



Communication Induction of Promoter DNA Methylation Upon High-Pressure Spraying of Double-Stranded RNA in Plants

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Abstract: Exogenous application of RNA molecules is a potent method to trigger RNA interference (RNAi) in plants in a transgene-free manner. So far, all exogenous RNAi (exo-RNAi) applications have aimed to trigger mRNA degradation of a given target. However, the issue of concomitant epigenetic changes was never addressed. Here, we report for the first time that high-pressure spraying of dsRNAs can trigger de novo methylation of promoter sequences in plants.

Keywords: DNA methylation; promoter; dsRNA; siRNAs; RNAi; bisulfite sequencing

1. Introduction

Eukaryotic DNA methylation is an important epigenetic modification governing a broad range of developmental aspects and refers to the addition of a methyl group to the fifth carbon of the six-ring cytosine residue. In plants, de novo DNA methylation is mediated by RNA molecules, thus aptly coined RNA-directed DNA methylation (RdDM) [1]. RdDM is tightly connected to RNA interference (RNAi), likely being its epiphenomenon [2,3]. Briefly, RNAi in plants is initiated by double-stranded RNAs (dsR-NAs) that are processed by Dicer-like (DCL) endonucleases into small interfering RNAs (siRNAs), which are loaded into argonaute (AGO) proteins [4]. In general, DCL4 generates 21-nt siRNAs that are loaded onto AGO1 and recognize complementary transcripts for cleavage in the cytoplasm [5,6], while DCL3 generates 24-nt siRNAs that are loaded onto AGO4 and are involved in the RdDM process in the nucleus [7,8]. According to the current models, 24-nt siRNAs (canonical RdDM) or even 21/22-nt siRNAs (noncanonical RdDM) interact with the DNA itself or with its nascent transcript produced by RNA polymerase v (POLV) [9–11]. This interaction is believed to recruit domains-rearranged methyltransferase 2 (DRM2) to de novo methylate the cytosines of the cognate DNA [12]. However, POLV has been suggested to be recruited to an already methylated DNA template [13], thus cannot possibly be involved in the very first step of de novo methylation of a completely unmethylated DNA. Moreover, RdDM was not eliminated in an Arabidopsis thaliana quadruple dcl1 dcl2 dcl3 dcl4 mutant [14], suggesting that DCL-produced siRNAs are dispensable for RdDM. Accordingly, some models propose that the very first step of de novo methylation is triggered not by siRNAs but by long dsRNAs instead [15,16]. According to this scenario, dsRNA interacts with cognate DNA and recruits DRM2 to impose a first wave (perhaps incomplete) of methylation marks. To this newly-but-incompletely methylated DNA, POLIV and POLV are recruited. POLIV generates short transcripts (~40 nt) that are further copied by RNA-directed RNA polymerase 2 (RDR2) into ~40 bp dsRNAs [17]. These short POLIV/RDR2 dsRNAs are then processed by DCL3 into 24-nt siRNAs that are loaded onto AGO4 and hybridize with POLV transcript, recruiting once more DRM2 to reinforce/amplify the methylation marks. When the dsRNA-directed de novo and siRNA-directed amplification steps are finished, all cytosines in both strands of the target DNA are methylated in any sequence context: CG, CHG, CHH (H being A,



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). T, or C). Upon cell division and in the absence of dsRNAs/siRNAs, CHH methylation cannot be re-established, thus being the hallmark of de novo RdDM [18]. However, CG and CHG methylation can be mitotically and meiotically maintained even in the absence of dsRNAs/siRNAs by the action of methyltransferase 1 (MET1) [19] and chromomethylase 3 (CMT3) [12], respectively.

Whereas RdDM of (transcribed) coding regions has been routinely achieved by conventional dsRNA-expressing transgenes [20,21], RdDM of (non-transcribed) promoter regions has been more troublesome [22,23]. In general, the most potent inducers of promoter methylation have been transgenes generating either dsRNAs lacking a polyadenylation tail [24,25] or residing in an intron [26]. Both approaches lead to the nuclear localization of the corresponding dsRNAs. However, the use of transgenes and transgenic plants is currently under strict regulation, at least in European Union. Yet, considerable progress over the last decade has suggested alternative methods to trigger RNAi in a transgene-free manner, simply by the exogenous application of dsRNA/siRNA molecules in plants by methods such as foliar spraying, trunk injection, and root drenching [27]. Exogenous RNAi (exo-RNAi) approaches have led to the silencing of plant genes and resistance against a diverse array of pests and pathogens [28–30]. Yet, in all of these cases, the application of dsRNAs/siRNAs aimed to target a given mRNA for degradation, and with a notable exception [28], no study addressed the issue of concomitant epigenetic modifications in the corresponding coding region. What is more, no study so far has analyzed whether exo-RNAi approaches can trigger RdDM of promoter sequences. In this report, we provide clear evidence that spraying of dsRNAs can trigger promoter RdDM in plants.

2. Materials and Methods

2.1. In Vitro Transcription of dsRNA

In order to generate the template for the in vitro transcription of the dsRNA that would target the 35S promoter, PCR was performed using genomic DNA of *N. benthamiana* 16C and the primers 5-ATA CAG AGT CTC TTA CGA CTC-3 and 5-GTC TTC TTT TTC CAC GAT GCT-3. The occurring 333 bp amplicon was gel excised and used as a template in a subsequent PCR with the T7 promoter-including primers 5-TAA TAC GAC TCA CTA TAG GGA GAA TAC AGA GTC TCT TAC GAC TC-3 and 5-TAA TAC GAC TCA CTA TAG GGA GAG TCT TTT TCC ACG ATG CT-3. Finally, the occurring 379 bp amplicon was used as a template for the generation of the double-stranded RNA with the MEGA Script RNAi kit (ThermoFisher Scientific, www.thermofisher.com, accessed on 15 December 2020), following the manufacturer's instructions.

2.2. High-Pressure Spraying of dsRNA

Before each spraying application, the dsRNA aliquot was removed from -80 °C and diluted with annealing buffer (1× working concentration: 60 mM KCl, 6 mM HEPES-pH 7.5, 0.2 mM MgCl₂), denatured at 70 °C for 7 min and re-annealed by being left to cool slowly at room temperature for 1 h. For each plant, a solution of 500 µL containing 50 µg dsRNA was used for high-pressure spraying (8 bar) of 3 leaves to which a small amount of carborundum was merely applied (but not rubbed), so any carborundum-mediated mechanical damage would take place during (and not before) the high-pressure spraying. High-pressure spraying (8 bar) conditions were provided by a MicroLux Airbrush Set and a Bormann BAT5060 compressor.

2.3. Genomic DNA Extraction

Genomic DNA from *N. benthamiana* leaves was extracted with a DNEasy Plant Pro kit (Qiagen, www.qiagen.com, accessed on 15 December 2020) according to the manufacturer's instructions.

2.4. Bisulfite Sequencing

Prior to bisulfite reaction, the genomic DNA was fragmented with AseI digestion and cleaned up with phenol/chloroform extraction and ethanol precipitation. The AseIdigested DNA (1 μ g/20 μ L) was then subjected to bisulfite conversion with the EZ DNA Methylation Gold Kit (Zymo Research, www.zymoresearch.com, accessed on 15 December 2020) according to the manufacturer's instructions. The recovered bisulfite-treated DNA was then subjected to PCR amplification with the degenerate primers 5-AGT YTY AGA YYA AAG GGY AAT-3 and 5-TCT TRC RAA RRA TAR TRR RAT TRT-3 (HPLC-grade purification) and the ZymoTaq Premix (Zymo Research, www.zymoresearch.com, accessed on 15 December 2020) at the gradient temperatures of 46 °C, 50 °C, and 54 °C, in order to ensure unbiased amplification of methylated and nonmethylated DNA, as described before [31]. The 327 bp amplicons were pooled together and used for TA-cloning in pGEM T easy vector (Promega, worldwide.promega.com, accessed on 15 December 2020) according to the manufacturer's instruction. For each treatment, 10–12 independent clones were subjected to Sanger sequencing. For the depiction of bisulfite sequencing data of the 279 bp promoter fragment (which occurs when omitting the primer sequences from the 327 bp amplicon), CyMATE software was used (www.cymate.org, accessed on 15 December 2020) [32].

3. Results and Discussion

For the dsRNA spraying experiments, we used the Nicotiana benthamiana 16C line (Nb-16C), which harbors the green fluorescent protein (GFP) transgene under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator [33] (Figure 1a,b). We have previously shown that spraying of siRNA molecules designed to target the GFP coding sequence in Nb-16C triggered local and systemic silencing of the GFP transgene [34]. In this study, we aimed to investigate whether spraying of dsRNA molecules targeting the 35S promoter (35S-dsRNA) could trigger RdDM on the cognate sequences. In the promoter region, the Nb-16C contains an 835 bp region of the CaMV, which contains the actual 343 bp promoter sequence (GenBank accession number KY464890) [35]. For 35S-dsRNA spraying, we generated a 333 bp dsRNA designed to target a part of the 343 bp 35S promoter by in vitro transcription, covering the essential regions for promoter activity but mapping upstream of the TATA box and the transcriptional start site (TSS) (Figure 1b). In doing so, we ensured that the sprayed dsRNA would target only non-transcribed regions. A recent study showed that exogenous application of RNA molecules is greatly affected by conditions such as plant age, soil moisture, and time of application [36]. In our case, Nb-16C plants were grown in standard conditions in a growth chamber (16 h/8 h light/dark photoperiod, 25 °C, 67% humidity). Six-leaf stage plants were used for all spraying experiments, which were conducted early in the morning. In each plant, the abaxial surface (approximately 1–2 cm diameter) of 3 leaves was sprayed with the 333 bp 35S-dsRNA. The 'control' plants were sprayed with the annealing buffer into which the 35S-dsRNA was dissolved.



Figure 1. Analysis of promoter methylation upon high-pressure spraying of double-stranded RNAs (dsRNA). (a) Green fluorescent protein (GFP)-expressing *Nicotiana benthamiana* plants (line 16C, Nb-16C) were sprayed with 333 bp dsRNA. (b) Schematic representation of the transgene present in Nb-16C, depicting the *Cauliflower Mosaic Virus* (CaMV) 35S promoter (P-35S), the GFP coding region, and the nopaline synthase terminator (T-NOS). The TATA motif and the transcriptional start site (TSS) are indicated. The dsRNA (333 bp) mapping region is indicated in the red line and the region chosen for bisulfite sequencing (279 bp) with the black line. (c) Bisulfite sequencing data at 3 dps. (d) Bisulfite sequencing data at 10 dps. The data represent sequenced clones (5 clones for control plants, 10 clones for each of the two plants). Circles denote cytosines in the CG context, squares denote cytosines in CHG context and triangles denote cytosines in CHH context. Empty symbols refer to nonmethylated cytosine, whereas filled symbols to methylated cytosine. Bisulfite analysis was performed with CyMATE software.

We chose to perform a time course analysis of the methylation status of the 35S promoter at 3- and 10-days post spraying (dps). At each of these timepoints, the sprayed tissue of each plant was pooled and used for DNA extraction and DNA methylation analysis by bisulfite sequencing. Treatment of DNA with sodium bisulfite results in the conversion of nonmethylated cytosine to uracil, and during PCR amplification, the uracil is replaced by thymine. Thus, sequences of PCR products from bisulfite-treated DNA exhibit thymines for nonmethylated cytosines. Bisulfite sequencing allows for strand-specific detection of DNA methylation, and we chose to analyze a 279 bp part of the upper strand of the 35S promoter (Figure 1b). When analyzing tissue obtained at 3 dps, no methylation of the 35S promoter was detected (Figure 1c). This suggested that, at least, the region we analyzed did not display inherent features rendering it resistant to bisulfite treatment and that the 35S promoter did not display any methylation prior to dsRNA-spraying. However, when we analyzed tissue obtained 10 dps, bisulfite sequencing revealed the onset of significant methylation of the 35S promoter (Figure 1d). However, not all bisulfite clones showed methylation. Almost 60% of the clones were completely devoid of methylation, while 40% of the clones exhibited very dense cytosine methylation marks at CG, CHG, and CHH context, in both plants 1 and 2 (Figure 1d). The reasons underlying this stark difference in methylation are not clear, but it is likely that the methylated clones (40%) reflect DNA retrieved from cells that efficiently received the dsRNA, whereas the others (60%) not. It has been shown that in contrast to siRNA spraying, long dsRNA spraying is less efficient [37]. We thus hypothesize that a significant portion of the dsRNA remained on

the leaf surface and/or the leaf apoplast and that only a few dsRNA molecules managed to be efficiently delivered inside the plant cell. Moreover, the dsRNAs would need to be transported to the nucleus to trigger RdDM. Thus, despite the huge amount of dsRNA sprayed on each plant (50 μ g), perhaps only a small fraction of these molecules managed to finally reach the nucleus of a limited number of cells. Nevertheless, it is of interest that in the methylated clones (40%), the methylation pattern was restricted to the region defined by the dsRNA and did not spread towards the 3' (Figure 1d), as observed in other cases [38]. The 3' spreading of methylation involves the concerted action of POLIV and RDR2, but it is not clear how rapid this process is. The gradual onset of unavoidable mechanical damage imposed upon the sprayed tissue 10 dps did not allow us to continue our analysis to subsequent time points (e.g., 20 dps or beyond). Thus, we could not analyze whether 3' spreading of methylation would eventually take place.

In this short communication, we show for the first time that dsRNA spraying triggers de novo methylation of a promoter sequence in plants. These perhaps preliminary data are nevertheless important, given the complete absence of similar reports on epigenetic phenomena induced upon exo-RNAi applications. Future research should elucidate the mechanistic details underlying our observations, e.g., investigating whether the dsRNA was processed by DCLs into siRNAs (small RNA deep sequencing), and if yes, what size class of siRNAs was generated and whether they homogeneously covered the dsRNA region and fully matched the regions of methylation. Additional insights would be provided by analyzing whether such putative DCL processing takes place in the cytoplasm or in the nucleus where DCLs seem to co-localize [39], as well as whether other intermediate dsRNA molecules (besides siRNAs) are formed that may have a role in the whole process, as shown before [40]. Finally, dsRNA-spraying in *dcl* knockout plants and/or 24-nt siRNA spraying and subsequently evaluating their ability to trigger methylation could provide valuable insights on the still elusive mechanism of RdDM.

In a more applied context, our data suggest that dsRNA spraying could potentially serve as a useful tool in epigenetics-based crop breeding platforms [41,42]. DNA methylation of promoter sequences ensues histone modifications, such as H3K9 methylation and H3/H4 deacetylation, that result in transcriptional gene silencing (TGS) [43,44]. Indeed, when certain criteria are met (see below), plants sprayed with dsRNAs against a promoter sequence may exhibit RdDM and TGS. Since, in plants, gametes are formed from somatic cells, in the sprayed-free progeny, CG/CHG methylation and perhaps even TGS (depending on the promoter) can be maintained. It needs to be noted, though, that, in our case, we did not observe TGS (absence of GFP fluorescence upon ultraviolet lamp detection) in 3 dps or even at 10 dps. In some cases, dense RdDM and concomitant TGS may require up to 4 generations of the continuous presence of the dsRNA trigger to be established [23,45]. However, optimization of dsRNA spraying assays may reach the desired goal. Such optimizations could include (i) the development of methods for efficient dsRNA delivery in meristematic tissues, which give rise to all developing plant organs and gametes, not just in easily sprayable plants, such as A. thaliana and N. benthamiana, but also in agronomically more important crops, (ii) the conjugation of dsRNAs with nanoparticles, such as clay nanosheets [46], so as to increase dsRNA delivery inside the plant cell, (iii) importantly, the introduction of nucleus-localization RNA signals, such as the C/D box motif [47], in the dsRNA (e.g., in the spacer of hairpin RNAs), so as to increase dsRNA delivery in the nucleus, and (iv) the consideration of tandem dsRNA spraying across 3–4 consecutive generations in order to increase the likelihood for efficient RdDM and TGS. If so, one could envisage that dsRNA spraying targeting endogenous sequences in various crops could lead to trans-generationally maintained DNA methylation and even TGS. This aspect is of particular interest in classical breeding programs that suffer from the loss of genetic variation (genetic erosion) [48]. Resorting to the epigenome and modifying it in a specific and transgene-free method by exo-RNAi approaches could reveal the much needed cryptic diversity that could invigorate breeding platforms. For reasons that are not very clear, endogenous promoters are not as prone to RdDM/TGS as transgenic

promoters are [22,49]. However, several endogenous promoters, especially tissue- and organ-specific ones, are susceptible to RdDM/TGS [3,50–53] and can be considered for the aforementioned approaches.

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