Chapter 16
Theoretical and Applied Epigenetics in Plants

Yuhya Wakasa, Taiji Kawakatsu, and Fumio Takaiwa

Abstract Artificial regulation of gene expression through RNA-directed DNA methylation (RdDM)-mediated epigenome editing is one the most important and attractive next-generation technologies for plant trait improvement, often called "new plant breeding techniques" (NPBTs). RdDM can induce transcriptional gene silencing (TGS) of a target gene via modification of the cytosine methylation levels of its promoter region; thus, RdDM is useful as a method for suppression of gene expression without changing the genomic DNA sequence. Likewise, several types of strict epigenetic regulation occur at both the DNA and chromatin levels under normal growth conditions in plants. Recent studies have revealed genome-wide and organ-specific landscapes of epigenetic modifications and their close relationship to plant growth regulation. Therefore, understanding recent findings concerning epigenetic regulation in plants is very important to the future application of epigenome editing in plant breeding. In this chapter, we illustrate several aspects of theoretical and applied epigenetics in plants through discussion of recent studies.

Keywords Epigenetics · Chromatin · Histone · Methylation · New plant breeding techniques · RNA-directed DNA methylation · Transcriptional gene silencing

16.1 Mechanisms of Epigenetic Regulation in Plants

16.1.1 DNA Methylation

Cytosine DNA methylation (5mC) is a covalent modification of the fifth carbon residue of cytosine. 5mC is conserved in eukaryotes including mammals and plants, although absent in some organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Law and Jacobsen 2010). There are three main strategies to assay DNA methylation levels: (1) digestion of methylated/unmethylated DNA

Y. Wakasa (✉) · T. Kawakatsu · F. Takaiwa
Plant Molecular Farming Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan
e-mail: ywakasa@affrc.go.jp

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fragments with methylation-sensitive restriction enzymes (MSREs), (2) methylated DNA immunoprecipitation (MeDIP) using an antibody against methylated cytosine, and (3) bisulfite sequencing, in which unmethylated cytosine is converted to uracil by sodium bisulfite, which is reported as thymine in sequence reads. Each strategy can be combined with high-throughput sequencing technology and extended to genome-wide analysis (Cokus et al. 2008; Down et al. 2008; Lister et al. 2008; Maunakea et al. 2010). However, bisulfite sequencing is the most comprehensive and accurate way to quantify DNA methylation levels, so it is widely used for genome-wide analysis (Urich et al. 2015).

In plants, 5mC occurs in three distinct sequence contexts: CG and CHG, which are both symmetric, and CHH (H = C, A, or T), which is asymmetric (Law and Jacobsen 2010). Based on extensive studies in *Arabidopsis thaliana*, four distinct DNA methylation pathways are known. CG methylation is catalyzed by DNA METHYLTRANSFERASE 1 (MET1) and maintained in a semiconservative manner during DNA replication (Kankel et al. 2003). CHG methylation is catalyzed by CHROMOMETHYLASE3 (CMT3), which recognizes methylation at the 9th lysine residue of the H3 tail (H3K9) (Bartee et al. 2001; Lindroth et al. 2001). CMT2, a homolog of CMT3, catalyzes CHG and CHH methylation in deep heterochromatic regions (Zemach et al. 2013; Stroud et al. 2014). DOMAINS OF REARRANGED METHYLTRANSFERASE 2 (DRM2) catalyzes cytosine methylation in all three sequence contexts, in a process referred to as RNA-directed DNA methylation (RdDM) (Cao and Jacobsen 2002; Law and Jacobsen 2010; Kawashima and Berger 2014; Cuerda-Gil and Slotkin 2016). In RdDM, 21- or 24-nucleotide (nt) small interfering RNAs (siRNAs) guide DRM2 to target regions, marked with DNA methylation and H3K9me, through association with three conserved Argonaute proteins, AGO4, AGO6, and AGO9 (Gao et al. 2010; Havecker et al. 2010; McCue et al. 2015). Non-CG DNA methylation and histone methylation (see Sect. 16.1.2) form a self-reinforcing loop, in which H3K9me controls non-CG DNA methylation and non-CG DNA methylation controls H3K9me (Stroud et al. 2014). Interplays between these pathways have been implicated by comprehensive methylome analysis of components in these pathways (Stroud et al. 2013).

DNA methylation can be actively removed by DNA demethylases. In *Arabidopsis*, DNA glycosylases DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1)/DEMETER-LIKE 1 (DML1), DML2, and DML3, are involved in DNA demethylation (Choi et al. 2002; Gong et al. 2002; Penterman et al. 2007; Ortega-Galisteo et al. 2008). In contrast to mutants of DNA methylation components, which are usually viable, *dme* is embryo lethal, highlighting the importance of active demethylation (Bartee et al. 2001; Cao and Jacobsen 2002; Choi et al. 2002; Kankel et al. 2003). DNA methylation and demethylation activities are balanced by a feedback loop between RdDM and ROS1 (Lei et al. 2015; Williams et al. 2015). *ROS1* expression is promoted by RdDM in the *ROS1* promoter region and repressed by ROS1 activity itself.

CG methylation is broadly distributed across the genome and often resides within gene bodies (transcribed regions); this type of methylation is called gene body methylation (gbM). The distributions of gbM and histone variant H2A.Z are

mutually exclusive, and gbM is associated with higher gene expression (Tran et al. 2005; Zhang et al. 2006; Zilberman et al. 2007). Therefore, gbM is thought to exclude H2A.Z and allow constitutive expression (Zilberman et al. 2008; Coleman-Derr and Zilberman 2012). However, recent studies on intra- and interspecies variations of DNA methylation indicate that gbM does not have a great effect on gene expression or affect H2A.Z distribution within genes (Bewick et al. 2016; Kawakatsu et al. 2016a). Currently, the role of gbM is unclear. Co-localization of CG and non-CG methylation is a characteristic of heterochromatin and transposable elements (TEs) and contributes to gene and TE silencing (Law and Jacobsen 2010). Population-wide methylome variations are largely associated with structural variations such as TE insertion or deletion (Kawakatsu et al. 2016a) and are enriched near signaling pathway genes or immune response genes. TE transposition has shaped the epigenome of *Arabidopsis* and has introduced variation in environmental responses during diversification.

DNA methylome studies are not limited to *Arabidopsis*, currently extending to nearly 100 species (Gent et al. 2013; Project 2013; Schmitz et al. 2013a; Stroud et al. 2013; Zhong et al. 2013; Seymour et al. 2014; Ong-Abdullah et al. 2015; Ausin et al. 2016; Niederhuth et al. 2016; Takuno et al. 2016). In addition, transgenerational, populational methylome variations, tissue- and cell-type-specific methylomes, and stress-responsive methylomes have been reported (Hsieh et al. 2009; Schmitz et al. 2011; Calarco et al. 2012; Dowen et al. 2012; Ibarra et al. 2012; Schmitz et al. 2013a, b; Garg et al. 2015; Secco et al. 2015; Hsieh et al. 2016; Kawakatsu et al. 2016a, b; Narsai et al. 2016; Park et al. 2016; Wibowo et al. 2016; Hossain et al. 2017). Recent advances in single-molecule real-time sequencing enable detection of methylated cytosines from long reads without bisulfite conversion (Rand et al. 2017; Simpson et al. 2017). These technologies potentially offer a paradigm shift in DNA methylome analysis, especially in crop species with large genomes and/or multiploidy.

16.1.2 Histone Modification

Histone proteins package genomic DNA into nucleosomes, which in turn form chromatin (Roudier et al. 2009). Histones are conserved in eukaryotes. Four major histones (H2A, H2B, H3, and H4) act as core histones and H1 acts as a linker histone (Kornberg 1974; Thoma and Koller 1977; Luger et al. 1997). The histone core is an octamer complex: two H2A-H2B dimers and an H3-H4 tetramer. Approximately 147 bp of DNA wraps around each histone core and forms a nucleosome. Several histone variants share homology with major histone proteins (Deal and Henikoff 2011a). In addition, histone tails can be posttranslationally modified through methylation (me) and acetylation (ac). These modifications are implicated in flowering, leaf development, seed maturation, flower development, circadian rhythm, and chloroplast development (Deal and Henikoff 2011a; Merini and Calonje 2015; Mozgova et al. 2015). Chromatin immunoprecipitation followed by

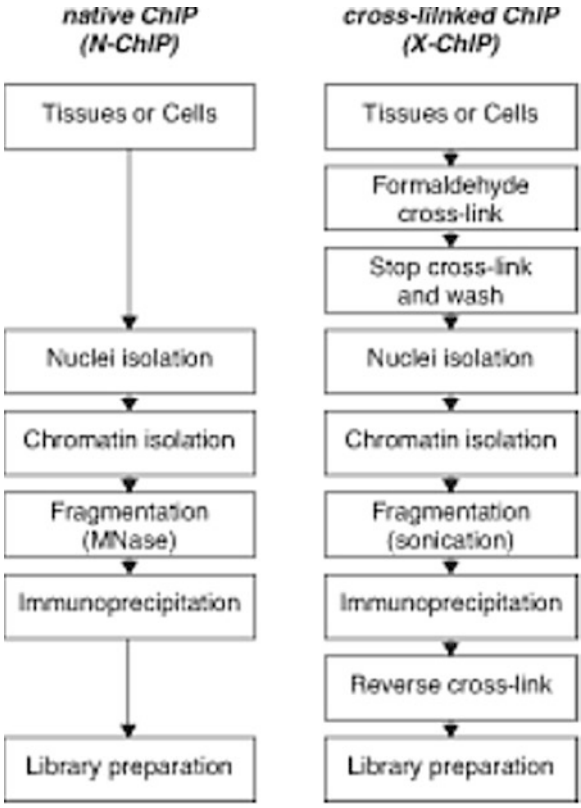

Fig. 16.1 Comparison of ChIP-seq protocols for genome-wide chromatin mark(s) distribution. Native chromatin is digested by micrococcal nuclease (MNase) in native ChIP (N-ChIP). Cross-linked chromatin is fragmented by sonication in cross-linked ChIP (X-ChIP). Nucleosomes are subjected to immunoprecipitation with specific antibodies

high-throughput sequencing (ChIP-seq) is widely used for analyzing the genome-wide distribution of histones, histone variants, and histone modifications (Luo and Lam 2014). Native chromatin digested by micrococcal nuclease (MNase) or cross-linked chromatin fragmented by sonication can be subjected to immunoprecipitation (N-ChIP [native ChIP] or X-ChIP [cross-linked ChIP]) using histone-, histone variant-, or histone modification-specific antibodies (Jackson 1978; O'Neill and Turner 1995; Barski et al. 2007; Schmid and Bucher 2007) (Fig. 16.1).

Although histone modifications are conserved in eukaryotes, their distribution patterns and functions vary. For example, mono-methylation of H3 (H3K9me1) is enriched in heterochromatin in *Arabidopsis* but is enriched at the transcription start sites (TSS) of active genes in animals (Fransz et al. 2006; Fuchs et al. 2006). H3K9 di-methylation (H3K9me2) is also enriched in heterochromatin (Turck et al. 2007). In contrast, H3K9 tri-methylation (H3K9me3) is enriched in euchromatin and is associated with active genes (Turck et al. 2007). H3K9 methylation is catalyzed by SET- and RING-associated (SRA) domain-containing SU(VAR) HOMOLOGUE 1 (SUVH1), SUVH4–6, and SET- and WIYLD-domain-containing SU(VAR)3–9 related 4 (SUVR4) (Ebbs et al. 2005; Ebbs and Bender 2006). INCREASED BONSAI METHYLATION 1 (IBM1) demethylates H3K9 (Inagaki et al. 2010).

H3K27 methylation tends to be associated with low-expression genes and tissue-specific genes (Turck et al. 2007; Roudier et al. 2011). H3K27me1 and H3K27me2 are distributed in both euchromatin and heterochromatin, and H3K27me3 is mainly observed in euchromatin (Roudier et al. 2011). H3K27me1, me2, and me3 mark distinct sets of genes. H3K27me1 and H3K27me3 are enriched in the transcribed regions of marked genes, relative to flanking regions. However, H3K27me2 levels are uniformly higher in both the transcribed and flanking regions of marked genes than in unmarked genes. H3K27me1 is catalyzed by ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5 (ATXR5) and ATXR6 (Jacob et al. 2009). H3K27m3 is catalyzed by Polycomb Repressive Complex 2 (PRC2) (Margueron and Reinberg 2011). *Drosophila* PRC2 consists of four subunits: (1) enhancer of zeste (E(z)), (2) suppressor of zeste 12 (Su(z)12), (3) nucleosome-remodeling factor 55 kDa subunit (NURF55), and (4) extra sex combs (ESC). *Arabidopsis* possesses three E(z) homologs (CURLY LEAF [CLF], MEDEA [MEA], and SWINGER [SWN]), three Su(z)12 homologs (EMBRYONIC FLOWER 2 [EMF2], VERNALIZATION 2 [VRN2], and FERTILIZATION-INDEPENDENT SEED 2 [FIS2]), five NURF55 homologs (MULTI-SUBUNIT SUPPRESSOR OF IRA 1–5 [MSI1–5]), and one ESC homolog (FERTILIZATION-INDEPENDENT ENDOSPERM [FIE]) (Ach et al. 1997; Goodrich et al. 1997; Grossniklaus et al. 1998; Kenzior and Folk 1998; Luo et al. 1999; Gendall et al. 2001; Yoshida et al. 2001; Hennig et al. 2003; Chanvivattana et al. 2004; Jullien et al. 2006; Makarevich et al. 2006; Zhang et al. 2007; Jiang et al. 2008; Kim et al. 2010; Lafos et al. 2011; Pazhouhandeh et al. 2011; Derkacheva et al. 2013). PRC2 target genes especially depend on E(z) homologs.

PRC1 is also required for transcriptional repression of H3K27me3-marked genes. PRC1 can catalyze histone H2A mono-ubiquitination (H2Aub) of target genes (de Napoles et al. 2004; Wang et al. 2004). *Drosophila* PRC1 consists of four subunits (Gil and O'Loghlen 2014): (1) chromodomain protein Polycomb (Pc), (2) RING-finger protein Posterior sex comb (Psc), (3) RING-finger protein *Drosophila* RING1 (dRING1), and (4) Polyhomeotic (Ph). In *Arabidopsis*, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)/TERMINAL FLOWER 2 (TFL2) plays a similar role to Pc (Turck et al. 2007). *Drosophila* Psc possesses a RING-finger domain, a RING-finger and WD40-associated ubiquitin-like (RAWUL) domain, and a long intrinsically disordered C-terminal region (CTR). The CTR domain of Psc is involved in inhibition of nucleosome remodeling, gene repression, and chromatin compaction; however, it is missing from *Arabidopsis* Psc homologs AtBMI1A, AtBMI1B, and AtBMI1C (Sanchez-Pulido et al. 2008). EMBRYONIC FLOWER 1 (EMF1) is similar to the CTR domain and acts in a similar manner (Aubert et al. 2001; Calonje et al. 2008). AtRING1A and AtRING1B correspond to dRING1 (Schoorlemmer et al. 1997; Xu and Shen 2008). No plant homolog of Ph has yet been identified.

JUMONJI (JMJ) proteins RELATIVE OF EARLY FLOWERING 6 (REF6)/JMJ12, EARLY FLOWERING 6 (ELF6)/JMJ11, JMJ30, and JMJ32 are H3K27 demethylases (Noh et al. 2004; Lu et al. 2011; Gan et al. 2014). Notably, four C2H2 zinc finger domains of REF6 recognize a CTCTGYTY motif and guide REF6 to its binding sites to modulate H3K27me3 levels (Cui et al. 2016; Li et al. 2016).

A hierarchical model for gene repression, in which PRC2 acts upstream of PRC1, has been widely accepted (Mozgova et al. 2015). In this model, PRC2 methylates H3K27 (to H3K27me3) in target genes as the first step. Second, PRC1 is guided there through H3K27me3 recognition by Pc or LHP1, and target genes are marked with H2Aub, leading to gene repression. However, the hierarchical order of PRC2 and PRC1 is more complicated than once thought (Merini and Calonje 2015). For example, PRC1 is required for H3K27me3 at many PRC2 target genes (Kim et al. 2012; Yang et al. 2013), and PRC2 is not necessarily required for H2Aub at target genes (i.e., PRC1 recruits) (Pengelly et al. 2015). H2Aub can recruit PRC2 and promote H3K27me3 in animals (Blackledge et al. 2014; Cooper et al. 2014; Kalb et al. 2014). In addition, some PRC1 and PRC2 components interact with each other (Xu and Shen 2008; Derkacheva et al. 2013; Wang et al. 2014). Therefore, the interactions between PRC1 and PRC2 may include a positive feedback loop, direct interplay, and mutually independent mechanisms.

H3K36me3 is associated with actively expressed genes (Roudier et al. 2011). H3K36me3 is most prevalent within TSSs but is distributed throughout transcribed regions. SDG8, and possibly SDG4 and SDG25/ATXR7, catalyze H3K36me2 and H3K36me3 (Zhao et al. 2005; Cartagena et al. 2008; Xu et al. 2008; Berr et al. 2009). H3K36me3 and H3K27me3 play antagonistic roles—activation and repression—and rarely co-exist on the same histone tail (Roudier et al. 2011; Yang et al. 2014).

H3K4 methylation is mostly found in genes and other euchromatin (Roudier et al. 2011). In contrast to enhancer-associated H3K4me1 in animals, H3K4me1 in *Arabidopsis* is distributed inside transcribed regions but is less prevalent near TSSs and transcription end sites (TESs). Both H3K4me2 and H3K4me3 are enriched around TSSs but depleted around TESs. H3K4me3 is associated with highly expressed genes, whereas H3K4me1 and H3K4me2 are associated with tissue-specific genes (Roudier et al. 2011). ARABIDOPSIS TRITHORAX 1 (ATX1)/SET DOMAIN GROUP 27 (SDG27) and Complex Proteins Associated with Set1 (COMPASS)-like complex catalyze H3K4me3, and ATX2/SDG30 catalyzes H3K4me2 (Saleh et al. 2008; Jiang et al. 2011). SDG2 catalyzes H3K4me1, H3K4me2, and H3K4me3 in vitro, but in vivo, *sdg2* shows reduction only in H3K4me3 (Berr et al. 2010; Guo et al. 2010). JMJ14 and homologs of LYSINE-SPECIFIC HISTONE DEMETHYLASE 1 (LSD1), FLOWERING LOCUS D (FLD)/LSD1-like 1 (LDL1), and LDL2 are required for H3K4 demethylation (Deleris et al. 2010; Lu et al. 2010; Greenberg et al. 2013).

H3K9ac and H3K27ac are associated with active gene expression (Charron et al. 2009). Levels of both H3K9ac and H3K27ac peak near TSSs and are distributed inside gene bodies. The H3K9ac and H3K27ac target regions are largely the same but distinct from H3K27me3 target regions. H3K9ac and H3K27ac are catalyzed by histone acetyltransferase (HAT) family proteins, such as homologs of general control non-derepressible (GCN5) and TATA binding protein-associated factor 1 (TAF1) (Pandey et al. 2002; Benhamed et al. 2006). AtGCN5 also catalyzes H4K14ac. Histone deacetylase (HDAC) family proteins, such as HDA6, are responsible for histone deacetylation

(Pandey et al. 2002; Earley et al. 2006; To et al. 2011; Liu et al. 2014). HATs and HDACs act as transcriptional co-activators and co-repressors, respectively.

Combinations of histone modifications are thought to be important for the precise expression state and responsiveness of a gene. Two opposing histone marks, for example, H3K27me3 (repressing) and H3K4me3 (activating), can be co-localized in the same genomic regions (Roudier et al. 2011). As in animals, bivalent chromatin regions in plants are associated with several transcription factors (TFs) that are normally expressed at low levels but are induced at specific timing and/or in specific tissues by developmental cues (Saleh et al. 2007; Jiang et al. 2008; Berr et al. 2010; Roudier et al. 2011). It is also possible that a mixture of different cell types with different chromatin modification states could be misinterpreted as co-localization of opposing histone marks. Cell-type-specific profiling would promote further understanding not only of cell-type-specific properties but also of the combinatorial functions of histone modifications. Several recent developments show considerable promise in this area. For example, low-input ChIP-seq methods and high-throughput sequencing technologies are evolving (Adli and Bernstein 2011; Brind'Amour et al. 2015; Schmidl et al. 2015). Recently developed simple but highly efficient INTACT (Isolation of Nuclei Tagged in specific Cell Types) is feasible for cell-type-specific profiling (Deal and Henikoff 2011b).

16.1.3 Chromatin Accessibility

Transcriptional activation is primarily regulated by TF binding to regulatory DNA elements, where chromatin is open or accessible. Genome-wide chromatin accessibility can be assayed directly or indirectly through a combination of nuclease digestion and high-throughput sequencing (Meyer and Liu 2014). As in N-ChIP, MNase digests bare DNA that is not protected by nucleosomes, whereas DNase I cleaves unprotected DNA. Therefore, MNase digestion followed by sequencing (MNase-seq) identifies nucleosome positioning and indirectly detects open chromatin regions (Schones et al. 2008), whereas DNase I cleavage followed by sequencing (DNase-seq) directly detects open chromatin regions (Boyle et al. 2008). FAIRE-seq (Formaldehyde-Assisted Isolation of Regulatory Elements) also directly detects open chromatin regions by isolating un-cross-linked DNA with nucleosomes (Giresi et al. 2007). Transposase Accessible Chromatin sequencing (ATAC-seq) uses Tn5 transposase to insert sequencing-ready adaptor sequences into open chromatin regions, starting with as few as 500 cells (Buenrostro et al. 2013). Genome-wide chromatin accessibility studies have been limited in plants (Zhang et al. 2012a, 2012b; Li et al. 2014; Wu et al. 2014; Zhang et al. 2015; Lu et al. 2016). Nevertheless, these studies clearly demonstrate that the identified open chromatin regions are associated with gene expression and TF binding sites. ChIP-seq has been used for assays of genome-wide TF binding sites in vivo (Song et al. 2016). However,

preparing antibodies against a wide variety of TFs or transgenic plants expressing tagged TFs with native promoters is time-, cost-, and labor-consuming. DNA affinity purification sequencing (DAP-seq) is a new technology to cost-effectively identify TF binding sites in vitro (O'Malley et al. 2016). The combination of chromatin accessibility assays and DAP-seq is expected to greatly advance our knowledge of transcriptional regulatory networks.

16.2 Application Studies on Epigenetics in Plants

16.2.1 Application of Epigenome Editing to Plant Breeding

Gene manipulation (GM) techniques have been used as molecular breeding tools to develop various GM crops with excellent traits such as resistance to insect pests, plant diseases, and specific herbicides. Furthermore, "golden rice," which accumulates provitamin A in the seed, will be practical to use in the Philippines in the near future. GM crops are at present commercially cultivated in 28 countries worldwide. The total global cultivated area of transgenic crops was estimated to have reached approximately 179.7 million hectares by 2015 (James 2016). On the other hand, conventional GM techniques are still sometimes viewed as a serious issue in numerous countries because the transgene is integrated into the genome of the target plant, and a number of people are concerned about gene flow from GM crops to the environment.

In recent years, technologies referred to as "new plant breeding techniques" (NPBTs) have been proposed as a solution for issues surrounding conventional GM crops. Genome editing (ZFN, TALEN, CRISPR/Cas9), "grafting with GM plants," "reverse breeding," *Agrobacterium* infiltration, and RdDM can all be classified as NPBTs (Lusser et al. 2012; Schaart et al. 2016). When these NPBTs are applied, it is difficult to distinguish between the newly introduced artificial mutation and natural mutations. In particular, changes to genomic DNA caused by RdDM cannot be detected by conventional molecular analysis methods such as PCR and DNA sequencing because there is no change to the DNA sequence. A key characteristic of RdDM-mediated transcriptional gene silencing (TGS) is the production of double-strand RNA (dsRNA) with homology to the promoter sequence of the target gene. The dsRNA is cleaved into 21–24 nt pieces of siRNA by DICER like (DCL) protein. In plants, these siRNAs become a cause of epigenetic modification of cytosine residues in CG, CHG, and CHH contexts into methylated cytosine (5mC). Two types of DNA-dependent RNA polymerases (Pol IV and Pol V) are necessary to advance the process of RdDM (Matzke et al. 2015). The increased methylation levels in the target gene promoter induce TGS, which is associated with changes in chromatin structure through histone modification (see Sect. 16.1.2).

Although posttranscriptional gene silencing (PTGS) has been used for functional analyses of target genes for about 20 years, TGS has an important advantage over

PTGS. Artificially induced methylation and TGS via RdDM may be preserved and inherited after removal of the trigger gene cassette; thus RdDM-mediated TGS could be considered as a type of NPBT. However, there are few research papers that discuss the relationship between the preservation or loss of TGS and the presence or absence of the trigger gene (Kasai and Kanazawa 2013).

The remainder of this chapter describes the application of epigenetic modification to improvement of plant traits through various strategies of RdDM-mediated TGS. CRISPR/Cas9-mediated epigenome editing is also briefly discussed.

16.2.2 Viral Vector-Mediated TGS in Plants

Plant viral vectors have been used to induce RdDM-mediated TGS (Fig. 16.2a). In this strategy, the plant defense response toward virus infection (called recovery) is applied to the production of dsRNA from target gene promoters. Viral vector-mediated TGS in plants has been successfully induced in both reporter genes and endogenous genes. Kanazawa and co-authors used recombinant *Cucumber mosaic virus* (CMV) to induce TGS of *Chalcone synthase-A* and *LeSPL-CNR* genes in petunia and tomato, respectively (Kanazawa et al. 2011). Both plants showed clear phenotypic changes in association with epigenetic modification of the target gene promoters (Kanazawa et al. 2011). Interestingly, these new traits have been observed in subsequent generations even though the viral vector was not detected in these progenies (Kanazawa et al. 2011). These authors further reported that 2b protein, one of the endogenous proteins derived from CMV, is useful for stable induction of RdDM because it increases the expression of TGS induction-related genes and decreases the expression of demethylation-related genes. The 2b protein functions as an RNA silencing suppressor that can inhibit PTGS and virus-induced gene silencing (VIGS) (Goto et al. 2007). These results suggest that the combination of dsRNA and the 2b protein leads to highly efficient induction of RdDM-mediated TGS in a viral vector system.

As another example, TGS induction of *Chalcone synthase-A* gene in petunia was achieved via the use of apple latent spherical virus (ALSV) as a viral vector (Kon and Yoshikawa 2014). *Tobacco rattle virus* (TRV) vectors have also been used for induction of TGS, but most of the target genes have been reporter genes such as GFP and GUS under the control of the CaMV 35S promoter (Jones et al. 2001). However, recently, Bond and Baulcombe reported that TGS induction of endogenous gene (*FLOWERING WAGENINGEN*, *FWA*) using TRV viral vector system in *Arabidopsis* and this report deeply discussed about initiation, establishment, and maintenance of TGS in endogenous gene, using *FWA* TGS *Arabidopsis* and various mutants of gene silencing-related genes (Bond and Baulcombe 2015).

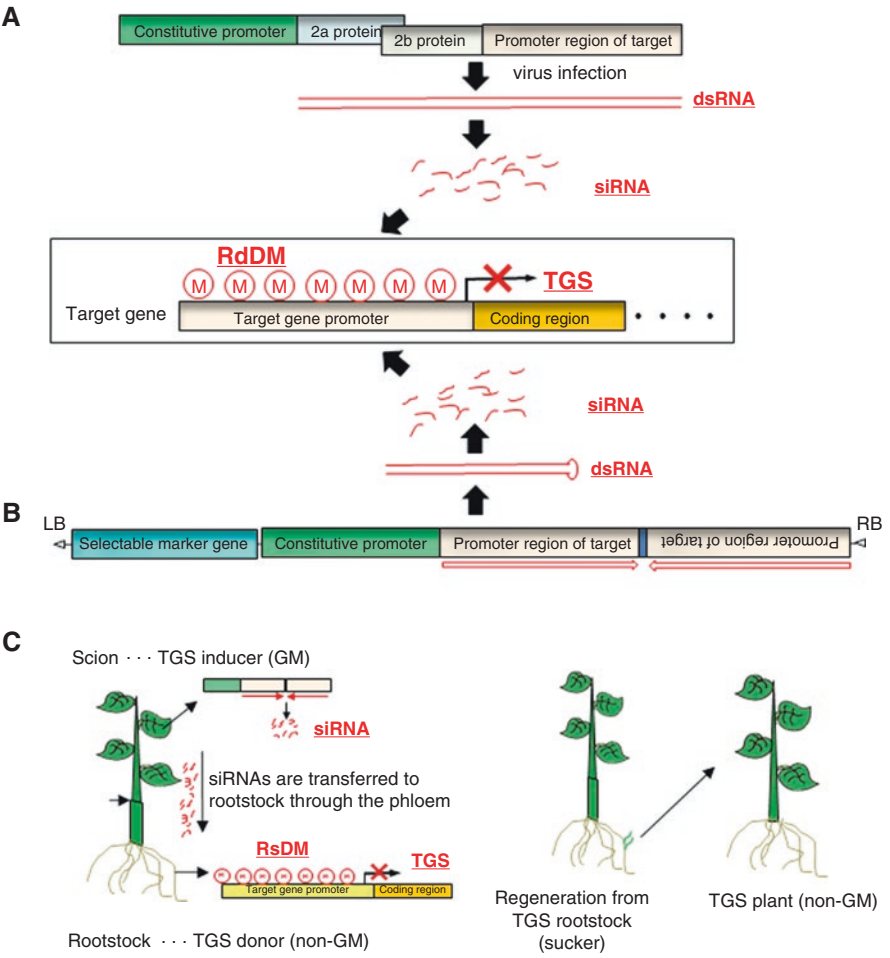

Fig. 16.2 Various strategies for induction of RdDM-mediated TGS in plants. Viral vector-mediated TGS (**a**), *Agrobacterium* (T-DNA)-mediated TGS (**b**), and grafting-mediated TGS (**c**) are shown. *M* methylation, *RB* right border, *LB* left border

16.2.3 Agrobacterium *(T-DNA)-Mediated TGS in Plants*

RdDM-mediated TGS in plants is generally induced by using T-DNA harboring gene cassettes to express dsRNA directed toward the promoter region of the target gene (Fig. 16.2b). Gene cassettes to express the dsRNA are introduced into the plant genome via *Agrobacterium*-mediated transformation. T0 plants expressing dsRNA derived from a foreign gene cassette should be treated as GM plants, whereas progenies of TGS plants after removal of T-DNA by segregation can be treated as non-GM plants in some world areas.

Although T-DNA-mediated TGS is very simple and easy to use, most research papers describe TGS of a reporter gene under the control of the CaMV 35S promoter. There are only a few reports of T-DNA-mediated TGS of endogenous genes in plants. In rice, RdDM-mediated TGS was attempted using a reporter gene (GFP under the control of the CaMV 35S promoter) and several endogenous genes. In these experiments, TGS could be easily induced for GFP; TGS of most endogenous genes could not be induced in spite of highly efficient induction of cytosine methylation of the target gene promoters (Okano et al. 2008). Although different levels of chromatin modification were observed between the CaMV 35S promoter and the endogenous gene promoter, it is not yet understood why reporter gene constructs such as CaMV 35S promoter::GFP can be silenced by TGS more easily than endogenous genes. On the other hand, successful induction of TGS of an endogenous gene by T-DNA-mediated expression of dsRNA corresponding to the target promoter region has been reported in petunia (Sijen et al. 2001), *Arabidopsis* (Deng et al. 2014), and potato (Kasai et al. 2016; Heilersig et al. 2006). However, it seems that a reproducible method for stable induction of TGS via T-DNA has not yet been found in plants.

As an alternative *Agrobacterium*-mediated strategy, T-DNA harboring a viral vector sequence is used to deliver the viral vector to plant cells. After *Agrobacterium*-mediated transformation or *Agrobacterium* infiltration, the viral vector is transferred into the nuclei of plant cells as a part of the T-DNA and can function independently as a viral vector. Viral vectors released from the T-DNA induce RdDM-mediated TGS (Ju et al. 2016).

16.2.4 Grafting-Mediated TGS in Plants

Grafting is a plant-specific strategy for inducing TGS. In vascular plants, the vascular bundle system functions to transport water, minerals, nutrients, proteins, and photosynthate from sink to source organs (or from source to sink). Some RNA molecules such as siRNA and microRNA (miRNA) are also transported by vascular bundle system. Specifically, these small RNA molecules are exclusively transported from sink to source organs through the phloem (Melnyk et al. 2011; Ham and Lucas 2017) and can move from cell to cell through the plasmodesmata (Melnyk et al. 2011; Ham and Lucas 2017). When a scion artificially expressing siRNA toward a target gene promoter (GM plant) is grafted onto a wild-type rootstock (non-GM), RdDM-mediated TGS can be induced in the rootstock via siRNA movement through the vascular bundle system and plasmodesmata. If a regenerated plantlet is obtained from the TGS rootstock, it would be transcriptionally silenced without insertion of foreign DNA into the genome. For this reason, "grafting on GM" is considered a type of NPBT (Fig. 16.2c). Bai and co-authors grafted transgenic tobacco scions expressing dsRNA directed toward parts of the CaMV 35S promoter region and under the control of the companion cell-specific *Commelina* yellow mottle virus (CoYMV) promoter onto transgenic tobacco rootstocks expressing GFP under the control of the CaMV 35S promoter. GFP fluorescence was drastically suppressed in

lateral roots of the rootstock, indicating that TGS was epigenetically induced in the rootstock (Bai et al. 2011). This study showed that the movement of siRNA from scion to rootstock was more efficient than from rootstock to scion (Bai et al. 2011). These same authors have also produced an epigenetically improved potato by grafting with transgenic tobacco as the TGS inducer, resulting in modified amylose content through suppression of granule-bound starch synthase I (*GBSSI*) gene without changes in the genomic DNA sequences of the host potato (Kasai et al. 2016).

Vegetatively propagated crops such as potato and apple may have an advantage over seed-propagated crops with respect to the use of RdDM-mediated TGS because vegetatively propagated crops do not require meiosis for self-reproduction; thus, the modified methylation level may be preserved more stably than in seed-propagated crops. However, further investigation would be necessary to clarify this point.

16.2.5 CRISPR/Cas9-Mediated Epigenome Modification

The CRISPR/Cas9 system is a convenient and powerful tool for genome editing in many organisms (Cong et al. 2013; Mali et al. 2013; Fauser et al. 2014). This system is very simple, consisting of the combination of a guide RNA and Cas9 nuclease (Fig. 16.3a). A modified CRISPR/Cas9 system can be applied to epigenome editing. A nuclease-activity-disrupted Cas9 (dCas9) fused with enzymes to modify genomic DNA or histone can be used as an epigenome editing inducer (Fig. 16.3b). For example, a fusion protein consisting of nuclease-disrupted Cas9 protein and human acetyltransferase p300 successfully catalyzed the acetylation of histone H3 at target sites in human cells, resulting in the robust transcriptional activation of target genes (Hilton et al. 2015). Further modifications of CRISPR/Cas9-mediated epigenome editing will continue to be developed in animals and plants (Johnson et al. 2014; Konermann et al. 2013).

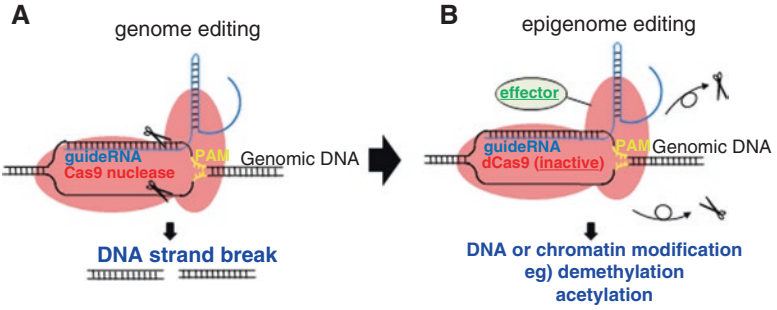

Fig. 16.3 CRISPR/Cas9-mediated epigenome editing. (**a**), Normal scheme of CRISPR/Cas9 system to induce double-strand break toward target genomic DNA. (**b**) Epigenome editing technology using fusion protein consists of effector and catalytically dead Cas9 (dCas9) lacking only endonuclease activity to induce the other modification such as demethylation or acetylation toward target genomic DNA or chromatin

16.3 Future Perspectives

This chapter describes new findings from both basic and applied studies on epigenetics in plants. Recently, the study of epigenetics has developed rapidly because of an increase in the precision of genome-wide association studies (GWAS), which have received a lot of attention in both animals and plants. At present, application of RdDM-mediated epigenome editing to plant breeding is not yet practical owing to the need to obtain stable induction of TGS toward endogenous genes and stable inheritance of the modified epigenome after removal of the trigger gene. However, we expect that many interesting findings will continue to be reported in the epigenetics field, with the result that RdDM-mediated epigenome editing will become a promising technology to produce trait-improved plants in the near future.

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