Plant Biotechnology 30, 233–241 (2013)
DOI: 10.5511/plantbiotechnology.13.0319a

Review

Induction of RNA-directed DNA methylation and heritable transcriptional gene silencing as a tool to engineer novel traits in plants

Megumi Kasai, Akira Kanazawa*

Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan
*E-mail: kanazawa@res.agr.hokudai.ac.jp Tel: +81-11-706-3873 Fax: +81-11-706-4933

Received January 7, 2013; accepted March 19, 2013 (Edited by T. Ariizumi)

Abstract Gene silencing through transcriptional repression can be induced by double-stranded RNA (dsRNA) that targets a gene promoter. This phenomenon, termed RNA-mediated transcriptional gene silencing (TGS), was first discovered in plants using a transgene that transcribes an inverted repeat of promoter sequence. However, endogenous genes differ from transgenes in the feasibility of TGS induction, by being more resistant to silencing. Heritable, transgenerational silencing of an endogenous gene has been induced by targeting dsRNA to the promoter in petunia and tomato plants, using a vector based on *Cucumber mosaic virus*. Efficient TGS depends on the function of a viral protein, which can facilitate epigenetic modifications through the transport of short interfering RNA to the nucleus. The efficiency of the TGS also depends on the length and nucleotide composition of the promoter RNA segments. Such epigenetic changes induced by the viral vector results in a novel class of modified plant, a plant that does not carry a transgene but has altered traits. Thus, TGS to modify the epigenetic state of a plant is now a feasible tool to engineer novel traits. Here we review epigenetic changes induced in a particular gene through RNA-directed DNA methylation and those induced randomly on the genome in terms of their use for plant biotechnology.

Key words: Epigenetic changes, RNA-directed DNA methylation, RNA silencing, transcriptional gene silencing, virus vector.

RNA has long been regarded as an intermediary in the process of gene expression. The discovery that eukaryotic cells have a mechanism to suppress gene expression by recognizing and processing homologous double-stranded RNA (dsRNA) has changed this view. RNA-guided, sequence-specific inhibition of gene expression occurs either at the posttranscriptional or transcriptional level (reviewed by Brodersen and Voinnet 2006; Vaucheret 2006). Posttranscriptional gene silencing (PTGS) was first discovered in transgenic petunia plants whose flower color pattern changed when the gene that encodes the key enzyme for anthocyanin biosynthesis was overexpressed (Napoli et al. 1990, van der Krol et al. 1990). Similar phenomena have also been reported for other transgenic plants including tomato and tobacco plants transformed with a construct transcribing a gene involved in a metabolic pathway (de Carvalho et al. 1992; Goring et al. 1991; Smith et al. 1990; Vaucheret 1993) or tobacco plants that acquired resistance against viruses as a consequence of transcribing genes or gene segments derived from the viruses (Dougherty et al. 1994; Lindbo et al. 1993; Mueller et al. 1995; Sijen et al. 1996; Smith et al. 1994). The mechanism of PTGS is also identical to similar gene silencing phenomena in other organisms such as quelling in *Neurospora crassa* (Cogoni and Macino 1997) and RNA interference in *Caenorhabditis*

Abbreviations: AGO, Argonaute; CaMV, *Cauliflower mosaic virus*; CLSY1, CLASSY 1; CMT3, CHROMOMETHYLASE 3; CMV, *Cucumber mosaic virus*; DCL, Dicer-like; DMS3, DEFECTIVE IN MERISTEM SILENCING 3; DDM1, DECREASE IN DNA METHYLATION 1; DNMT, DNA methyltransferase; DRD1, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1; DRM, DOMAINS REARRANGED METHYLTRANSFERASE; dsRNA, double-stranded RNA; FWA, FLOWERING WAGENINGEN; GFP, green fluorescent protein; GUS, *β*-glucuronidase; HDA6, HISTONE DEACETYLASE 6; IDN2, INVOLVED IN DE NOVO 2; IR, inverted repeat; KTF1, KOW domain-containing transcription factor 1; KYP, KRIPTONITE; LeSPL-CNR, *Lycopersicon esculentum* SQUAMOSA promoter binding protein-like colorless non-ripening; m^5C, methylation at the carbon-5 position of cytosine; MET1, METHYLTRANSFERASE 1; NRPD1, NUCLEAR RNA POLYMERASE D1; NRPE1, NUCLEAR RNA POLYMERASE E1; nt, nucleotide; Pol, RNA polymerase; PTGS, post-transcriptional gene silencing; PVX, *Potato virus X*; RdDM, RNA-directed DNA methylation; RISC, RNA-induced silencing complex; Se5, Phtoperiodic sensitivity 5; SINE, short interspersed element; siRNA, short interfering RNA; SMC, structural maintenance of chromosome; SPT5L, SUPPRESSOR OF TY INSERTION 5-LIKE; SUVH4, SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE 4; TALENs, transcription activator-like effector nucleases; TGS, transcriptional gene silencing; TRV, *Tobacco rattle virus*; VIGS, virus-induced gene silencing; ZFNs, zinc finger nucleases.

This article can be found at http://www.jspcmb.jp/
Published online August 7, 2013

elegans (Fire et al. 1998).

The PTGS pathway involves processing of dsRNA into small RNA molecules of 20–26 nucleotides (short interfering RNA; siRNA) by an enzyme called Dicer or Dicer-like (DCL), and the siRNA then guides the cleavage of RNA at a complementary nucleotide sequence in an RNA-induced silencing complex (RISC) containing the Argonaute (AGO) protein (reviewed by Matzke et al. 2001). The formation of dsRNA from single-stranded sense RNA has been explained by the synthesis of its complementary strand by RNA-dependent RNA polymerase (RDR) (Baulcombe 2004). Small RNA molecules called micro RNAs (miRNAs) are also involved in the negative regulation of gene expression in a posttranscriptional process and in the control of development (reviewed by Mallory and Vaucheret 2006). In addition to its role in sequence-specific RNA degradation, dsRNA is also responsible for epigenetic changes involving DNA methylation and histone modification in the nucleus, which leads to transcriptional gene silencing (TGS) (reviewed by Matzke et al. 2009). Gene silencing phenomena that are induced by a sequence-specific interaction involving RNA are forms of RNA silencing (reviewed by Matzke et al. 2004; Voinnet 2002).

RNA-directed DNA methylation and TGS

RNA-guided epigenetic modification of the genome was first discovered in tobacco plants that carried a cDNA derived from a viroid (Wassenegger et al. 1994). When these transgenic plants were infected with the viroid, the cDNA in the plant genome became methylated de novo. Because a viroid is a self-replicating RNA that does not code a protein, the phenomenon indicated that the replicating viroid RNA had induced de novo methylation of its homologous DNA, which was called RNA-directed DNA methylation (RdDM). Similar phenomena were also observed in virus-infected plants that had a cDNA of the virus (Jones et al. 1998). The viral RNA that replicated in the cytoplasm or the RNA derived from it was assumed to have moved to the nucleus and induced the methylation of cytosine in the homologous DNA sequence in the nuclear genome. Cytosine methylation, in particular that occurring in a gene promoter, was found to be correlated with a repressed state of transcription in plants and animals (Kass et al. 1997).

On the bases of these observations, a gene construct that produces promoter dsRNA by transcribing an inverted repeat of the promoter was introduced by Mette et al. (2000) to transgenic plants that had a transgene driven by the promoter. Cytosine methylation of the promoter as well as suppression of transgene expression was discovered, suggesting that dsRNA can induce DNA methylation and TGS. This phenomenon was accompanied by siRNA production, thus implicating the involvement of siRNAs in the process. Induction of TGS by targeting dsRNA to a gene promoter has also been reported in cultured human cells and in *Schizosaccharomyces pombe* (Morris et al. 2004; Schramke et al. 2003; Ting et al. 2005; Volpe et al. 2002).

Promoter-targeted gene silencing has been used to modify gene expression in plants (Table 1) and other organisms (Hawkins and Morris 2008; Suzuki and Kelleher 2009). In some instances, RdDM in plants (Shibuya et al. 2009) and promoter-targeted dsRNA in human can induce transcriptional activation (Suzuki and Kelleher 2009).

Factors involved in RdDM and maintenance of DNA methylation

Factors involved in RdDM and TGS have been identified by analyzing mutants of *Arabidopsis thaliana* that are defective in the process. RdDM induces de novo methylation of cytosine in all sequence contexts (CG, CHG, and CHH, where H is A, C or T) at the region of siRNA–DNA sequence homology (Matzke et al. 2009). In *Arabidopsis*, DOMAINS REARRANGED METHYLTRANSFERASES (DRM1 and DRM2), orthologues of mouse de novo methyltransferase DNA methyltransferase 3 (DNMT3) (Goll and Bestor 2005), establish de novo methylation independently of a CG or non-CG context (Cao et al. 2003) mediated by 24-nt siRNAs (Matzke et al. 2009). In addition to the canonical RNA silencing machinery that includes DCL and AGO family proteins, RdDM requires two plant-specific RNA polymerases, Pol IV and Pol V, and proteins that can interact with them (Matzke et al. 2009) (Figure 1). Mutation in the largest subunit of Pol IV, NUCLEAR RNA POLYMERASE D1 (NRPD1), resulted in a reduction of siRNA (Pikaard et al. 2008), which suggested that Pol IV initiates siRNA biogenesis by producing single-stranded RNA transctripts. DsRNAs are synthesized by RDR2 using Pol IV transcripts as a template, processed into 24-nt siRNAs by DCL3, and loaded into AGO4 (Matzke et al. 2009). AGO4 interacts with the Pol V subunit NUCLEAR RNA POLYMERASE E1 (NRPE1) possibly through a base-pairing interaction between AGO4-loaded siRNA and nascent Pol V transcripts (El-Shami et al. 2007), which is aided by the SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L, also known as KOW domain-containing transcription factor 1; KTF1) (Bies-Etheve et al. 2009). These RNA-mediated protein interactions may be recognized by INVOLVED IN DE NOVO 2 (IDN2), which may recruit methylation machinery including DRMs to establish de novo methylation (Ausin et al. 2009). Pol IV and Pol V are also known to act with chromatin remodeling factors, CLASSY 1 (CLSY1, also known as CHR38) (Smith et al. 2007) and DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) (Kanno et al. 2004), and a

Table 1. Examples of RdDM and TGS induced by dsRNA targeting a gene promoter in plants.

Target	Plant species	Source of promoter RNA	Effects of gene silencing	Inheritance of TGS*	Reference
Endogenous gene promoter					
Dihydroflavonol-4-reductase gene	Petunia	Transgene transcribing promoter IR	Reduction in flower pigmentation	−	Sijen et al. 2001
Anther expressed genes	Maize	Transgene transcribing promoter IR	Reduction in male fertility	−	Cigan et al. 2005
Granule-bound starch synthase I gene	Potato	Transgene transcribing promoter IR	Reduction in amylose content	−	Heilersig et al. 2006
FWA (SINEs in the promoter)	*A. thaliana*	Transgene transcribing promoter IR	Restoration of FWA imprinting and reversion from late-flowering in an epimutant	+	Kinoshita et al. 2006
Se5	Rice	Transgene transcribing promoter IR	Not detected	−	Okano et al. 2008
Chalcone synthase-A gene	Petunia	Recombinant virus (CMV)	Reduction in flower pigmentation and male fertility	+	Kanazawa et al. 2011a
LeSPL-CNR	Tomato	Recombinant virus (CMV)	Inhibition of fruit ripening	+	Kanazawa et al. 2011a
Transgene promoter					
Agrobacterium nopaline synthase gene	Tobacco and *A. thaliana*	Transgene transcribing promoter IR	Loss of transgene function	−	Mette et al. 2000
CaMV 35S	*N. benthamiana*	Recombinant virus (TRV)	Loss of transgene function	+	Jones et al. 2001
Soybean β-conglycinin α' subunit gene	*A. thaliana*	Transgene transcribing promoter IR	Loss of transgene function	−	Kanno et al. 2004
CaMV 35S	*N. benthamiana*	Recombinant virus (CMV)	Loss of transgene function	+	Otagaki et al. 2006, 2011
CaMV 35S	Rice	Transgene transcribing promoter IR	Loss of transgene function	+	Okano et al. 2008
CaMV 35S	*N. benthamiana*	Transgene transcribing promoter IR	Loss of transgene function	+	Bai et al. 2011

Abbreviations: CaMV, *Cauliflower mosaic virus*; CMV, *Cucumber mosaic virus*; FWA, FLOWERING WAGENINGEN; IR, inverted repeat; LeSPL-CNR, *Lycopersicon esculentum* SQUAMOSA promoter binding protein-like colorless non-ripening; Se5, Phtoperiodic sensitivity 5; SINE, short interspersed element; TRV, *Tobacco rattle virus*.

*Inheritance of TGS independent of the RNA trigger: +, the TGS is heritable; −, the TGS is not heritable or the inheritance of the TGS is not reported.

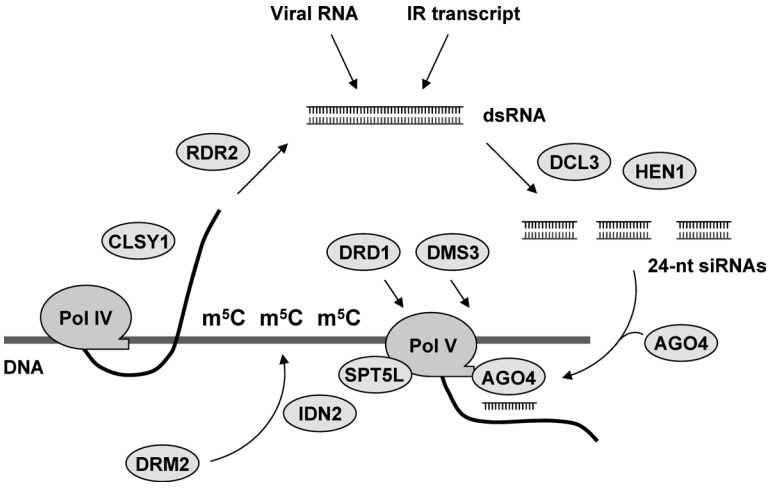

Figure 1. Model for RNA-directed DNA methylation via production of dsRNA. Transcripts from transgenes that have an inverted repeat (IR) sequence can form dsRNA. The replication intermediate or duplex structures formed within single-stranded RNA of the viral genome can also provide dsRNA. The 24-nt siRNAs produced from the dsRNA bind to AGO4 and, together with Pol V and accessory factors, form a guiding complex that recruits DRM2 for de novo methylation at the carbon-5 position of cytosine (m^5C). Pol IV may transcribe the methylated DNA, which leads to secondary siRNA production and spreading of cytosine methylation. All protein names in this figure are those in *Arabidopsis*, identified by screening mutants deficient in the RdDM pathway.

protein with structural maintenance of chromosome (SMC) hinge domain, DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) (Kanno et al. 2008). The entire picture regarding RdDM and factors known so far has been reviewed elsewhere (Law and Jacobsen 2010; Matzke et al. 2009; Zhang and Zhu 2011).

When dsRNA is provided, for example, from a transgene that produces an inverted repeat RNA of a target sequence or by a recombinant virus containing a target sequence, 24-nt siRNAs are produced by DCL3 cleavage of the dsRNA, which subsequently induces de novo methylation. A current hypothetical model posits that the primary methylation elicits transcription by Pol IV, which leads to secondary siRNA production and spreading of cytosine methylation (Lorkovic et al. 2012; Matzke et al. 2009).

Cytosine methylation established through RdDM can be maintained by factors including methyltransferases (Chan et al. 2005). The maintenance of CG methylation requires METHYLTRANSFERASE 1 (MET1) (Finnegan and Kovac 2000; Kankel et al. 2003), an orthologue of mouse DNMT1, a chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (DDM1) (Jeddeloh et al. 2001; Vongs et al. 1993) and HISTONE DEACETYLASE 6 (HDA6) (Aufsatz et al. 2002). The maintenance of non-CG methylation requires DRM1, DRM2, the plant-specific methyltransferase CHROMOMETHYLASE3 (CMT3) (Bartee et al. 2001; Lindroth et al. 2001), which function redundantly, and a histone methyltransferase SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE 4 (SUVH4, also known as KRIPTONITE; KYP) (Jackson et al. 2002; Malagnac et al. 2002). On the other hand, a family of DNA glycosylases can demethylate cytosine in plants (Chan et al. 2005; Gong et al. 2002).

Use of a virus vector as a tool to induce RNA silencing and epigenetic changes

One of the mechanisms that plants use to cope with viruses is RNA silencing. When plants are infected with an RNA virus, dsRNA of the viral genome is degraded by the infected plants via an RNA silencing pathway (Al-Kaff et al. 1998; Covey et al. 1997). The dsRNA in the virus-infected cells is thought to be the replication intermediate of the viral RNA (Lu et al. 2003) or a duplex structure formed within single-stranded viral RNA (Molnar et al. 2005). If a segment of the host plant gene is inserted in the viral genome, then siRNAs would also be produced from the segment. Therefore, infection by the virus results in the production of siRNAs corresponding to the plant gene and subsequently induces RNA degradation of the gene. These discoveries led to the use of a virus vector to induce silencing of a specific gene in the plant genome, which is referred to as virus-induced gene silencing (VIGS; Kumagai et al. 1995; Purkayastha and Dasgupta 2009; Ruiz et al. 1998). Dozens of plant virus vectors have been developed for inducing PTGS, as listed previously (Kanazawa 2008; Senthil-Kumar and Mysore 2011).

PTGS of both transgenes and endogenous genes has been induced efficiently in plants. In contrast, there is a marked difference between transgenes and endogenous genes in the feasibility of TGS induction by targeting dsRNA to a promoter (Okano et al. 2008). Transgenes in plant genome can be easily silenced, and the silenced state is heritable in the presence or absence of the silencing inducer (Jones et al. 2001). On the other hand, several previous reports have shown that endogenous genes can be silenced only in the presence of the silencing inducer (Cigan et al. 2005; Heilersig et al. 2006; Sijen et al. 2001). The *Potato virus X* (PVX) and *Tobacco rattle virus* (TRV) vectors have been shown to induce heritable RNA-mediated TGS against transgenes such as the green fluorescent protein (*GFP*) and *β*-glucuronidase (*GUS*) genes (Jones et al. 1999; 2001), but no RdDM and RNA-mediated TGS against an endogenous gene was induced (Jones et al. 1999).

We have developed an RNA virus vector based on *Cucumber mosaic virus* (CMV), which is able to rapidly induce PTGS and TGS of transgene in *Nicotiana benthamiana*, a model plant for plant viral research (Otagaki et al. 2006) or PTGS of endogenous genes in soybean (Nagamatsu et al. 2007; 2009). Subsequently, we reported for the first time that TGS of endogenous genes can be induced by the vector in petunia and tomato plants and that the induced TGS is heritable (Kanazawa et al. 2011a). The TGS resulted in phenotypic changes and was accompanied by epigenetic changes including cytosine methylation and histone modification.

Mechanisms behind the efficient induction of epigenetic changes by the CMV vector

In our analyses of the mechanism(s) behind the induction of TGS of endogenous genes by the CMV vector, we found that the 2b protein encoded by the vector virus bound to siRNAs and was localized in the nucleus in tobacco protoplasts. When the protoplasts had been transfected with siRNAs, they accumulated the siRNAs in the nucleus efficiently in the presence of 2b. We also found that promoter-targeted TGS was induced more efficiently in both virus-infected plants and dsRNA-transfected protoplasts when the 2b gene is expressed. Thus, efficient TGS depends on the function of the 2b protein, which has the ability to facilitate epigenetic modifications through the transport of siRNA to the nucleus (Kanazawa et al. 2011a).

The 2b protein was originally identified as a suppressor of viral RNA degradation via RNA silencing pathway (Lucy et al. 2000). Viral suppressor proteins can prevent the incorporation of siRNAs into RISCs or interfere

with RISCs, which allows viral accumulation (reviewed by Silhavy and Burgyan 2004). Interestingly, because common reactions of RNA silencing are involved in the degradation of both viral RNA and plant mRNA, infection of plants with CMV suppresses PTGS of a plant gene. In fact, posttranscriptional silencing of the *chalcone synthase* genes in petunia (Kasai et al. 2012; Koseki et al. 2005) and soybean (Senda et al. 2004) is suppressed in CMV-infected plants. Because of this function, viral infection can be used as a tool to "diagnose" an RNA-silencing induced phenotype (Kasai and Kanazawa 2012). Therefore, the induction of TGS by the CMV vector should be regarded as a mixed outcome of different reactions involving siRNAs: the promotion of epigenetic changes and the suppression of RNA degradation. We also found that the expression of genes involved in RdDM is upregulated and that of genes for demethylation is downregulated in *Arabidopsis* plants infected with CMV (Kanazawa et al. 2011b). These changes may also contribute to the efficient induction of RdDM and TGS.

Although genes involved in the RdDM pathway have been identified, whether dsRNAs of different promoter regions differ in the extent of their effects on RdDM and/or TGS had not been known. We addressed this question by targeting the *Cauliflower mosaic virus* (CaMV) 35S promoter in the genome of *N. benthamiana* using a recombinant CMV that contained various portions of the promoter (Otagaki et al. 2011). We found that the efficiency of the induction of TGS depends on the length of the promoter segment that triggers the RdDM and that there is a lower size limit for TGS induction of 81–91 nt. In addition, the frequency of cytosine at symmetric sites in the region targeted by dsRNA is the major factor that allows the induction of heritable TGS via RdDM (described below in detail). We also discovered several intriguing events whose mechanisms are unsolved: TGS was induced when 70-nt fragments were connected in tandem, none of which solely induced TGS; TGS induction did not simply depend on the production of siRNAs corresponding to the promoter; along with the induction of RdDM, DNA methylation spread from the originally targeted site to adjacent regions; cytosine methylation at CHH sites was present in the progeny plants that had no RNA trigger of RdDM, implying the presence of unknown mechanism(s) to maintain CHH methylation (Otagaki et al. 2011). Some of these phenomena may be relevant to the efficiency of TGS induction.

The advantage of the use of CMV vector is that it allows efficient induction of heritable epigenetic changes on a target gene. Another advantage of this silencing system is that the progeny plants do not have any transgene because the virus is eliminated during meiosis. Plants that are produced by this system have altered traits but do not carry a transgene, thus constituting a novel class of modified plants (Kanazawa et al. 2011b) (Figure 2).

Factors allowing the induction of heritable TGS

When a strong and heritable TGS of a transgene was induced by targeting CaMV 35S promoter using the CMV vector, a high level of methylation was detected at cytosines in all sequence contexts (i.e., CG, CHG and CHH). In the progeny of virus-infected plants, the frequencies of methylation, especially those at symmetrical sites (CG and CHG), were as high as or even higher than those in the former generation (Otagaki et al. 2011). In contrast, the frequency of methylcytosine was lower in plants in which a weak level of TGS was induced. In addition, methylcytosines were barely detected in the progeny, in which transgene expression was restored. The lower level of RdDM and TGS induction by a vector construct is correlated with a lower frequency (3.4/100 nt) of cytosines at symmetrical sites in the target DNA region, while the value of the

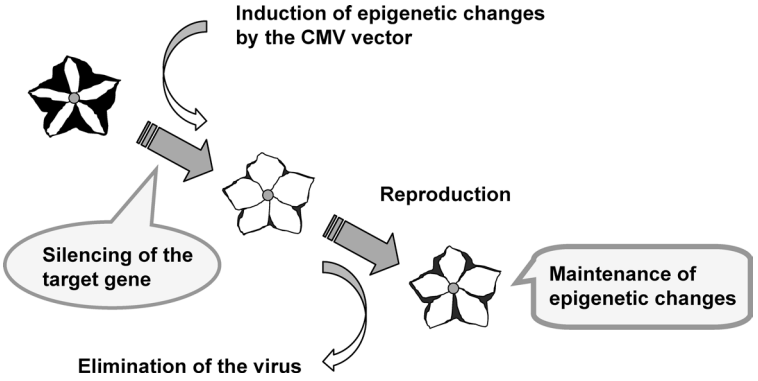

Figure 2. Induction of epigenetic changes by the CMV vector and transmission of the altered traits to progeny plants. The CMV vector containing an endogenous gene promoter sequence produces siRNAs in the infected plants via the RNA silencing machinery. The siRNAs are targeted to the promoter of the endogenous gene in the nucleus, which induces epigenetic changes. Progeny plants maintain the epigenetic changes but do not carry any foreign nucleic acids because the virus is eliminated during meiosis and thus constitute a novel class of modified plants.

other constructs that induced a higher extent of TGS was 5.4–9.8/100 nt (Otagaki et al. 2011). Thus, in addition to the length of the promoter segment above the threshold (81–91 nt) for triggering the RdDM, the frequency of cytosine at symmetrical sites in the region targeted by dsRNA is the major factor that allows the induction of heritable TGS via RdDM. The importance of cytosines at symmetrical sites on heritable TGS is consistent with a previous report of Diéguez et al. (1998): on the basis of experiments using a modified CaMV 35S promoter devoid of CG and CHG sequences, they suggested that CG/CHG methylation is essential for the maintenance of established silenced states, although it is not a prerequisite for the initiation of TGS.

Another epigenetic mark correlated with heritable TGS was histone modification. When RdDM and TGS of plant endogenous genes were induced by the CMV vector, an altered state of histone modification, the presence of dimethylation at Lys9 of histone H3 and the absence of acetylation of histone H3, was detected and the altered state was inherited by the progeny (Kanazawa et al. 2011a). The altered state of histone modification was maintained in the progeny even when the frequency of cytosine methylation decreased.

TGS can be transmitted through multiple generations. For example, TGS of the *GFP* gene induced by targeting dsRNA to the CaMV 35S promoter using the CMV vector was stable for at least four generations with no detectable reversion to a nonsilenced state (Otagaki et al. 2011). TGS of the same gene induced by the TRV vector was also heritable, although 70% of the progeny of virus-infected plants reverted to a nonsilenced state (Jones et al. 2001). Transmission of acquired epigenetic states between generations is restricted by establishment of a default epigenetic state during the process of reproduction: extensive DNA demethylation has been detected in pollen and endosperm (Hauser et al. 2011). Transgenerational inheritance of an epigenetic state reflects the presence of epigenetic marks that remained through epigenetic resetting during gametogenesis, condition(s) of which remains largely elusive. A recent study has shown that RdDM can be enhanced by simultaneously downregulating a *ROS1* orthologue using the CMV vector in *N. benthamiana* (Otagaki et al. 2013). This method can be used to control the level of cytosine methylation in a targeted DNA region via RdDM and may potentially be useful for enhancing transgenerational epigenetic inheritance.

Perspectives on the use of epigenetic changes in plant biotechnology

RdDM, together with synthetic restriction endonucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), is one of seven new plant breeding techniques highlighted in a recent review (Lusser et al. 2012). While genomic engineering by ZFNs or TALENs involves changes in nucleotide sequence, controlling gene expression via RdDM is not accompanied by changes in nucleotide sequence and can be regarded as an acceleration of gene expression control intrinsic to eukaryotes.

Bai et al. (2011) recently reported that TGS of a transgene is induced after infiltration of plant leaves with *Agrobacterium* that expresses an inverted repeat of promoter RNA and that the TGS is transmitted to a grafting partner. The transmissible TGS is maintained through tissue culture and inherited by the progeny. Accordingly, in addition to the induction of RdDM and TGS of plant endogenous genes by the CMV vector, this method may also provide a tool to produce transgene-less plants with altered traits via gene-specific epigenetic changes, if this method is applied to TGS of an endogenous gene.

In addition to inducing gene-specific epigenetic changes via RdDM, changes in epigenetic state can also be induced randomly in the genome by inhibiting cytosine methylation. Although a method for targeted demethylation has never been developed for any organism, transgenerational inheritance of a state of decreased methylation with an increased transcriptional activity has been noted for limited loci in plants (Paszkowski and Grossniklaus 2011). On the basis of such a notion, plants with an altered phenotype and epigenetic state have been produced (Akimoto et al. 2007; Boyko et al. 2010; Reinders et al. 2009). Similarly, plant lines that have distinct characteristics and different epigenetic states were selected from an isogenic plant population (Hauben et al. 2009). Plants can be treated with a demethylating agent (Akimoto et al. 2007) or crossed with methyltransferase gene mutants (Reinders et al. 2009) to produce plants with a genome with an altered epigenetic state. Through an *in planta* assay system to assess the inhibition of cytosine methylation using transgenic petunia and *N. benthamiana* plants, a novel inhibitor of cytosine methylation in plants has been found (Arase et al. 2012). Different methylation inhibitors might confer different spectra of epi-mutagenesis and thus will be useful for producing novel epigenetic states.

Both gene-specific and gene-nonspecific inductions of epigenetic changes have become a feasible tool to engineer novel traits in plants. The concept shared by these methods is to produce and/or utilize novel epi-alleles. Using these approaches, scientists may be able to exploit novel resources for plant breeding. At present, for both the promoter-targeting method and the random induction method, we do not know whether epigenetic changes can be induced at an equal efficiency between different genes, and this issue may be important in terms of using this technology for plant breeding. Considering

the fact that epigenetic control of gene expression is ubiquitous, the role of epigenetic modification in plant biotechnology will undoubtedly increase.

Acknowledgements

We appreciate the efforts of Hiroshi Ezura and other members of organizing committees for holding SOL & ICuGI 2011 and arranging this publication. We also thank coauthors of our research on RNA silencing cited in this review for their cooperation. Our work is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Akimoto K, Katakami H, Kim HJ, Ogawa E, Sano CM, Wada Y, Sano H (2007) Epigenetic inheritance in rice plants. *Ann Bot* (Lond) 100: 205–217

Al-Kaff NS, Covey SN, Kreike MM, Page AM, Pinder R, Dale PJ (1998) Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* 279: 2113–2115

Arase S, Kasai M, Kanazawa A (2012) *In planta* assays involving epigenetically silenced genes reveal inhibition of cytosine methylation by genistein. *Plant Methods* 8: 10

Aufsatz W, Mette MF, van der Winden J, Matzke M, Matzke AJ (2002) HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. *EMBO J* 21: 6832–6841

Ausin I, Mockler TC, Chory J, Jacobsen SE (2009) IDN1 and IDN2 are required for de novo DNA methylation in *Arabidopsis thaliana*. *Nat Struct Mol Biol* 16: 1325–1327

Bai S, Kasai A, Yamada K, Li T, Harada T (2011) A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. *J Exp Bot* 62: 4561–4570

Bartee L, Malagnac F, Bender J (2001) *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev* 15: 1753–1758

Baulcombe D (2004) RNA silencing in plants. *Nature* 431: 356–363

Bies-Etheve N, Pontier D, Lahmy S, Picart C, Vega D, Cooke R, Lagrange T (2009) RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family. *EMBO Rep* 10: 649–654

Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, Hollunder J, Hollander J, Meins F, Kovalchuk I (2010) Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of Dicer-like proteins. *PLoS ONE* 5: e9514

Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. *Trends Genet* 22: 268–280

Cao X, Aufsatz W, Zilberman D, Mette MF, Huang MS, Matzke M, Jacobsen SE (2003) Role of the *DRM* and *CMT3* methyltransferases in RNA-directed DNA methylation. *Curr Biol* 13: 2212–2217

Chan SW, Henderson IR, Jacobsen SE (2005) Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat Rev Genet* 6: 351–360

Cigan AM, Unger-Wallace E, Haug-Collet K (2005) Transcriptional gene silencing as a tool for uncovering gene function in maize. *Plant J* 43: 929–940

Cogoni C, Macino G (1997) Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc Natl Acad Sci USA* 94: 10233–10238

Covey S, AlKaff N, Langara A, Turner D (1997) Plants combat infection by gene silencing. *Nature* 385: 781–782

de Carvalho F, Gheysen G, Kushnir S, Van Montagu M, Inzé D, Castresana C (1992) Suppression of β-1,3-glucanase transgene expression in homozygous plants. *EMBO J* 11: 2595–2602

Diéguez MJ, Vaucheret H, Paszkowski J, Mittelsten Scheid O (1998) Cytosine methylation at CG and CNG sites is not a prerequisite for the initiation of transcriptional gene silencing in plants, but it is required for its maintenance. *Mol Gen Genet* 259: 207–215

Dougherty WG, Lindbo JA, Smith HA, Parks TD, Swaney S, Proebsting WM (1994) RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation. *Mol Plant Microbe Interact* 7: 544–552

El-Shami M, Pontier D, Lahmy S, Braun L, Picart C, Vega D, Hakimi MA, Jacobsen SE, Cooke R, Lagrange T (2007) Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev* 15: 2539–2544

Finnegan EJ, Kovac KA (2000) Plant DNA methyltransferases. *Plant Mol Biol* 43: 189–201

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806–811

Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74: 481–514

Gong Z, Morales-Ruiz T, Ariza RR, Roldán-Arjona T, David L, Zhu JK (2002) *ROS1*, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. *Cell* 111: 803–814

Goring DR, Thomson L, Rothstein SJ (1991) Transformation of a partial nopaline synthase gene into tobacco suppresses the expression of a resident wild-type gene. *Proc Natl Acad Sci USA* 88: 1770–1774

Hauben M, Haesendonckx B, Standaert E, Van Der Kelen K, Azmi A, Akpo H, Van Breusegem F, Guisez Y, Bots M, Lambert B, et al. (2009) Energy use efficiency is characterized by an epigenetic component that can be directed through artificial selection to increase yield. *Proc Natl Acad Sci USA* 106: 20109–20114

Hauser MT, Aufsatz W, Jonak C, Luschnig C (2011) Transgenerational epigenetic inheritance in plants. *Biochim Biophys Acta* 1809: 459–468

Hawkins PG, Morris KV (2008) RNA and transcriptional modulation of gene expression. *Cell Cycle* 7: 602–607

Heilersig BH, Loonen AE, Janssen EM, Wolters AM, Visser RG (2006) Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat. *Mol Genet Genomics* 275: 437–449

Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* 416: 556–560

Jeddeloh JA, Stokes TL, Richards EJ (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat Genet* 22: 94–97

Jones AL, Thomas CL, Maule AJ (1998) *De novo* methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO J* 17: 6385–6393

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11: 2291–2301

Jones L, Ratcliff F, Baulcombe DC (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* 11: 747–757

Kanazawa A (2008) RNA silencing manifested as visibly altered phenotypes in plants. *Plant Biotechnol* 25: 423–435

Kanazawa A, Inaba J, Shimura H, Otagaki S, Tsukahara S, Matsuzawa A, Kim BM, Goto K, Masuta C (2011a) Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants. *Plant J* 65: 156–168

Kanazawa A, Inaba J, Kasai M, Shimura H, Masuta C (2011b) RNA-mediated epigenetic modifications of an endogenous gene targeted by a viral vector: a potent gene silencing system to produce a plant that does not carry a transgene but has altered traits. *Plant Signal Behav* 6: 1090–1093

Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddeloh JA, Riddle NC, Verbsky ML, Richards EJ (2003) Arabidopsis *MET1* cytosine methyltransferase mutants. *Genetics* 163: 1109–1122

Kanno T, Mette MF, Kreil DP, Aufsatz W, Matzke M, Matzke AJ (2004) Involvement of putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation. *Curr Biol* 14: 801–805

Kanno T, Bucher E, Daxinger L, Huettel B, Böhmdorfer G, Gregor W, Kreil DP, Matzke M, Matzke AJ (2008) A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. *Nat Genet* 40: 670–675

Kasai M, Kanazawa A (2012) RNA silencing as a tool to uncover gene function and engineer novel traits in soybean. *Breed Sci* 61: 468–479

Kasai M, Koseki M, Goto K, Masuta C, Ishii S, Hellens RP, Taneda A, Kanazawa A (2012) Coincident sequence-specific RNA degradation of linked transgenes in the plant genome. *Plant Mol Biol* 78: 259–273

Kass SU, Pruss D, Wolffe AP (1997) How does DNA methylation repress transcription? *Trends Genet* 13: 444–449

Kinoshita Y, Saze H, Kinoshita T, Miura A, Soppe WJ, Koornneef M, Kakutani T (2007) Control of *FWA* gene silencing in *Arabidopsis thaliana* by SINE-related direct repeats. *Plant J* 49: 38–45

Koseki M, Goto K, Masuta C, Kanazawa A (2005) The star-type color pattern in *Petunia hybrida* 'Red Star' flowers is induced by sequence-specific degradation of *chalcone synthase* RNA. *Plant Cell Physiol* 46: 1879–1883

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci USA* 92: 1679–1683

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11: 204–220

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* 5: 1749–1759

Lindroth AM, Cao X, Jackson JP, Zilberman D, McCallum CM, Henikoff S, Jacobsen SE (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* 292: 2077–2080

Lorković ZJ, Naumann U, Matzke AJ, Matzke M (2012) Involvement of a GHKL ATPase in RNA-directed DNA methylation in *Arabidopsis thaliana*. *Curr Biol* 22: 933–938

Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003) Virus-induced gene silencing in plants. *Methods* 30: 296–303

Lucy AP, Guo HS, Li WX, Ding SW (2000) Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J* 19: 1672–1680

Lusser M, Parisi C, Plan D, Rodríguez-Cerezo E (2012) Deployment of new biotechnologies in plant breeding. *Nat Biotechnol* 30: 231–239

Malagnac F, Bartee L, Bender J (2002) An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J* 21: 6842–6852

Mallory AC, Vaucheret H (2006) Functions of microRNAs and related small RNAs in plants. *Nat Genet* 38(Suppl): S31–S36

Matzke M, Matzke AJ, Kooter JM (2001) RNA: guiding gene silencing. *Science* 293: 1080–1083

Matzke M, Aufsatz W, Kanno T, Daxinger L, Papp I, Mette MF, Matzke AJ (2004) Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim Biophys Acta* 1677: 129–141

Matzke M, Kanno T, Daxinger L, Huettel B, Matzke AJ (2009) RNA-mediated chromatin-based silencing in plants. *Curr Opin Cell Biol* 21: 367–376

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J* 19: 5194–5201

Molnár A, Csorba T, Lakatos L, Várallyay E, Lacomme C, Burgyán J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J Virol* 79: 7812–7818

Morris KV, Chan SW, Jacobsen SE, Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 305: 1289–1292

Mueller E, Gilbert J, Davenport G, Brigneti G, Baulcombe D (1995) Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J* 7: 1001–1013

Nagamatsu A, Masuta C, Senda M, Matsuura H, Kasai A, Hong JS, Kitamura K, Abe J, Kanazawa A (2007) Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. *Plant Biotechnol J* 5: 778–790

Nagamatsu A, Masuta C, Matsuura H, Kitamura K, Abe J, Kanazawa A (2009) Down-regulation of flavonoid 3′-hydroxylase gene expression by virus-induced gene silencing in soybean reveals the presence of a threshold mRNA level associated with pigmentation in pubescence. *J Plant Physiol* 166: 32–39

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* 2: 279–289

Okano Y, Miki D, Shimamoto K (2008) Small interfering RNA (siRNA) targeting of endogenous promoters induces DNA methylation, but not necessarily gene silencing, in rice. *Plant J* 53: 65–77

Otagaki S, Arai M, Takahashi A, Goto K, Hong JS, Masuta C, Kanazawa A (2006) Rapid induction of transcriptional and post-transcriptional gene silencing using a novel *Cucumber mosaic virus* vector. *Plant Biotechnol* 23: 259–265

Otagaki S, Kawai M, Masuta C, Kanazawa A (2011) Size and

positional effects of promoter RNA segments on virus-induced RNA-directed DNA methylation and transcriptional gene silencing. *Epigenetics* 6: 681–691

Otagaki S, Kasai M, Masuta C, Kanazawa A (2013) Enhancement of RNA-directed DNA methylation of a transgene by simultaneously downregulating a *ROS1* orthologue using a virus vector in *Nicotiana benthamiana*. *Front Genet* 4: 44

Paszkowski J, Grossniklaus U (2011) Selected aspects of transgenerational epigenetic inheritance and resetting in plants. *Curr Opin Plant Biol* 14: 195–203

Pikaard CS, Haag JR, Ream T, Wierzbicki AT (2008) Roles of RNA polymerase IV in gene silencing. *Trends Plant Sci* 13: 390–397

Purkayastha A, Dasgupta I (2009) Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. *Plant Physiol Biochem* 47: 967–976

Reinders J, Wulff BB, Mirouze M, Marí-Ordóñez A, Dapp M, Rozhon W, Bucher E, Theiler G, Paszkowski J (2009) Compromised stability of DNA methylation and transposon immobilization in mosaic *Arabidopsis* epigenomes. *Genes Dev* 23: 939–950

Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10: 937–946

Schramke V, Allshire R (2003) Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* 301: 1069–1074

Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong JS, MacFarlane S (2004) Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *Plant Cell* 16: 807–818

Senthil-Kumar M, Mysore KS (2011) New dimensions for VIGS in plant functional genomics. *Trends Plant Sci* 16: 656–665

Shibuya K, Fukushima S, Takatsuji H (2009) RNA-directed DNA methylation induces transcriptional activation in plants. *Proc Natl Acad Sci USA* 106: 1660–1665

Sijen T, Wellink J, Hiriart JB, Van Kammen A (1996) RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell* 8: 2277–2294

Sijen T, Vijn I, Rebocho A, van Blokland R, Roelofs D, Mol JN, Kooter JM (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr Biol* 11: 436–440

Silhavy D, Burgyán J (2004) Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci* 9: 76–83

Smith CJ, Watson CF, Bird CR, Ray J, Schuch W, Grierson D (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Mol Gen Genet* 224: 477–481

Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell* 6: 1441–1453

Smith LM, Pontes O, Searle I, Yelina N, Yousafzai FK, Herr AJ, Pikaard CS, Baulcombe DC (2007) An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis*. *Plant Cell* 19: 1507–1521

Suzuki K, Kelleher AD (2009) Transcriptional regulation by promoter targeted RNAs. *Curr Top Med Chem* 9: 1079–1087

Ting AH, Schuebel KE, Herman JG, Baylin SB (2005) Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* 37: 906–910

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2: 291–299

Vaucheret H (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequences are sufficient for trans-inactivation. *C R Acad Sci III* 316: 1471–1483

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* 20: 759–771

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. *Curr Opin Plant Biol* 5: 444–451

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297: 1833–1837

Vongs A, Kakutani T, Martienssen RA, Richards EJ (1993) *Arabidopsis thaliana* DNA methylation mutants. *Science* 260: 1926–1928

Wassenegger M, Heimes S, Riedel L, Sänger HL (1994) RNA-directed de novo methylation of genomic sequences in plants. *Cell* 76: 567–576

Zhang H, Zhu JK (2011) RNA-directed DNA methylation. *Curr Opin Plant Biol* 14: 142–147