and RNA methylation (Mardis 2013). Two major strategies, including affinity

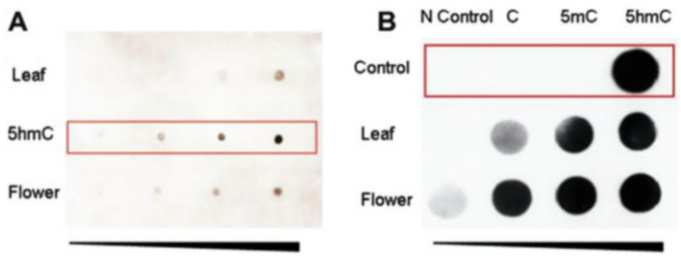

Fig. 3 Dot-blot detection of 5-hmC in DNA of *Arabidopsis thaliana*. (**a**) Dot-blot assay of synthetic DNA and *Arabidopsis thaliana* genomic DNA containing biotin-N3-5-gmC. The amount of loaded DNA increased from left to right. Detection *top row*: leaf DNA (25, 50, 100, 200 ng); *middle row*: 5-hmCcontaining DNA standard (boxed) (1–8 ng); *bottom row*: flower DNA (25, 50, 100, 200 ng). (**b**) Dot-blot assay using antibody to 5-hmC to detect 5-hmC in synthetic DNA or *Arabidopsis thaliana* genomic DNA. Spotted samples in *top row* (boxed): N Control: H_2O; C: Synthetic DNA with C; 5-mC: Synthetic DNA with 5mC; 5-hmC: Synthetic DNA with 5-hmC; 0.5 ng of each synthetic DNA were loaded; *middle row* and *bottom rows* (leaf and flower DNA, respectively): The amount of *Arabidopsis thaliana* DNA (0.5, 1, 2, 4 ng) increased from left to right (Reprinted with permission from Yao Q, Song CX, He C, Kumaran D, Dunn JJ. *Protein Expr Purif*, 2012, 83:104–111)

enrichment and bisulfite conversion followed by next-generation sequencing, have been widely used to profile the location of 5-mC in DNA and m^6A in RNA of plants.

3.1 Affinity Enrichment-Sequencing Analysis

Affinity enrichment of modified DNA and RNA using antibodies or affinity binding proteins has been proved to be a powerful tool for comprehensive profiling of 5-mC and its derivatives (Thu et al. 2010). The enriched DNA-antibody/protein complex can be analyzed using sequencing-based technologies (Down et al. 2008). However, affinity enrichment-sequencing methods do not provide location information at single-base resolution.

Methylated DNA immunoprecipitation sequencing (MeDIP-seq) relies on the use of antibody to precipitate fragments of DNA containing methylated cytosine (Down et al. 2008). Related methods include MBD-seq (Serre et al. 2010) and MethylCap-seq (Brinkman et al. 2010), which employ methyl CpG binding domain protein to precipitate DNA fragments containing methylated CpG sites. These approaches have the advantage that a greatly reduced portion of the genome needs to be sequenced. However, the major drawback of these approaches is the low resolution for identifying methylation sites.

Genome-wide profiles of DNA methylation for maize (*Zea mays*) inbred lines by MeDIP-seq demonstrated that DNA methylation variation is influenced by genetic and epigenetic changes that are often stably inherited and can affect the expression

of nearby genes (Eichten et al. 2013). Hu et al. (2015) used MeDIP-seq to profile DNA methylation in the rice PTGMS line PA64S under two different phenotypes (sterility and fertility). The results revealed that hypermethylation was observed in PA64S (sterility), and 1258 differentially methylated regions were found between PA64S (sterility) and PA64S (fertility).

In addition to 5-mC in DNA, Luo et al. (2014) established m^6A-targeted antibody enrichment coupled with next-generation sequencing to map transcriptome-wide m^6A in *Arabidopsis thaliana*. The results showed that m^6A is a highly conserved modification of mRNA in plants. Distinct from mammals, m^6A in *Arabidopsis thaliana* is enriched around both the stop codon and the start codon. The distribution pattern of m^6A in *Arabidopsis thaliana* is associated with plant-specific pathways involving the chloroplast. A positive correlation between m^6A deposition and mRNA abundance was observed, suggesting a regulatory role of m^6A in plant gene expression. The m^6A transcriptome-wide study of *Arabidopsis thaliana* provides a starting roadmap for uncovering m^6A functions that may regulate plant metabolism.

Recently, Shen et al. (2016) employed anti-m^6A polyclonal antibody enrichment coupled with next-generation sequencing that further map the m^6A sites in mRNA of *Arabidopsis thaliana*. The results suggested an indispensable role of FIP37 (a core component of the m^6A methyltransferase complex) in mediating m^6A mRNA modification, which is required for maintaining the shoot meristem as a renewable source for continuously producing aerial organs in plants.

3.2 Bisulfite Conversion-Sequencing Analysis

The discovery that treatment of DNA with sodium bisulfite revolutionized DNA methylation analysis since 1990s (Clark et al. 1994; Frommer et al. 1992). And various methodologies have been developed based on bisulfite treatment that leads to the conversion of unmethylated cytosine to uracil, while methylated cytosine remains unchanged in DNA and RNA (Plongthongkum et al. 2014; Schaefer 2015). Amplification by polymerase chain reaction of converted DNA followed by sequencing can reveal positions of 5-mC in DNA and RNA. Since bisulfite conversion-sequencing strategy can provide single-base resolution for DNA and RNA cytosine methylation, the technique has been widely utilized in various plant samples, including *Arabidopsis thaliana* (Cokus et al. 2008; Feng et al. 2010; Ibarra et al. 2012; Lister et al. 2008; Shen et al. 2012; Stroud et al. 2013; Yu et al. 2013), rice (Li et al. 2012), soybean (Song et al. 2013), and *Marchantia polymorpha* (Takuno et al. 2016).

With applications of bisulfite conversion-sequencing to wild-type *Arabidopsis thaliana* and mutants defective in DNA methyltransferase or demethylase activity, Lister et al. (2008) observed local sequence effects upon methylation state and revealed a direct relationship between the location of smRNAs and DNA

methylation. Cokus et al. (2008) identified sequence motifs that associate with high and low methylation for each different context of methylation in *Arabidopsis thaliana*.

Using the bisulfite conversion-sequencing, Stroud et al. (2013) substantially extended and refined the characterization of regulatory factors of the methylome by examining 86 *Arabidopsis thaliana* mutants, suggesting that individual sites of methylation may be regulated by novel RNA-directed pathways. Feng et al. (2010) carried out bisulfite conversion-sequencing in the flowering plants rice and *Arabidopsis thaliana* and found that the patterns of methylation were similar in flowering plants with methylated cytosines detected in all sequence contexts. Shen et al. (2012) found that genome-wide remodeling of DNA methylation mediated by the RNA-directed DNA methylation pathway in *Arabidopsis thaliana* may play a role in heterosis. Yu et al. (2013) found some transposable elements are demethylated and transcriptionally reactivated during antibacterial defense in *Arabidopsis thaliana*, which provides evidence that DNA demethylation is part of a plant-induced immune response and can potentially act to transcriptional activation of some defense genes linked to transposable elements and repeats. Ibarra et al. (2012) demonstrated that demethylation in companion cells reinforces transposon methylation in *Arabidopsis thaliana* gametes and contributes to stable silencing of transposable elements across generations.

In addition to the most studied model plant of *Arabidopsis thaliana*, Li et al. (2012) generated single-base resolution DNA methylome maps by bisulfite conversion-sequencing for Asian cultivated rice *Oryza sativa* ssp. *japonica*, *indica,* and their wild relatives, *Oryza rufipogon* and *Oryza nivara*. The overall methylation level of rice genomes is four times higher than that of *Arabidopsis thaliana*. Interestingly, the authors discovered that methylation in gene transcriptional termination regions can significantly repress gene expression, and the effect is stronger than that of promoter methylation.

Song et al. (2013) also analyzed the DNA methylation status in soybean roots, stems, leaves, and cotyledons of developing seeds at single-base resolution. Profiling of DNA methylation in different organs revealed 2162 differentially methylated regions among organs. Recently, Takuno et al. (2016) studied the single-base resolution methylome that span the phylogenetic breadth of land plants using bisulfite conversion-sequencing. The results showed that a basal land plant, *Marchantia polymorpha*, lacks evident signal of gene-body methylation within exons, but conifers have high methylation levels in both CG and CHG sites in expressed genes, which indicated the evolutionary forces acting on DNA methylation vary substantially across species, genes, and methylation contexts.

The advantage of bisulfite-converted strategy is that it can provide single-base resolution for DNA and RNA methylation analysis. However, the sample preparation associated with bisulfite sequencing can be time-consuming, and the conversion process may result in DNA and RNA degradation and reduce sequence complexity. As bisulfite analysis depends on the complete conversion of unmethylated cytosines to uracil, incomplete conversion will cause error or inaccurate results. In addition, discrimination between dC, 5-mC, and 5-hmC cannot be

accomplished by bisulfite sequencing. Therefore, these exiting issues need to be further addressed.

3.3 Single-Molecule Detection

DNA methylation analysis by single-molecule, real-time (SMRT) sequencing without bisulfite conversion was first established in 2010 (Flusberg et al. 2010). The SMRT sequencing is considered to be the third-generation sequencing technology and can realize the direct distribution study of 5-mC in DNA. In SMRT sequencing, DNA polymerases catalyze the incorporation of fluorescently labeled nucleotides into complementary DNA strands. The recording of nucleotide incorporations generates the sequence readout and information about the polymerase kinetics, which are used to discriminate different nucleotides.

In addition to SMRT method, nanopore sequencing technology also has been established to single-molecule detection of modified nucleosides (Branton et al. 2008). Nanopore analysis uses a voltage to drive molecules through a nanoscale pore and monitors how the ionic current through the nanopore changes as single molecules pass through it (Venkatesan and Bashir 2011). Different nucleotides passing through nanopores generate different electric currents, which can be measured and designated to the corresponding nucleotides or modified nucleotides. The methodology is successfully used to distinguish methylated from unmethylated cytosines without bisulfite conversion (Clarke et al. 2009; Mirsaidov et al. 2009).

Using the SMRT sequencing, Kim et al. (2014) obtained high-coverage SMRT sequence datasets from five organisms including *Arabidopsis thaliana*. Later, Berlin et al. (2015)introduced the MinHash Alignment Process (MHAP) and integrated MHAP with the Celera Assembler, enabling reference-grade de novo assemblies of *Arabidopsis thaliana* from SMRT sequencing. Although the SMRT sequencing and nanopore sequencing techniques haven't been employed for DNA methylation analysis in plants so far, these powerful techniques are qualified for mapping of DNA methylation and we expect the technique will be used in plant samples in the near future.

4 Conclusions and Perspectives

DNA and RNA methylation are important epigenetic modifications in eukaryotes to maintain genome integrity and regulate gene expression. DNA methylation in plants is species-, tissue-, organelle- and age-specific. Although DNA cytosine methylome and RNA adenine methylome have been profiled in plants, however, these previous studies mainly used the mixture of different cell types for methylome analysis, which may shade somemethylation patterns in specific cell types. Therefore, the improvement on the isolation of cell type-specific nucleic acids and

methylome sequencing in a single cell would facilitate to understand the dynamics of DNA and RNA methylation in plants.

The application of next-generation sequencing technology in DNA and RNA methylation studies has greatly contributed to our knowledge of DNA and RNA methylation. Future applications of some newly developed sequencing approaches such as single-molecule sequencing approaches are particularly well suited for the location study of DNA and RNA methylation. SMRT and nanopore sequencing offer the potential for direct sequencing of nucleic acid modifications without complicated pretreatment. The advancement of new technologies and methods may also lead to the discovery of novel epigenetic modifications in both DNA and RNA that will enhance our understanding of the fundamental issues in cellular developmental processing in plants.

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