Indian J. Genet. Plant Breed., (2024); 84(2): 143-155
https://doi.org/10.31742/ISGPB.84.2.1
ISSN: 0975-6906
www.isgpb.org

REVIEW ARTICLE

Recent advances in epigenomic techniques: Analysis of DNA base modifications

Suresh Kumar*, Pallavi$, Ashok K. Singh[1] and Trilochan Mohapatra[2]

Abstract

Epigenetics refers to the study of modifications in DNA bases, histone proteins and/or alterations in non-coding-RNA biogenesis that cause changes in gene expression without affecting the underlying nucleotide sequence. Epigenetic variation is emerging as one of the regulators of developmental processes and stress responses in animals and plants. Identification and interrogation of epigenetic changes provide unprecedented opportunities for resolving some of the enigmas that could not be solved based on genetic principles. Moreover, epigenome editing might become a preferred technique for the manipulation of gene expression in the desired organism. Although epigenetics is still in its infancy, the need of the day is to identify epigenetic marks and associate them with heritable phenotypes. Several techniques have been developed to analyze the epigenetic changes and associate them with molecular/physiological processes. It is necessary to examine them in a site- and cell-specific manner. Though considerable progress has been made in the last decade towards mapping DNA modifications as well as other epigenetic changes, there is a need to improve the specificity, sensitivity, and resolving power of the techniques to conduct epigenetic research in a cell-specific manner. The recent technological advancements are currently driving the field of epigenetics, and facilitating a better understanding of the field of functional genomics. The present review outlines the recent advances in epigenomic tools and techniques and presents the future perspectives of epigenomic studies.

Keywords: Epigenetics, epigenomics, DNA methylation, bisulfite sequencing, 5-methylcytosine, N^6-methyladenine, SMRT sequencing, nanopore sequencing

Introduction

Epigenetics is described differently by different researchers. However, with the identification of molecular mechanisms involved and a better understanding of the genetic phenomena, epigenetics can be defined as the study of molecular changes in and around the DNA that control genome activity independent of the alteration in the nucleotide sequence that is inherited through mitosis or meiosis. Epigenomic changes are being reported throughout the development of an organism and exposure to environmental stresses, which may correlate with the expression level of genes (Kumar and Singh 2016; Kumar 2018a; Kumar and Mohapatra 2021a). The genome of an individual is considered to be stable; however, the epigenome continuously gets altered during the developmental processes and due to environmental cues. Thus, the epigenome is highly dynamic, with varying levels of modified DNA bases and chromatin architecture, which affect gene expression (transcriptome dynamics) (Kumar et al. 2018). Therefore, epigenetic changes include the biological phenomena wherein macromolecules like DNA, RNA, protein, and chromatin get modified chemically/

Division of Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.
[1]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.
[2]Protection of Plant Varieties and Farmers' Rights Authority, New Delhi 110 012, India.
[$]Present adresss: J.N.L. College, Patliputra University, Patna 800 020, Bihar, India
***Corresponding Author:** Suresh Kumar, Division of Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India, E-Mail: sureshkumar@iari.res.in
How to cite this article: Kumar S., Pallavi, Singh A.K. and Mohapatra T. 2024. Recent advances in epigenomic techniques: Analysis of DNA base modifications. Indian J. Genet. Plant Breed., **84**(2): 143-155.
Source of support: National Agricultural Science Fund (NASF/ABP-70161/2018-19), and Extramural Research grant [18(3)/2018-O&P] from ICAR, Government of India.
Conflict of interest: None.
Received: Aug. 2023 **Revised:** March 2024 **Accepted:** April 2024

structurally without any alteration in their primary structure (Kumar and Mohapatra 2021a; Kumar and Mohapatra 2021b; Kumar et al. 2021). Cellular processes like cell cycle, DNA replication, abiotic/biotic stress tolerance, growth, and development are regulated through epigenetic modifications. Some of the epigenetic changes might be heritable, leading to transgenerational changes in phenotype. Therefore, epigenetics is considered to be the connecting link between the genotype and the acquired character. Such epigenetic changes include modification in DNA bases, histone proteins, and alteration in non-coding-RNA (ncRNA) biogenesis (Kumar 2017a).

Many of the epigenetic changes occur due to covalent modifications in DNA bases, among which the most common is methylation of cytosine at the 5th carbon of the pyrimidine ring (5-mC, Fig. 1a). Because of its ubiquitous occurrence in the eukaryotic genome, 5-mC is now considered the 5th base of DNA. In the human genome, 60 to 80% of the cytosines present in the CG context were reported to be methylated (Smith and Meissner 2013). In angiosperms, the highest (43%) level of genome-wide 5-mC has been detected in *Beta vulgaris* (Alonso et al. 2015; Shi et al. 2017). Recently, we reported >17% 5-mC in rice under P-starvation stress (Kumar et al. 2022). After the discovery of 5-mC in calf thymus DNA, subsequent studies have identified its role in the regulation of gene expression. Gene expression is not only affected directly by DNA base modifications but it is also affected through histone modification and modulation in chromatin architecture. The enzymes that transfer the methyl (CH_3) group to the DNA base (DNA methyltransferases, DNMT1 and DNMT3a) also bind to the histone methyltransferase (SUV39H1) and help selective methylation of histone tails, which suppress transcription of the gene (Fuks et al. 2003). Methylated DNA is recognized by methyl-binding protein (MBD) that recruits DNMT and enzymes for histone modification (Moore et al. 2013). Thus, methylation of cytosine (5-mC) in DNA may result in the compaction of the chromatin (formation of heterochromatin), which creates an additional layer of genetic information.

With the advent of high-throughput and more sensitive techniques, the occurrence of other modifications of cytosine [5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC)] as well as adenine [N^6-methyladenine(6-mA), 8-oxo-adenine (8-oxoA)]are also being discovered (Fig. 1). The role of ten-eleven translocation (TET) enzyme in the oxidation of 5-mC to 5-hmC (<0.002%) was observed in the genomic DNA of mouse embryonic stem (mES) cells as well as in several tissues of an adult mouse (He et al. 2011; Ito et al. 2011; Pfaffeneder et al. 2011). Since 5-hmC is often associated with open chromatin and found in the promoter region (Ficz et al. 2011), it has been suggested to play an important role in the regulation of gene expression. Although the occurrence of other DNA base modifications such as 5-hmC, 5-fC, 5-caC, N^6-methyladenine (6-mA), 6-hmA, and 8-oxoGhave been reported to be less frequent, they are proposed to play important roles in regulating gene expression (Wion and Casadesus 2006; Kriaucionis and Heintz 2009; Tahiliani et al. 2009; He et al. 2011; Ito et al. 2011; Pfaffeneder et al. 2011; Liu et al. 2016).

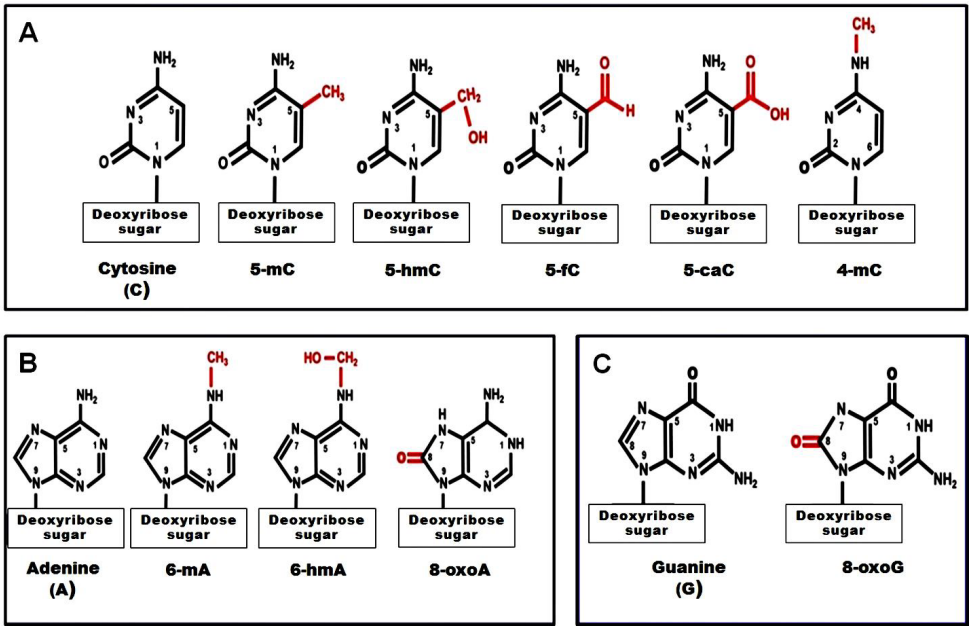

Fig. 1. Modified DNA bases observed in eukaryotes. (A) Modified cytosine: 5=methylcytosine (5-mC), 5=hydroxymethyl cytosine (5-hmC), 5=formylcytosine (5-fC), 5=carboxylcytosine (5-caC), N^4=methylcytosine (4-mC) and (B) Modified adenine: N^6=methyladenine (6-mA), N^6=hydroxymethyladenine (6-hmA), 8=oxo-adenine (8-oxoA). (C) Modified guanine: 8=oxo-guanine (8-oxoG)

After the discovery of various modified DNA bases and dynamic changes in their content, it is evident that epigenetic modification of DNA is more important than they were initially thought. The epigenetic changes may create stable variation(s) in the (epi)genome which can pass on to the next generation, resulting in the inheritance of the associated phenotype/trait(Johannes et al. 2009). Thus, epigenetics acts as a connecting link between the genotype and phenotype as it regulates various cellular processes like gene expression, cell cycle, DNA replication, recombination, growth, and development (Reik et al. 2007). Richards et al. (2010) emphasized the need for epigenetic variations in the adaptation and evolution of natural populations. To date, our knowledge of epigenetic modifications and their functions in gene expression is limited mainly because of the limitations of tools and techniques required for the detection, characterization, and validation of epigenetic features (Tiwari 2017). Epigenetic studies on animals have been comparatively far ahead compared to those on plants. However, it is gaining interest to understand the mechanisms that plants adapt to withstand continuous and diverse environmental stresses. Hence, we need to understand epigenetic diversity, identify the epigenetic marks, their association with the trait of interest, and their inheritance, and to explore the possibilities to utilize them in crop improvement programs (Álvarez-Venegas et al. 2016; Kumar et al. 2017; Kumar and Mohapatra 2021a).

The significance of 5-mC, one of the most commonly modified DNA bases in the genome, is not only determined by its abundance but also by its occurrence in asymmetric (CHH) and symmetric (CG and CHG) contexts (Kumar et al. 2018). In addition, its location in different parts of the gene has different effects on the expression of the gene (Kumar et al. 2022). In plants, it has been reported to occur in all three contexts (CG, CHG, and CHH) (Wang et al. 2016; Kumar et al. 2018). The 5-mC may affect the accessibility of the genomic region to the transcriptional machinery regulatory proteins/complexes; thus, it influences chromatin architecture and affects the transcriptional rate of the gene. Though 6-mAwasfirst detected in a bacterial genome, its occurrence, significance and functions are being established in eukaryotic genomes. Considerable progress has been made in the identification and quantification of modified DNA bases, which broadens the area of epigenomic research and applications. Several methods for determination of 5-mC content, even at single-base resolution, have been devised; several of them take advantage of sodium-bisulfite conversion reaction to distinguish cytosine from 5-mC wherein unmethylated cytosine (C) gets converted to uracil (U) which finally gets converted to thymine (T) during the synthesis of the complementary strand on polymerase chain reaction (PCR). To detect the presence of 6-mA in the genome, liquid chromatography coupled with tandem mass spectrometry was used **(**Wion and Casadesus 2006) to detect it even at a very low level. Recent studies report the genome-wide distribution of 6-mA even in many flowering plants. Liang et al. (2018) deployed a single-molecule real-time (SMRT) sequencing technology to detect 6-mA in a strand-specific manner at single-nucleotide resolution in *Arabidopsis*. Zhou et al. (2018) presented a 6-mA genomic profile of rice by immune precipitation and sequencing. In *Arabidopsis*, 6-mA is enriched in the gene body compared to that in the intergenic regions and it shows a dynamic change in different tissues and at different developmental stages. Studies indicate that the occurrence of 6-mA in the gene body is associated with the actively expressed genes, while its occurrence in the promoter represses the gene expression. Though studies have established that 6-mA is an epigenetic mark in plant genomes, its distribution pattern and detailed functions in plants remain to be deciphered.

This review aims to provide an update on the recent methodological developments in the detection and quantification of covalent base modifications in DNA bases. Different methods with several variations are being employed for the characterization of modified DNA bases. Each method has its advantages and disadvantages, which become evident with its specificity, sensitivity, resolution, and potential artifacts. The choice of a technique to be used for epigenomic analysis would depend on the quality and quantity of DNA, and the number of samples to be analyzed with the required coverage and resolution. It would also be important to take into account the organism and its (epi)genome size to be studied. For epigenomic studies, high-throughput sequencing techniques are used for whole-genome deep sequencing; hence, the availability of good-quality reference genome sequences becomes necessary for comparative analyses.

Importance of modifications in DNA bases

Though nine different types of modifications in DNA bases (Fig. 1) have been reported so far in eukaryotic organisms (compared to about 170 distinct modifications identified in RNAs) (Kumar and Mohapatra 2021b), only little is known about their role and importance except for those of the 5-mC. Interestingly, 5-mC is known to play important roles in the regulation of gene transcription, X chromosome inactivation, genomic imprinting, cell differentiation, tumorigenesis, etc. (Table 1). Though potential functions of 5-hmC at promoter and gene-body are not clearly understood, it is known to play a role in maintaining and/or promoting gene expression. The occurrence of 6-mA is common in microbial genomes which plays important functions in regulating numerous biological processes. It might act as a carrier of heritable epigenetic information in *C. elegans* (Greer et al. 2015) and transposon suppression in Drosophila during embryogenesis (Zhang et al. 2015). Methylome profiling for 6-mA in *Arabidopsis* suggests that the occurrence of 6-mA positively correlates with the

level of gene expression and transition from vegetative to reproductive growth (Liang et al. 2018). In addition to these modified bases, the presence of 5-hydroxymethyluracil (5-hmU) in diverse organisms (from bacteriophages to mammals) (Gommers-Ampt and Borst 1995) and N^7-methylguanine (7-mG) in insect and fish (Chao et al. 2007) has been reported. The occurrence of enzyme-mediated pathways for the synthesis of 5-hmU in eukaryotes suggests its functional significance (Pfaffeneder et al. 2014); however, their biological significance is not yet fully understood.

Epigenetic modifications in DNA bases are reported to affect gene expression levels in controlling growth, development, and tolerance to various abiotic and biotic stresses in different organisms. However, the prevalence, context/location, and dynamics of modified DNA bases are important for affecting the expression of the trait. Therefore, the detection of epigenetic variations in a different cell or tissue in a dynamic manner becomes critically important for scientific/diagnostic purposes. More importantly, information on epigenetic marks associated with a trait of interest is crucial for the detection of stress/problems. For example, the initiation of cancerous activity can be detected at the early stage of its development even when none of the symptoms has appeared if some of the epigenetic marks associated with the cancer are known. Technological advances in the detection/analysis of DNA base modifications have been exciting developments in the field of epigenomics (Cokus et al. 2008; McIntyre et al. 2017; Kumar et al. 2018). A better understanding of DNA base modifications and knowledge about non-covalent interactions between the epigenetic modifications would be required to gain insights into the functional diversity. Though considerable progress is being made every day in understanding various DNA base modifications, deeper insights into the dynamics, functions, interactions and newly identified modifications would be needed for applied purposes. Rapid developments in high-throughput sequencing techniques, together with the use of conventional methods (Table 2), are expected to further improve our understanding of epigenetics and its possible applications for the benefit of humankind.

Techniques for profiling DNA base modification

A specific technique is needed not only to identify the modified DNA base but also to distinguish a particular modified base from the unmodified as well as other modified bases. The resolution power of the technique is important to identify the modified base at the single-base level to determine the context of modification. While some of the methods can determine the presence or abundance of a modified base within the genome, other methods, like bisulfite treatment of DNA followed by DNA sequencing, can detect specific modified bases in a precise context-specific manner virtually in any stretch of DNA. Thus, a method that can identify a wide range of base modifications has a lower horizontal resolution, while the method having a higher horizontal resolution is specific to a particular base modification. A technique does not pose any difficulty in analyzing animal or plant (epi) genome for a modified base unless the presence of the modified base has been established by analyzing the total base composition. Unfortunately, the conventional methods have been laborious and time-consuming to scan a large rgenome. Such issues could be addressed, to some extent, using modification-sensitive restriction endonucleases (MSREs), which provide a relatively simple method for mapping methylated-cytosine and -adenine at specific sites. Such a strategy was initially used for large-scale screening to map the genomic locations for the modified bases in specific restriction endonuclease recognition sites/sequences.

Initially, the detection of 5-mC was dependent on the methylation-sensitive amplified polymorphism (MSAP) technique (Xiong et al. 1999). Subsequently, a monoclonal antibody specific to the 5-mC was used to quantify methylcytosine at the whole-genome level, which offers reliable detection at a lesser cost with high throughput in comparison to the results gained through HPLC-based analysis. Then, a number of efficient tools and techniques have been developed (based on sodium-bisulfite conversion reaction) to determine 5-mC content at single-base resolution. Quantification of 5-hmC has been based on liquid chromatography and mass spectrometric techniques (LC-MS, HPLC-MS); however, immunoassay is also being developed and used for the purpose. The occurrence of 6-mA was initially quantified using high-pressure liquid chromatography (HPLC) (Eick et al. 1983; Woodcock et al. 1984). Some of the modified bases, like 6-mA and 4-mC occur at a low level. They are detectable using highly sensitive techniques (Heyn et al. 2015; Liu et al. 2019). Most of the common methylated DNA bases (5-mC, 6-mA and 4-mC) can be detected by SMRT sequencing, requiring only 25-fold coverage to obtain higher confidence in their detection (Davis et al. 2013). DA-6mA-seq (*Dpn*I-Assisted 6-mA sequencing) approach was used to cleave methylated adenine sites, wherein *Dpn*I cuts duplex DNA in other sequence motifs besides the canonical GATC restriction sites. Thus, DA-6mA-seq was considered to be more sensitive, requiring only nanograms of input DNA and lower sequencing depth than conventional techniques (Luo et al. 2016). Recently, the existence of 6-hmA was reported in mammalian cells by using LC-MS analysis (Xiong et al. 2019). Significant technological advancements have been made in the detection/quantification of modifications in DNA bases that resulted in enriched knowledge/interest in epigenomics. Here, we present an overview of the recent technological advancements in the detection of modified DNA bases, their applications and limitations.

Table 1. DNA base modifications and their role in the biological process

Modified DNA base	Modification process	Role	References
5-methylcytosine (5-mC)	Methylation of Cytosine by DNA methyl transferases (DNMT)	Genomic imprinting, X-chromosome inactivation, transposon suppression, gene regulation and epigenetic memory maintenance	Luo et al. 2016
5-hydroxymethylcytosine (5-hmC)	Oxidation of 5-mC by Ten Eleven Translocation (TET) dioxygenases	Presence of 5-hmC in promoter seems to inhibit transcription. Also, studies suggest that 5-hmC functions as an epigenetic mark in mammalian neuronal development	Breiling and Lyko 2015; Klungland and Robertson 2017
5-formylcytosine (5-fC)	Oxidation of 5-hmC by TET dioxygenases	Role in recruiting DNA repair machinery	Breiling and Lyko 2015
5-carboxylcytosine (5-caC)	Oxidation of 5-fC by TET dioxygenases	Role in recruiting DNA repair machinery	Breiling and Lyko 2015
N^4-methylcytosine (4-mC)	Methylation of Cytosine by DNA methyl transferases (DNMT)	Play role in bacterial restriction–modification (R-M) systems as defense mechanisms	Ehrlich et al. 1987
N^6-methyladenine (6-mA)	Methylation of adenine, DNA N^6 adenine methyltransferases (DAMT-1)	Regulate transcription, transposable elements and trans-generational epigenetic inheritance	Luo et al. 2016
N^6-hydroxymethyladenine (6-hmA)	hydroxylation of 6-mAby ALKBH1 in genomic DNA of mammals	Potential role remains undeciphered	Xiong et al. 2019
8-oxo-adenine (8-oxoA)	Modification of adenine by reactive oxygen species	Affect DNA replication and gene expression during oxidative stress	Malins et al. 2000
8-oxo-guanine (8-oxoG)	Modification of guanine by reactive oxygen species	Involved in modulating gene expression during oxidative stress	Ba et al.2014; Park et al. 2019
8-oxo-2′-deoxyguanosine (8-oxodG)	Oxidation of deoxyguanosine	Biomarker for oxidative DNA damage under oxidative stress	Karahan et al. 2019; Chiorcea-Paquim 2022

Advances in the detection of modified DNA bases

Considerable technological advances in detecting the modified DNA bases, as well as their effects on phenotype, have been achieved since it was discovered that modification in DNA bases also happens in eukaryotic cells. The techniques currently used for the detection of DNA base modification can be categorized into two broad categories based on the results they generate. The first is relative quantification, while the other is detection at the level of single-base resolution. Initially, thin-layer chromatography was used to quantify the modified DNA base (Gunthert et al. 1976); however, several other advanced techniques are being used to detect the modified DNA bases.

Though the HPLC-UV technique, used by Kuo and coworkers (Kuo et al. 1980), is considered to be the "gold standard" assay for quantitative analysis of 5-mC content, this method has certain limitations like the need for specialized equipment and requiring a larger amount (3–10 µg) of genomic DNA sample. In this method of analysis, the DNA sample is hydrolyzed to its constituent bases, C and 5-mC are separated chromatographically, and then the fractions are measured to quantify 5-mC content. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was then used as an alternative technique, which is highly sensitive and requires a much smaller amount of the DNA sample (Song et al. 2005; Thuc et al. 2011; Liu et al. 2009). Later on, an enzyme-linked immunosorbent assay (ELISA)-based kit for quick and comparative assessment of the level of modified DNA bases has become available; however, the technique is prone to give variable results. Though the technique is easier and faster, it is less sensitive and requires a larger difference in global DNA methylation level.

Subsequently, the techniques used to detect DNA methylation include enzyme-based methylome profiling. The enzyme-based method exploits methylation-sensitivity of restriction enzymes like *Msp*I [digestion blocked due to methylated cytosine (5-mC) in CHG context] and *Hpa*II (restriction blocked due to 5-mC in CG and CHG contexts). Such a method was initially used in plants to detect differentially methylated sites in wild-type and mutant *Arabidopsis* by microarray analysis of smaller DNA fragments (Tran et al. 2005). Methylation-specific enzyme *Mcr*BC, which preferentially digests methylated DNA, has also been used to detect DNA methylation. A combination of *Mcr*BC digestion and tiling microarray (Vaughn et al. 2007; Li et al. 2008) or high-throughput sequencing (Wang et al. 2009;

Table 2. Techniques used for the detection of modified DNA bases

Technique used	Modified base	Host system	References
Quantitative LC-MS	5-mC	Mouse brain cells	Münzel et al. 2010
Chemical isotopic labeling of genomic DNA coupled with ultra-sensitive analytical liquid chromatography-tandem mass spectrometry	5-hmC	Mammalian cells	Bachman et al. 2014
Reduced bisulfite sequencing (redBS-Seq) and oxidative bisulfite sequencing (oxBS-Seq)	5-mC, 5-hmC, 5-fC	Mouse embryonic stem cells	Booth et al. 2014
Nanopore sequencing	5-mC	Human breast cell lines	Simpson et al. 2017
NanoHPLC-MS/HRMS	5-fC	Mouse cell	Bachman et al. 2015
hmC-CATCH bisulphite-free sequencing.	5-hmC	Human embryonic stem cell	Zeng et al. 2018
4mC-Tet-assisted bisulfite-sequencing (4mC-TAB-seq).	4-mC	*Caldicellulosiruptor* sp.	Yu et al. 2015
Liquid chromatography-multistage mass spectrometry (LC-MS/MS/MS) followed by Illumina Hi-Seq 2000 system	5-hmC	Oryza sativa	Wang et al. 2015
Methyl flash hydroxylated DNA quantification (colormetric)	5-hmC	Brassica rapa	Golubov and Kovalchuk 2017
Single-molecule real-time (SMRT) sequencing	6-mA	*Tetrahymena thermophila*	Wang et al. 2017
Liquid chromatography–mass spectrometry (LC-MS/MS)	6-mA	Mammalian embryonic stem cell	Wu et al. 2016
Ultra-high-performance liquid chromatography coupled with a triple-quadrupole tandem mass spectrometry (UHPLC–QQQ–MS/MS) assay	6-mA	Zebrafish	Liu et al. 2016
UHPLC–QQQ–MS/MS	6-mA	Chlamydomonas	Fu et al. 2015
SMRT sequencing	6-mA	Arabidopsis thaliana	Liang et al. 2018
SMRT sequencing	6-mA, 4-mC	Rosaceae members	Liu et al. 2019
LC-MS analysis	6-hmA	Mammalian cell	Xiong et al. 2019
Oxford Nanopore long-read sequencing in coordination with Deep-Mod, a bidirectional recurrent neural network (RNN) with long short-term memory (LSTM) to detect DNA modifications	5-mC, 6-mA	*Escherichia coli*, *Chlamydomonas reinhardtii*, and human genomes	Liu et al. 2019

He et al. 2010) was used for genome-wide analysis of DNA methylation.

Affinity purification, deploying the protein that preferentially binds methylcytosine using immuno-precipitation (mCIP), is used to select/concentrate methylated DNA fragments. A widely used 5-mC-binding protein is MBD domain-containing human protein MeCP2, which specifically binds to methylated DNA but does not bind to unmethylated DNA at high salt concentrations. The MeCP2 recognizes only CG-methylated DNA and its binding affinity is positively correlated with the number of methylated CG sites in the DNA fragments. The mCIP enrichment of methylated DNA fragments was combined with Illumina high-throughput sequencing (mCIP-seq) to profile the DNA methylation in plants (Yan et al. 2010; Gohlke et al. 2013).

For context-specific analysis of 5-mC at single-base resolution, whole-genome bisulfite sequencing (WGBS) is considered to be of "gold standard". In WGBS analysis, the genomic DNA is treated with sodium bisulfite, which converts unmodified cytosine, 5-caC, and 5-fC to uracil, but 5-mC and 5-hmC remain unmodified. Since its discovery in 1992 (Frommer et al. 1992), bisulfite sequencing has been confidently used for DNA methylation studies. However, the potential of this technique was only realized when bisulfite-treated DNA could be subjected to shotgun sequencing (MethylC-seq) in deciphering 5-mC at single-base resolution (Urich et al. 2015). WGBS analysis allows genome-wide detection of DNA methylation patterns at single-base resolution in all three (CG, CHG, and CHH) sequence contexts, which is of particular interest in plants. Three distinct DNA methyl transferases methylate cytosine in different sequence contexts (i.e., MET1 at CG site, CMT3 at CHG site and DRM2 at CHH site) and they have distinct effects/importance in the regulation of gene expression (Kumar et al. 2022).

Next-generation sequencing to analyze modified DNA bases

Advances in third-generation sequencing facilitate the detection of modified DNA bases directly without any prior chemical or enzymatic treatment of DNA samples (Plongthongkum et al. 2014). Pacific Biosciences (PacBio) developed SMRT sequencing technology that enables the detection of 5-mC and its oxidation derivatives directly while sequencing based on the nucleotide incorporation time/kinetic signature (Eid et al. 2009; Flusberg et al. 2010). The SMRT sequencing uses a zeptolitre-volume cylindrical cavity (~100 nm diameter and height) known as a zero-mode waveguide (ZMW), which is immobilized with a DNA-polymerase complex. In SMRT, DNA polymerase is used for the replication of DNA strands, which is optically measured using fluorescently labeled dNTP analogs. For sequencing of a DNA fragment, a polymerase-bound DNA template is fixed to the bottom of the ZMW utilizing the biotin-streptavidin chemistry, which requires substantial time for DNA to pass through the ZMW. This allows preferential entry of short DNA templates over long ones under diffusive conditions. Although the requirement of input DNA is still higher (above nanogram levels), magnetic bead assays have been deployed to improve loading efficiency. Shorter DNA fragments are removed to minimize competitive binding. However, a smaller difference in the kinetic signature of the base variants may lead to poor accuracy in the quantification of the modified bases, which is considered to be one of the major shortcomings of SMRT. Moreover, in certain cases, chemical labeling or enzymatic conversion is used to improve signal detection efficiency. For example, conversion of 5-hmC to N3-5-gmC or HS-N3-5-gmC is performed before SMRT sequencing for enhanced signal detection efficiency (Eid et al. 2009). Nevertheless, SMRT is considered to be a promising and efficient technique for the detection of 6-mA and 4-mC at single-nucleotide resolution. A higher average read length of 8.5 kb enables SMRT to detect 5-mC and its oxidation derivatives more efficiently, even in the repetitive regions.

Further advances in the single-molecule-based method were made using the nanopore sequencing technique (Clark et al. 2013), which directly produces DNA methylome profiles without the need for bisulfite conversion to discriminate 5-mC from other bases (Flusberg et al. 2010). Nanopore sequencing utilizes an electrical field to mobilize the electrically charged biopolymer passing through a nanoscale aperture. When the biopolymer (DNA strand) is passed through the nanopore, it partially blocks the flow of ionic current, resulting in obstruction in the flow of ion current. Since the DNA strand passes through the nanopore in a single-file manner, it enables scanning the variation in the ion current obstruction, thereby detecting different bases, including modified DNA bases, direct along the DNA strand. The nanopore can be modified for different applications like DNA barcoding, binding of transcription factors to DNA, and label-free identification of single nucleotides (Feng et al. 2015). Engineered nanopores were utilized for profiling modified DNA bases by nanopore sequencing, and some such examples include MspA channel (Laszlo et al. 2013) and solid-state NP (ssNP) (Zahid et al. 2016). A modified method for directly detecting the modified base (e.g., 5-mC) on the basis of electrical readout, without any requirement of chemical treatment, using MinION of Oxford Nanopore Technology, was suggested by Simpson et al. (2017). Like the SMRT sequencing technology, nanopore sequencing also takes the advantages of longer-read length and rapid sequencing. Though the error rate in base identification is higher (18–20%) for SMRT (Ross et al. 2013), it is significantly lower (6–10%) for nanopore sequencing (Delahaye and Nicolas 2021). However, the throughput and depth of sequencing for third-generation sequencing need to be improved by orders of magnitude before they can be used for routine methylome analysis for larger genomes.

Recent advances in epigenetic and epigenomic studies

Epigenetic changes are the continuous process that occurs throughout the development of an organism as well as during exposure to various environmental stresses. The standard methods currently being used to analyze the epigenetic variations are discontinuous and destructive. Normally, for the analysis of epigenetic change, one is required to collect/fix the tissue sample (discontinuing the normal biological processes that have been happening inside the cells) and isolate the molecules of interest (DNA and/or histone proteins) by rupturing the cells (destroying the living cells). Moreover, the sample used for epigenetic analysis is the tissues containing a mixture of different types of cells (rather than a single cell). Generally, the diverse mixture of cells possesses different methylation states, which cannot be correlated with the phenotype of the organism. Since the epigenome is dynamic in nature, the cell-specific analysis would be necessary for analyzing an epigenetic change, and its functional assignment. Using a bimolecular anchor detector (BiAD) system, the epigenetic changes can be monitored in live tissue (Fig. 2). It contains a fluorescence-based sensor with two components. The first one is an anchor protein (e.g., a zinc finger protein) with a non-fluorescent fluorophore that binds to a specific part of DNA, but it cannot emit fluorescence until it joins a complementary fluorophore fragment. The second component is a detector protein (e.g., Methyl-CpG-binding domain protein 2) with the complementary fluorophore, which recognizes and binds specifically to an epigenetically modified DNA site. When the anchor and detector proteins are present in close vicinity, they fuse to form the complete

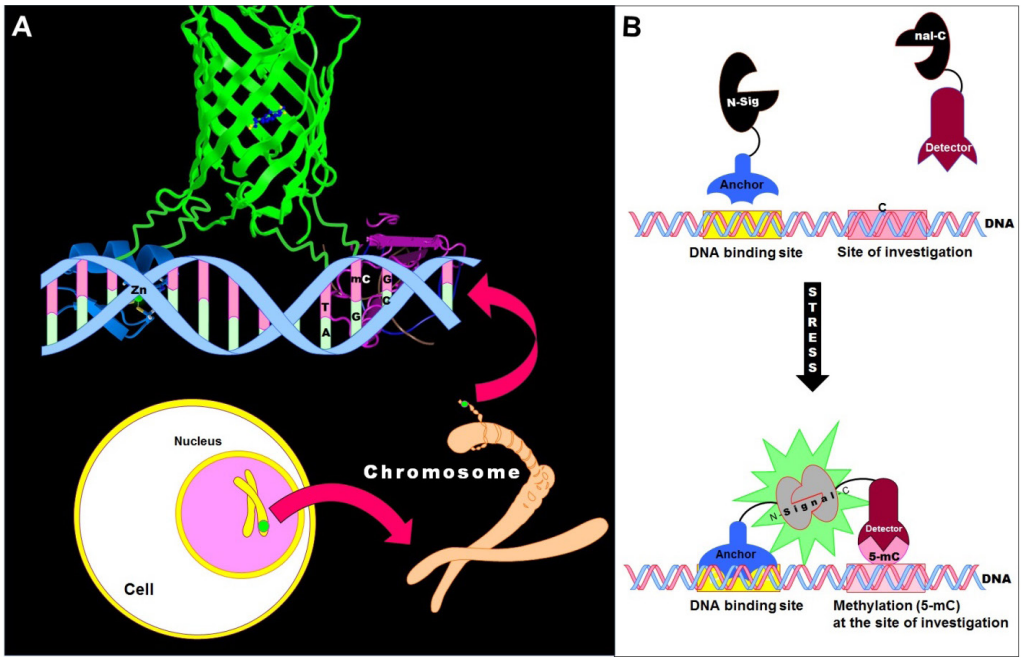

Fig. 2. Bimolecular anchor detector (BiAD) system detects methylation of DNA at a specific site (green) in the nucleus of a cell. (A) The BiAD sensor detects methylation of DNA at the site of investigation (green) in a gene. The anchor protein (blue) is positioned in the vicinity of the site of investigation for the epigenetic change. Only when the detector (purple) binds to the epigenetically modified site in the proximity of the anchor the fluorophore component proteins are coupled to produce a fluorescent signal (green), which can be detected with a fluorescence microscope; and (B) Working model of BiAD sensor system. The anchor (blue) recognizes a specific DNA fragment, and it is coupled to an N-terminal half (N-Sig) of a fluorophore (black). The detector protein (purple) recognizes an epigenetic modification (e.g., 5-methylcytosine, 5-mC), and it is coupled to the C-terminal (nal-C) half of the fluorophore (black). Under stress, cytosine residue in the site of investigation gets methylated (5-mC) and provides a binding site for the detector protein. This leads to the assembly of N- and C-terminal fluorophore components (grey) into an active fluorophore (green)

fluorescent protein, which happens when the DNA/gene to be examined gets methylated (providing the binding site for the detector). The fluorescent signal can be detected using a fluorescence microscope in the live cells. The anchor can be combined with different detector domains specifically designed for different types of epigenetic modifications, and it can work with different types of cells.

Quantification of modified DNA bases

Accumulating evidence indicates that epigenetic changes are important players in determining cellular differentiation, growth, development, as well as tolerance to various environmental stresses in most of living organisms. Association among environmental stresses, changes in DNA base modifications, and gene expression level is being demonstrated in animals as well as plants. Thus, quantitative analyses of epigenomic variations have become crucial for investigative applications. Some of the technical improvements in epigenetics have been the introduction of effective methods to identify/analyze modified DNA bases, particularly at single-base resolution. The progress made in the identification and quantification of modified DNA bases has considerably increased/broadened the area of epigenomic research in several fields of biology.

Quantification of 5-mC

Combining the bisulfite conversion along with the high-throughput DNA sequencing for estimation of DNA methylation in a context-specific manner is referred to as whole-genome bisulfite sequencing (WGBS). In WGBS, unmethylated cytosine is converted to uracil first and then to thymine during the PCR amplification process. The presence of cytosine in aWGBS read indicates that the cytosine was methylated (5-mC), as methylcytosine is secured from conversion due to bisulfite treatment. Moreover, the location/position, content, and context of cytosine(de) methylation need to be determined for meaningful interpretation of the findings. WGBS analysis generates computable data for DNA base methylation/demethylation at individual cytosine locations as multiple reads are aligned on the reference sequence. In the recent years, the Nanopore sequencing technique was used to detect 5-mC depending on the changes in electrolytic current due to the modified DNA base. Quantitative analysis of 5-mC was reported using Oxford Nanopore Technologies deploying MinION sequencer in the human genome (Simpson et al. 2017), wherein bisulfite conversion and its associated demerits could be minimized. Further advancements in Nanopore

sequencing techniques are likely to transform epigenetic investigations using high throughput NGS technologies.

Quantification of 5-hmC

Quantification of 5-hydroxymethylcytosine utilizing immunoassay principles (deploying 5-hmC-specific antibody) is being deployed. Though such methods are quantitative as well as reproducible; these are intricate, costly, less sensitive, and have less suitability for the desired high throughput epigenetic analysis. Although a number of methods have been available for the detection/quantification of 5-hmC, most of the methods do not meet the prerequisites for cutting-edge epigenomic investigation. Generally, a large amount of sample is needed when the sample tissue possesses lesser 5-hmC content. Usually detection capacity of such an assay is about 0.03% 5-hmC only which limits its utility in such studies. In addition, these assays provide only a relative value for 5-hmC quantity; therefore, quantification of 5-hmC requires a standard curve to be generated.

An alternative method uses an enzyme (β-glucosyltransferase) from phage T4 that catalyzes the attachment of β-D-glucosyl residues from uridine-diphosphoglucose (UDP-Glu) to the hydroxyl group of 5-hmC. In this method, 5-hmC is labeled with a reactive azide group; the azide group is labeled with a fluorescent alkyne tag (e.g. dibenzocyclooctyne-Cy5) as a reporter. Tough this method provided accurate and high-throughput quantification of 5-hmC, a large quantity (about 6 µg) of DNA is needed. To lower the requirement of DNA, the ultra-sensitive single-molecule method is utilized for directly visualizing and counting the fluorescent 5-hmC mark (Gilat et al. 2017).

Quantification of 6-mA/1-mA

Ultra-high performance, liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) has recently been used to detect 6-Ma (Boulias and Greer, 2021). While this is a qualitative technique for detecting 6-mA, it does not provide any information about the genomic location/context of modification. Combining UHPLC-MS/MS with methylated DNA immune precipitation (MeDIP) can differentiate 1-mA from 6-mA, which provides unambiguous detection of 1-mA in the genome (Greer et al. 2015). Therefore, accuracy in the detection of 6-mA requires combining complementary methods as an individual technique has certain limitations.

Challenges in the detection of modified DNA bases

One of the major challenges in the detection/mapping of modified DNA bases is its very low/low occurrence in the genome. While some of the techniques detect the presence/magnitude of the modified base (the first step in epigenetics of DNA base modification), others are used to map it at the single-nucleotide (context/location) level. However, the specificity, sensitivity, and resolving power of the technique are very important, and these must be considered while selecting the technique(s) to detect/map the modified DNA base. A meticulous selection of the technique(s) and inclusion of control(s) in the experimental design is essential for precision in the base modification studies. Mostly, a well-known DNA sequence (e.g., lambda/M13 phage DNA) not containing the modified base (λDNA^{-} ^{mC}) is used to spike the sample DNA aimed at checking the efficiency of the technique used. For example, bisulfite conversion efficiency needs to be checked while performing a WGBS study. Bisulfite conversion and subsequent PCR amplification may cause pronounced sequencing bias/artifact. Degradation of genomic DNA is a concurrent effect of bisulfite conversion, posing another challenge in its usage for analyzing DNA samples available in only a limited amount, such as single-cell epigenome profiling. Bisulfite treatment-induced fragmentation of genomic DNA was initially attributed to the loss of purines (Frommer et al. 1992; Raizis et al. 1995), but later on, it was found to result from random base losses at unmethylated cytosine, causing backbone breakage on exposure to heat and alkali (Tanaka and Okamoto 2007). Such cytosine-specific effects lead to biases like depletion of cytosine-rich DNA (resulting in a skewed representation of the genomic sequences) and depletion of fragments containing unmethylated cytosine (leading to the overestimation of 5-mC content) (Olova et al. 2018). Generally, epigenome analysis uses genomic DNA from millions of cells from a sample. As the epigenome is highly dynamic, a cell-specific analysis would be essential for analyzing base modification and its functional characterization. Epigenetic analysis of DNA methylation is also affected by the diversity and complexity of the technique used. In addition to the aforementioned challenges in the detection of the known modified DNA bases, we may encounter several other modified bases not yet known, and we may require devising a new strategy to analyse them. It would also be essential to determine the heritable component of DNA base modifications to identify the trait-associated epimarks.

Future perspectives

Continuous technological advances have provided unprecedented opportunities for the identification/detection of modified DNA bases and in the application of epigenomic tools for practical significance. We envisage that rapidly advancing modern techniques, particularly the newer chemical biology would be applied to expedite studies in the field of epigenomics. One of the limitations in epigenomic understanding has been the limited information about the writers, readers, and/or erasers of the modified DNA bases. Quick ablation of writer, reader, and eraser does not allow much investigation of the site-specific role of these factors and limits functional studies on DNA base modification. However, advances in genome/epigenome editing might prove to be an efficient approach

for site-specific manipulation of DNA (Kumar 2019) to better understand the role of base modification in a context-specific manner.

Plant system provides a better opportunity to elucidate the biological functions of modified DNA bases and study their regulatory aspects through easier and more rapid investigations of epigenetic alteration in higher eukaryotes, which are otherwise difficult to perform using animal system (Shen et al. 2019). Using a combination of techniques like genetic ablation and NGS-based mapping, regulatory functions of epigenetic modifications on developmental processes and stress tolerance in plants have been demonstrated. Readers of the base modifications have been reported to play more important roles in abiotic stress responses. This suggests that deciphering the context of base modification is more important than detecting the change (writing/erasing). Epigenetic understanding would be further improved with the discovery of other covalent modifications in DNA bases and their writers/readers/erasers.

Recent studies reveal the interaction between the epigenetic modifications (e.g., modified DNA base and histone modification) as well as between the epigenetic (histone protein) and epi transcriptomic (N^6-methyladenosine) modifications, which present a new regulatory mechanism (Kumar and Mohapatra 2021; Kumar et al. 2021). This indicates a linkage between epigenetic and epi transcriptomic modifications and suggests a complex role of base modifications in regulating gene expression. To understand the shared role of reversible biochemical modifications in DNA and RNA for the dynamic and effective regulation of gene expression, investigations on DNA methylation, histone modification, sRNA biogenesis, and epi transcriptomic modifications in rice under abiotic (drought and P deficiency) stress are being carried out by our team. Identification of epigenetic marks and their role in stress memory is another challenging task. However, once a heritable mark is identified, a CRISPR-based epigenome-editing tool might be utilized for targeted/site-specific alteration in the epigenome to achieve the desired phenotype (Seem et al. 2024). A comprehensive understanding of the epigenetic-epitranscriptomic regulation of gene expression might enable us the development of climate-smart crops in the near future. This would not only improve the adaptability/productivity of crops under the changing climatic conditions but would also ensure food security.

Considerable functional diversity of DNA base modifications has been deciphered during the last decade mainly because of the high-throughput NGS-assisted detection of modified bases and their functional validation. Several other dynamic base modifications in DNA (4-mC, 6-mA, and 8-oxoG) are also being detected/analyzed in different organisms, which would require functional characterization and validation to strengthen their epigenetic role as an additional factor in regulating gene expression. Other epigenetic modifications might be identified in the future, which may demonstrate positive/negative interaction with other modified bases in modulating gene expression. The biological functions of DNA base modifications are not yet fully understood, probably because of the lack of optimized/efficient methods for detection and functional characterization. Detection of the modified base at single-base resolution using third-generation direct sequencing technology such as nanowells (SMRT) and nanopore (Oxford Nanopore) sequencing is very promising for the rapid developments in epigenomics. However, proper experimental design with a sufficient number of replicates and controls is very important for confidence in the findings. Identification of the enzyme (writer) involved in base modification, readers for the modified base, and erasers for replacing it with the unmodified base are necessary for devising the strategy for epigenome editing. Moreover, several fundamental questions remain to be answered (Kumar 2017); some more questions may arise after getting answers to the existing questions, but our epigenomic understanding would certainly improve with such investigations on plants for growth, fitness, and survival under environmental stress (Kumar 2018b; Kaur and Kumar 2020).

Authors' contribution

All the authors have contributed equally.

Acknowledgment

The financial support from the National Agricultural Science Fund (NASF/ABP-70161/2018-19) and Extramural Research grant [18(3)/2018-O&P] from the Indian Council of Agricultural Research, Government of India, New Delhi is gratefully acknowledged.

References

Alonso C., Pérez R., Bazaga P. and Herrera C. M. 2015. Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. Front. Genet., **6**: 4.

Álvarez-Venegas R. and De-la-Peña C. 2016. Recent advances of epigenetics in crop biotechnology. Front. Plant Sci., **7**: 413.

Ba X., Bacsi A., Luo J., Aguilera-Aguirre L., Zeng X., Radak Z., Brasier A. R. and Boldogh I. 2014. 8-oxoguanine DNA glycosylase-1 augments proinflammatory gene expression by facilitating the recruitment of site-specific transcription factors. J. Immunol., **192**: 2384-94.

Bachman M., Uribe-Lewis S., Yang X., Burgess H. E., Iurlaro M., Reik W., Murrell A. and Balasubramanian S. 2015. 5-Formylcytosine can be a stable DNA modification in mammals. Nat. Chem. Biol., **11**: 555.

Bachman M., Uribe-Lewis S., Yang X., Williams M., Murrell A. and Balasubramanian S. 2014. 5-Hydroxymethylcytosine is a

predominantly stable DNA modification. Nat. Chemistry, **6**: 1049.

Booth M. J., Marsico G., Bachman M., Beraldi D. and Balasubramanian S. 2014. Quantitative sequencing of 5-formylcytosine in DNA at single-base resolution. Nat. Chemistry, **6**: 435.

Boulias K. and Greer E. L. 2021. Detection of DNA Methylation in Genomic DNA by UHPLC-MS/MS. Methods in molecular biology (Clifton, N.J.), **2198**: 79–90.

Breiling A., Lyko F. 2015. Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. Epigenetics Chromatin, **8**: 24.

Chiorcea-Paquim A.-M. 2022. 8-oxoguanine and 8-oxodeoxyguanosine biomarkers of oxidative DNA damage: A review on HPLC–ECD determination. *Molecules,* **27**: 1620. https://doi.org/10.3390/molecules27051620

Clark T. A., Lu X., Luong K., Dai Q., Boitano M., Turner S. W., He C. and Korlach J. 2013. Enhanced 5-methylcytosine detection in single-molecule, real-time sequencing via Tet1 oxidation. BMC Biol., **11**: 4.

Cokus S. J., Feng S., Zhang X., Chen Z., Merriman B., Haudenschild C. D., et al. 2008. Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. *Nature,* **452**: 215–219. https://doi.org/10.1038/nature06745

Davis B. M., Chao M. C., Waldor M. K. 2013. Entering the era of bacterial epigenomics with single molecule real time DNA sequencing. Curr. Opin. Microbiol., **16**: 192-198.

Delahaye C. and Nicolas J. 2021. Sequencing DNA with nanopores: Troubles and biases. PLoS ONE, **16**: e0257521. https://doi.org/10.1371/journal.pone.0257521

Ehrlich M. E., Wilson G. G., Kuo K. C. and Gehrke C. W. 1987. N4-methylcytosine as a minor base in bacterial DNA. J. Bacteriol., **169**: 939-43.

Eick D., Fritz H. J. and Doerfler W. 1983. Quantitative determination of 5-methylcytosine in DNA by reverse-phase high-performance liquid chromatography. Analytical Biochem., **135**: 165-71.

Eid J., Fehr A., Gray J., Luong K., Lyle J., Otto G., Peluso P., et al. 2009. Real-time DNA sequencing from single polymerase molecules. Science, **323**: 133-138.

Feng J., Liu K., Bulushev R. D., Khlybov S., Dumcenco D., Kis A. and Radenovic A. 2015. Identification of single nucleotides in Mos2 nanopores. Nat. Nanotechnol., **10**: 1070–1076.

Ficz G., Branco M. R., Seisenberger S., Santos F., Krueger F., Hore T. A., Marques C. J., Andrews S. and Reik W. 2011. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature, **473**: 398.

Flusberg B. A., Webster D. R., Lee J. H., Travers K. J., Olivares E. C., Clark T. A., Korlach J. and Turner S. W. 2010. Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat. Methods, **7**: 461.

Frommer M., Mcdonald L. E., Millar D. S., Collist C. M., Wattt F., Griggt G. W., et al. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. USA, **89**: 1827–31.

Fu Y., Luo G. Z., Chen K., Deng X., Yu M., Han D., Hao Z., Liu J., Lu X., Doré L. C. and Weng X. 2015. N6-methyldeoxyadenosine marks active transcription start sites in Chlamydomonas. Cell, **161**: 879-92.

Fuks F., Hurd P. J., Deplus R. and Kouzarides T. 2003. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res., **31**: 2305–2312.

Gilat N., Tabachnik T., Shwartz A., Shahal T., Torchinsky D., Michaeli Y., Nifker G., Zirkin S. and Ebenstein Y. 2017. Single-molecule quantification of 5-hydroxymethylcytosine for diagnosis of blood and colon cancers. Clin Epigenetics, **9**: 70. https://doi.org/10.1186/s13148-017-0368-9

Gohlke J., Scholz C. J., Kneitz S., Weber D., Fuchs J., Hedrich R. and Deeken R. 2013. DNA methylation mediated control of gene expression is critical for development of crown gall tumors. PLoS Genet., **9**: e1003267. https://doi.org/10.1371/journal.pgen.1003267

Golubov A. and Kovalchuk I. 2017. Analysis of DNA Hydroxymethylation Using Colorimetric Assay. Methods Mol. Biol., **1456**: 89-97. https://doi.org/10.1007/978-1-4899-7708-3_8. PMID: 27770360

Gommers-Ampt J. H. and Borst P. 1995. Hypermodified bases in DNA. FASEB J., **9**: 1034–42.

Greer E. L., Blanco M. A., Gu L., Sendinc E., Liu J., Aristizabal-Corrales D., et al. 2015. DNA Methylation on N(6)-Adenine in *C. elegans*. Cell, **161**: 868–78. https://doi.org/10.1016/j.cell.2015.04.005

Gunthert U., Schweiger M., Stupp M. and Doerfler W. 1976. DNA methylation in adenovirus, adenovirus-transformed cells, and host cells. Proc. Natl. Acad. Sci., **73**: 3923-27.

He G., Zhu X., Elling A. A., Chen L., Wang X., et al. 2010. Global epigenetic and transcriptional trends among two rice subspecies and their reciprocal hybrids. Plant Cell, **22**: 17–33.

He Y. F., Li B. Z., Li Z., Liu P., Wang Y., Tang Q., Ding J., Jia Y., Chen Z., Li L. and Sun Y. 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science, **333**: 1303-7.

Heyn H. and Esteller M. 2015. An adenine code for DNA: a second life for N6-methyladenine. Cell, **161**: 710-3.

Ito S., Shen L., Dai Q., Wu S. C., Collins L. B., Swenberg J. A., He C. and Zhang Y. 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science, **333**: 1300-3.

Johannes F., Porcher E., Teixeira F. K., Saliba-Colombani V., Simon M., Agier N., et al. 2009. Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genetics, **5**: e1000530.

Karahan M., Yildirim M., Kucuk H. F., Turunc V., Demir H., Salturk C., Yavuz A., Demir T. and Ari E. 2019. Oxidative DNA damage is increased in living kidney donors. Transplant Proc., **51**: 1049-1053.

Kaur S. and Kumar S. 2020. Nutriepigenomics: Need of the day to integrate genetics, epigenetics and environment towards nutritious food for healthy life. Food Sci. Nutri. Technol., **5**: 1-13.

Klungland A. and Robertson A. B. 2017. Oxidized C5-methyl cytosine bases in DNA: 5-hydroxymethylcytosine; 5-formylcytosine; and 5-carboxycytosine. Free Radical Biol. Med., **107**: 62-68.

Kriaucionis S. and Heintz N. 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science, **324**: 929-30.

Kumar S. 2017. Epigenetic control of apomixis: a new perspective of an old enigma. Adv. Plants Agric. Res., **7**: 227-233. https://doi.org/10.15406/apar.2017.07.00243

Kumar S. 2017. Epigenetic control of apomixis: A new perspective of an old enigma. Adv. Plants Agric. Res., **7**(1): 00243. https://doi.org/10.15406/apar.2017.07.00243

Kumar S. 2018a. Epigenomics of plant responses to environmental stress. Epigenomes, **2**: 6. https://doi.org/10.3390/epigenomes2010006

Kumar S. 2018b. Environmental stress, food safety, and global health: biochemical, genetic and epigenetic perspectives. Med. Safety Global Health, **7**: e145. https://doi.org/10.4172/2574-0407.1000145

Kumar S. 2019. Genome editing to epigenome editing: Towards unravelling the enigmas in developmental biology. Trends Develop. Biol., **12**: 31-38.

Kumar S. and Mohapatra T. 2021a. Dynamics of DNA methylation and its functions in plant growth and development. Front. Plant Sci., **12**: 596236. https://doi.org/10.3389/fpls.2021.596236

Kumar S. and Mohapatra T. 2021b. Deciphering epitranscriptome: Modification of mRNA bases provides a new Perspective for post-transcriptional regulation of gene expression. Front. Cell Dev. Biol., **9**: 628415. https://doi.org/10.3389/fcell.2021.628415

Kumar S. and Singh A. 2016. Epigenetic regulation of abiotic stress tolerance in plants. Adv. Plants Agric. Res., **5**: 517-521. https://doi.org/10.15406/apar.2016.05.00179

Kumar S., Chinnusamy V. and Mohapatra T. 2018. Epigenetics of Modified DNA bases: 5-methylcytosine and beyond. Front. Genet., **9**: 640. https://doi.org/10.3389/fgene.2018.00640

Kumar S., Kaur S., Seem K., Kumar S. and Mohapatra T. 2021. Understanding 3D genome organization and its effect on transcriptional gene regulation under environmental stress in plant: A chromatin perspective. Front. Cell Dev. Biol., **9**: 774719. https://doi.org/10.3389/fcell.2021.774719

Kumar S., Seem K., Kumar S., Vinod K. K., Chinnusamy V. and Mohapatra T. 2022. *Pup1* QTL regulates gene expression through epigenetic modification of DNA under phosphate starvation stress in rice. Front. Plant Sci. **13**: 871890. https://doi.org/10.3389/fpls.2022.871890

Kumar S., Singh A. K. and Mohapatra T. 2017. Epigenetics: History, present status and future perspective. Indian J. Genet. Plant Breed., **77**: 445-463.

Kuo K. C., McCune R. A., Gehrke C. W., Midgett R. and Ehrlich M. 1980. Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. Nucleic Acids Res., **8**: 4763–4776.

Laszlo A. H., Derrington I. M., Brinkerhoff H., Langford K. W., Nova I. C., Samson J. M., et al. 2013. Detection and mapping of 5-methylcytosine and 5-hydroxymethylcytosine with nanopore MspA. Proc. Natl. Acad. Sci., **110**: 18904-9.

Laszlo A. H., Derrington I. M., Ross B. C., Brinkerhoff H., Adey A., Nova I. C., et al. 2014. Decoding long nanopore sequencing reads of natural DNA. Nat. Biotechnol., **32**: 829–833.

Li X.,Wang X., He K., Ma Y., Su N., et al. 2008. High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression. Plant Cell, **20**: 259–76.

Liang Z., Shen L., Cui X., Bao S., Geng Y., Yu G., Liang F., Xie S., Lu T., Gu X. and Yu H. 2018. DNA N6-adenine methylation in *Arabidopsis thaliana*. Develop Cell, **45**: 406-16.

Liu J., Zhu Y., Luo G. Z., Wang X., Yue Y., Wang X., Zong X., Chen K., Yin H., Fu Y. and Han D. 2016. Abundant DNA 6mA methylation during early embryogenesis of zebrafish and pig. Nat. Commun., **7**: 13052.

Liu Q., Fang L., Yu G., Wang D., Xiao C. L. and Wang K. 2019. Detection of DNA base modifications by deep recurrent neural network on Oxford Nanopore sequencing data. Nat. Commun., **10**: 1-11.

Liu Z., Wu J., Xie Z., Liu S, Fan-Havard P., Huang T. H., Plass C., Marcucci G. and Chan K. K. 2009. Quantification of regional DNA methylation by liquid chromatography/tandem mass spectrometry. Anal. Biochem., **391**: 106–113.

Luo G. Z., Wang F., Weng X., Chen K., Hao Z., Yu M., Deng X., Liu J. and He C. 2016. Characterization of eukaryotic DNA N 6-methyladenine by a highly sensitive restriction enzyme-assisted sequencing. Nat. Commun., **7**: 11301.

Malins D. C., Polissar N. L., Ostrander G. K. and Vinson M. A. 2000. Single 8-oxo-guanine and 8-oxo-adenine lesions induce marked changes in the backboe structure of a 25-base DNA strand. Proc. Natl. Acad. Sci., **97**: 12442-12445.

McIntyre A. B. R., Alexander N., Burton A. S., Castro-Wallace S., Chiu C. Y., John K. K., et al. 2017. Nanopore detection of bacterial DNA base modifications. BioRxiv, https://doi.org/10.1101/127100

Moore L., Le T. and Fan G. 2013. DNA Methylation and its basic function. Neuropsychopharmacol., **38**: 23–38. https://doi.org/10.1038/npp.2012.112

Münzel M., Globisch D., Brückl T., Wagner M., Welzmiller V., Michalakis S., Müller M., Biel M. and Carell T. 2010. Quantification of the sixth DNA base hydroxymethylcytosine in the brain. Angewandte Chemie Int. Edition, **49**: 5375-77

Olova N., Krueger F., Andrews S., Oxley D., Berrens R. V., Branco M. R. and Reik W. 2018. Comparison of whole-genome bisulfite sequencing library preparation strategies identifies sources of biases affecting DNA methylation data. Genome Biol., **19**: 1-19.

Park J. W., Han Y. I., Kim S. W., et al. 2019. 8-OxoG in GC-rich Sp1 binding sites enhances gene transcription in adipose tissue of juvenile mice. Sci. Rep., **9**: 15618. https://doi.org/10.1038/s41598-019-52139-z

Pfaffeneder T., Hackner B., Truss M., Münzel M., Müller M., Deiml C. A., Hagemeier C. and Carell T. 2011. The discovery of 5-formylcytosine in embryonic stem cell DNA. Angewandte Chemie Int. Edition, **50**: 7008-12.

Pfaffeneder T., Spada F., Wagner M., Brandmayr C., Laube S. K., Eisen D., et al. 2014. Tet oxidizes thymine to 5-hydroxymethyluracil in mouse embryonic stem cell DNA. Nat. Chem. Biol., **10**: 574–81. https://doi.org/10.1038/nchembio.1532

Plongthongkum N., Diep D. H. and Zhang K. 2014. Advances in the profiling of DNA modifications: cytosine methylation and beyond. Nat. Rev. Genet., **15**: 647.

Raizis A. M., Schmitt F. and Jost J. P. 1995. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. Anal Biochem., **226**(1): 161–166.

Reik W. 2007. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature, **447**: 425.

Richards C. L., Bossdorf O. and Verhoeven K. J. 2010. Understanding natural epigenetic variation. New Phytologist, **187**: 562-4.

Ross M. G., Russ C., Costello M., Hollinger A., Lennon N. J., Hegarty R., Nusbaum C. and Jaffe D. B. 2013. Characterizing and measuring bias in sequence data. Genome Biol., **14**: R51.

Seem K., Kaur S., Kumar S. and Mohapatra T. 2024. Epigenome editing for targeted DNA (de)methylation: A new perspective

in modulating gene expression, Crit. Rev. Biochem. Mol. Biol., **59**(1): https://doi.org/10.1080/10409238.2024.2320659

Seiler C. and Kuhlmann M. 2019. Quantification of DNA methylation as biomarker for grain quality rice grain quality. Humana Press, New York, NY pp. 301-310.

Shi D.Q., Ali I., Tang J. and Yang W. C. 2017. New insights into 5hmC DNA modification: generation, distribution and function. Front. Genet., **8**: 100.

Simpson J. T., Workman R. E., Zuzarte P. C., David M., Dursi L. J., Timp W. 2017. Detecting DNA cytosine methylation using nanopore sequencing. Nat. Methods, **14**: 407–410.

Smith Z. D. and Meissner A. 2013. DNA methylation: roles in mammalian development. Nat. Rev. Genet., **14**: 204.

Song L., James S. R., Kazim L. and Karpf A. R. 2005. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. Anal. Chem., **77**: 504–510.

Tahiliani M., Koh K. P., Shen Y., Pastor W. A., Bandukwala H., Brudno Y., et al. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science, **324**: 930-35.

Tanaka K. and Okamoto A. 2007. Degradation of DNA by bisulfite treatment. Bioorg Med. Chem. Lett., **17**: 1912–5.

Thuc L., Kim K. P., Fan G., Faull K. F. 2011. A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. Anal. Biochem., **412**: 203–209.

Tiwari S. 2017. Recent advancement in methodology for understanding epigenetic modifications. J. Clin. Epidemiol., **3**: 21.https://doi.org/10.21767/2472-1158.100055.

Tran R. K., Henikoff J. G., Zilberman D., Ditt R. F., Jacobsen S. E., Henikoff S. 2005. DNA methylation profiling identifies CG methylation clusters in Arabidopsis genes. Curr. Biol., **15**: 154-9.

Urich M. A., Nery J. R., Lister R., Schmitz R. J. and Ecker J. R. 2015. MethylC-seq library preparation for base-resolution whole-genome bisulfite sequencing. Nat. Protocols, **10**: 475.

Vaughn M. W., Tanurdzić M., Lippman Z., Jiang H., Carrasquillo R., Rabinowicz P. D., et al. 2007. Epigenetic natural variation in *Arabidopsis thaliana*. PLoS Biol., **5**: e174. https://doi.org/10.1371/journal.pbio.0050174

Wang X., Elling A. A., Li X., Li N., Peng Z., et al. 2009. Genome-wide and organ-specific landscapes of epigenetic modifications and their relationships to mRNA and small RNA transcriptomes in maize. Plant Cell, **21**: 1053–69.

Wang X. L., Song S. H., Wu Y. S., Li Y. L., Chen T. T., Huang Z. Y., et al. 2015. Genome-wide mapping of 5-hydroxymethylcytosine in three rice cultivars reveals its preferential localization in transcriptionally silent transposable element genes. J. Exp. Bot., **66**: 6651-6663.

Wang Y., Sheng Y., Liu Y., Pan B., Huang J., Warren A., et al. 2017. N6-methyladenine DNA modification in the unicellular eukaryotic organism Tetrahymena thermophila. Eur. J. Protistol., **58**: 94–102. https://doi.org/10.1016/j.ejop.2016.12.003

Wion D. and Casadesús J. 2006. N6-methyl-adenine: an epigenetic signal for DNA–protein interactions. Nat. Rev. Microbiol., **4**: 183.

Woodcock D. M., Crowther P. J., Simmons D. L., Cooper I. A. 1984. Sequence specificity of cytosine methylation in the DNA of the Chinese hamster ovary (CHO-K1) cell line. Biochimica et Biophysica Acta-Gene Structure and Expression, **783**: 227-33.

Wu T. P., Wang T., Seetin M. G., Lai Y., Zhu S., Lin K., et al. 2016. DNA methylation on N6-adenine in mammalian embryonic stem cells. Nature, **532**: 329.

Xiong J., Ye T. T., Ma C. J., Cheng Q. Y., Yuan B. F. and Feng Y. Q. 2019. N6-Hydroxymethyladenine: a hydroxylation derivative of N 6-methyladenine in genomic DNA of mammals. Nucleic Acids Res., **47**: 1268-77.

Xiong L. Z., Xu C. G., Saghai Maroof M. A. and Zhang Q. 1999. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. Mol. Gen. Genet., **261**: 439-446.

Yan H., Kikuchi S., Neumann P., Zhang W., Wu Y., Chen F. and Jiang J. 2010. Genome-wide mapping of cytosine methylation revealed dynamic DNA methylation patterns associated with genes and centromeres in rice. Plant J., **63**: 353–365.

Yu M., Ji L., Neumann D. A., Chung D. H., Groom J., Westpheling J., He C., Schmitz R. J. 2015. Base-resolution detection of N 4-methylcytosine in genomic DNA using 4mC-Tet-assisted-bisulfite-sequencing. Nucleic Acids Res., **43**: e148.

Zahid O. K., Zhao B. S., He C., Hall A. R. 2016. Quantifying mammalian genomic DNA hydroxymethylcytosine content using solid-state nanopores. Sci. Rep., **6**: 29565.

Zeng H., He B., Xia B., Bai D., Lu X., Cai J., et al. 2018. Bisulfite-free, nanoscale analysis of 5-hydroxymethylcytosine at single base resolution. J. Am. Chem. Society, **140**: 13190-13194.

Zhang G., Huang H., Liu D., Cheng Y., Liu X., Zhang W., et al. 2015. N6-methyladenine DNA modification in *Drosophila*. Cell, **161**: 893-906.

Zhou C., Wang C., Liu H., Zhou Q., Liu Q., Guo Y., Peng T., et al. 2018. Identification and analysis of adenine N6-methylation sites in the rice genome. Nat. Plants, **4**: 554-563. https://doi.org/10.1038/s41477-018-0214-x