An 11bp Region with Stem Formation Potential Is Essential for de novo DNA Methylation of the RPS Element

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Abstract

The initiation of DNA methylation in Arabidopsis is controlled by the RNA-directed DNA methylation (RdDM) pathway that uses 24nt siRNAs to recruit de novo methyltransferase DRM2 to the target site. We previously described the REPETITIVE PETUNIA SEQUENCE (RPS) fragment that acts as a hot spot for de novo methylation, for which it requires the cooperative activity of all three methyltransferases MET1, CMT3 and DRM2, but not the RdDM pathway. RPS contains two identical 11nt elements in inverted orientation, interrupted by a 18nt spacer, which resembles the features of a stemloop structure. The analysis of deletion/substitution derivatives of this region showed that deletion of one 11nt element RPS is sufficient to eliminate de novo methylation of RPS. In addition, deletion of a 10nt region directly adjacent to one of the 11nt elements, significantly reduced de novo methylation. When both 11nt regions were replaced by two 11nt elements with altered DNA sequence but unchanged inverted repeat homology, DNA methylation was not affected, indicating that de novo methylation was not targeted to a specific DNA sequence element. These data suggest that de novo DNA methylation is attracted by a secondary structure to which the two 11nt elements contribute, and that the adjacent 10nt region influences the stability of this structure. This resembles the recognition of structural features by DNA methyltransferases in animals and suggests that similar mechanisms exist in plants.

Introduction

Cytosine methylation is an ancient modification system that has diversified into different biological roles including restriction modification systems in bacteria, and epigenetic regulation of gene expression and genome structure in most eukaryotes, where cytosine methylation works in combination with histone modifications [1]. Research on the control and biological effects of 5mC has focussed on understanding how 5mC methylation in Arabidopsis is controlled by the RNA-directed DNA methylation (RdDM) pathway that uses 24nt siRNAs to recruit de novo methyltransferase DRM2 to the target site. We previously described the REPETITIVE PETUNIA SEQUENCE (RPS) fragment that acts as a hot spot for de novo methylation, for which it requires the cooperative activity of all three methyltransferases MET1, CMT3 and DRM2, but not the RdDM pathway. RPS contains two identical 11nt elements in inverted orientation, interrupted by a 18nt spacer, which resembles the features of a stemloop structure. The analysis of deletion/substitution derivatives of this region showed that deletion of one 11nt element RPS is sufficient to eliminate de novo methylation of RPS. In addition, deletion of a 10nt region directly adjacent to one of the 11nt elements, significantly reduced de novo methylation. When both 11nt regions were replaced by two 11nt elements with altered DNA sequence but unchanged inverted repeat homology, DNA methylation was not affected, indicating that de novo methylation was not targeted to a specific DNA sequence element. These data suggest that de novo DNA methylation is attracted by a secondary structure to which the two 11nt elements contribute, and that the adjacent 10nt region influences the stability of this structure. This resembles the recognition of structural features by DNA methyltransferases in animals and suggests that similar mechanisms exist in plants.
elements, and de novo methylation levels are especially high around a HhaI site located within a putative stemloop region [16]. RPS-specific DNA methylation differs from DNA methylation at heterochromatic regions as it does not involve the recognition of sequence repeats nor does it depend on the chromatin-remodelling ATPase DDM1 [17], which is required for H3K9 methylation and DNA methylation in heterochromatic regions [18]. RPS methylation is also independent of DCL3 and RDR2, but it requires the presence of all three DNA Methyltransferases MET1, CMT3 and DRM2. In wildtype, RPS-specific methylation is associated with homologous small RNAs, which are, however, absent in a RDR2 mutant where RPS methylation is reduced but still present. This suggested that RPS initiates DNA methylation via an RdRM-independent mechanism, and that methylation, once it has been established, is recognized and augmented by RdRM pathway functions [17]. To investigate the nature of the signal that initiates RPS methylation, we tested if DNA methylation was affected by deletions or substitutions within and around the putative stemloop region. We find that removal of one of the 11nt inverted repeat elements is sufficient to eliminate RPS methylation, while sequence changes within the 11 nt repeats that do not alter pairing homology don’t affect DNA methylation efficiency, which suggests that RPS-specific methylation is initiated via the recognition of structural features rather than specific sequence elements.

Results

We selected the potential stemloop region between position 559–598 of the RPS fragment [16] as a target for a deletion/substitution analysis, and designed five modification constructs RPSmod1-5 (Figure 1). In RPSmod1, the sequence of the loop region between the 11 nt Box2a and 2b stem elements was altered. In RPSmod2 and RPSmod3, DNA sequences of the 11 bp Box2a and 2b stem elements were modified while nucleotide composition and stem formation potential remained unchanged. In addition, 10 nucleotides of BoxB were deleted in RPSmod3. In RPSmod4, stem formation was weakened by nucleotide substitutions in the 11 bp Box2a and 2b stem elements, and in RPSmod5, one of the 11 nt Box2a stem elements was deleted.

We selected three single copy Arabidopsis thaliana transformants for each construct, for which we determined the DNA methylation levels within a 200 bp region around the stemloop region or its modifications (Figure 2). Sequence changes to the loop region in RPSmod1 transformants had no inhibitory effect on RPS methylation, which reached very high levels both at CG and non-CG targets. Sequence modifications to the Box2 stem region in RPSmod2 lines, which do not alter stem formation, had only a very minor effect in reducing DNA methylation, which was, however, significantly enhanced when coupled with a 10nt replacement of BoxB in RPSmod3 transformants. Resolution of the stem structure in RPSmod4 lowers but does not eliminate methylation, while deletion of one of the 11 nt stem elements in RPSmod5 abolishes methylation.

To investigate if the RdDM pathway contributed to the efficiency of methylation for those RPS derivatives that still attracted DNA methylation, we repeated the methylation analysis of three single copy transformants in a rdr2 mutant background (Figure 3). All modifications except RPSmod4 show a reduction in methylation compared to wild-type lines indicating an amplification role of RDR2 in RPS methylation. Only RPSmod4 methylation patterns remain largely unaffected by the absence of RDR2.

The loss of de novo methylation in RPSmod5 suggests that the putative stem region is crucial and possibly represents the initial target region for de novo methylation. The methylation analysis of RPS derivatives in rdr2 confirms our previous model that initial methylation is independent of RdRM functions but that it can be enhanced by the RdRM pathway leading to increased methylation levels and possibly also to spreading of methylation from the initial de novo methylation region.

The large error bars associated with methylation patterns of RPSmod4 transformants highlight that a standard deviation analysis is deceptive as RPSmod4 lines actually show a bimodal methylation profile (Figure 4). Individual RPSmod4 clones are either methylated at levels >80% or almost completely unmethylated. These data are in accordance with a ‘switch’ mechanism that alternates between almost complete hypomethylation and hypermethylation of RPSmod4, respectively. Individual transformants showed different ratios of almost fully methylated and fully unmethylated clones (Figure 5). To investigate if these observed differences in the

Figure 1. Modifications to the RPS stemloop. Unmodified RPSstemloop is shown at the top along with coloured regions for boxA and boxB (blue), and palindromic regions Box2a and Box2b (red). Modifications are indicated by: dashed region in RPSmod1 indicating change to loop sequence; green boxes in mod2 and mod3 indicate sequence changes that remain palindromic; modification of sequences in RPSmod2 and RPSmod3 (boxB), and also RPSmod4 (box2a and box2b) indicated by missing sections; and deletion of box2a in RPSmod5 indicated by dashed line. A schematic representation of the putative stem structure predicted by mfold (http://mfold.rna.albany.edu) is given to the right where applicable.

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Figure 2. Bisulfite analysis of *RPSmod* lines in a wild-type background. Bars show the percentage of DNA methylation at each cytosine residue indicated on the horizontal axis, with CG methylation in red, CNG methylation in blue and CNN methylation in green. The RPS stemloop region starts at position 64. Error bars represent variation between three single copy lines. doi:10.1371/journal.pone.0063652.g002

Figure 3. Bisulfite analysis of *RPSmod* lines in a *rdr2* background. CG, CNG and CNN methylation is indicated by red, blue and green bars, respectively. All modifications except *RPSmod4* show a reduction in methylation compared to wild-type lines indicating an amplification role of RDR2 in RPS methylation. Methylation in *RPSmod3* is almost eliminated. *RPSmod4* methylation remains largely unchanged from wild-type. doi:10.1371/journal.pone.0063652.g003
hypermethylation frequency represented locus-specific effects, we analysed methylation ratios in the F1 generation of three lines (Figure 5). Our results show that the frequencies at which individual RPSmod4 transgenes become hypermethylated or remain hypomethylated, are not line-specific but appear to be stochastic events.

Discussion

The RPS is an unusual DNA methylation target as its de novo methylation occurs independently of RdRM pathway functions, which only contribute to the augmentation of DNA methylation marks once they have been established [17]. This leaves the question of which regions and features within RPS are responsible for the initiation of DNA methylation. Our data identify the 11nt stem structure as a critical region for the recognition of RPS as a de novo methylation target, as DNA methylation is reduced to background levels in RPSmod5 transformants, which contain a RPS transgene from which one of the 11nt stem segments has been removed. For RPSmod2 transgenes, where both 11nt stem elements have been replaced with DNA elements with altered
Figure 5. Analysis of hypermethylation rates for RPSmod4 clones in the next generation. Classification of RPSmod4 clones according to percentage of total methylation for each independent line. T1 generations (solid bars) show a distinct bimodal distribution of <10% and >80% methylation. For line A, the majority of clones in the T1 generation are highly methylated. Line B shows fewer highly methylated clones and line C shows mostly unmethylated clones. All lines show an increase in unmethylated clones in the T2 generation (striped bars). Line A has no clones showing >80% methylation. Line B shows 2 out of 12 clones still having high levels of methylation and 2 clones with intermediate levels. For line C, no clones showed significant methylation in the T2 generation.

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Figure 6. Folding models for RPS derivatives. RPS (A) and the five RPSmod sequences (B–F) were submitted to the DNAfold programme [27] to calculate potential secondary structures and their energy levels. For RPSmod4 (E) two alternative folding options are shown.

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sequence but unchanged inverted repeat homology, no significant reduction in DNA methylation efficiency was detected, which supports the assumption that structural features rather than DNA sequence elements guide RPS methylation. While the 11nt region is required for RPS methylation, it is not sufficient, as maintenance of efficient de novo methylation is influenced by modifications to the adjacent BoxB region in the RPSmod3 constructs. This implies that BoxB contributes to the efficiency of secondary structure formation that attracts de novo methylation.

Conformational changes of DNA regions are powerful recognition signals for regulatory proteins [19]; [20]; [21] and can also significantly affect target selection of DNA methylation functions [22]. Sequence specificity of mammalian Dnmt1 is altered by supercoiling which might induce alternative secondary structures that affect their interaction with DNA methyltransferases or auxiliary nuclear proteins [23]. Structural features have also been proposed to play a role in the specific recognition of transposition and viral integration intermediates by DNA methylation systems that inactivate invasive DNA [24]. Unusual non-B structures at the adjacent BoxB region in the RPSmod3 constructs. This implies that functionality of the stem element is not dependent on a specific DNA sequence, and that methylation efficiency is significantly influenced by a small region directly adjacent to the stem region. We don’t know the nature of the structural signals responsible for the de novo methylation of RPS but we notice that the efficiency of attracting de novo methylation among the different RPS modifications correlates with the probability of stemloop formation (Figure 6). For RPSmod4, the programme calculates two folding options with strong and weak hairpin potential (Figure 6E), which would be in agreement with a conformational switch between structures that favour hypermethylation and hypomethylation, respectively. It will be interesting to investigate if and which endogenous loci provide structural signals that can establish novel DNA methylation patterns, independent of RdRM pathway functions, and if these regions can undergo conformational changes that makes them as efficient targets for de novo DNA methylation similar to some of the RPS variants we examined.

**Experimental Procedures**

DNA extraction was performed using a modified CTAB method. 1–2 g of tissue was ground in liquid nitrogen and then transferred to a 2 ml eppendorf tube. 560 µl Extraction buffer (2M NaCl; 20 mM sodium meta-bisulfite; 200 mM TrisHCl, pH 8.0; 70 mM EDTA) was added and the sample was vortexed briefly before adding 180 µl 5% sarsoyl. Samples were incubated at 65°C for 2 hours. Samples were phenol:chloroform extracted and DNA precipitated with 1/2 volume isopropanol and 1/2 volume high salt buffer (0.8M Sodium citrate; 1.2M NaCl). The resulting pellet was dissolved in 400 µl TE buffer (pH 8.0), and after addition of RNase (25 µg) was incubated at 65°C for 15 minutes. 400 µl of CTAB buffer (50 mM EDTA; 200 mM TrisHCl, pH 8.0; 125 mM NaCl; 0.04% w/v PVP; 5% β-mercaptoethanol) was added and samples were incubated at 65°C for a further 15 minutes. Then samples then underwent two rounds of phenol:chloroform:IAA extraction followed by a further chloroform:IAA (24:1) clean up. Samples were precipitated with isopropanol.

**RPSmod plasmids design and transformation**

Oligonucleotides containing the RPS stemloop modification sequences flanked by SnaBI and BsrGI restriction sites were ordered from MWG biotech. Formation of secondary structures of the modified regions was tested by importing modified regions into DNAfold [27]. To generate the RPShyg vector, pGreen0179 [28] was digested with HindIII (New England Biolabs www.neb.com) and ligated with a 1.6 kb RPS HindIII fragment isolated from p55SGUS/RPS [16]. To produce the RPSgygdel recipient vector, RPShyg was digested with SnaBI and BsrGI (New England Biolabs www.neb.com) to remove a 232 bp region containing the RPS stemloop. The vector was gel purified using Q-spin Gel Extraction kit (Genellow, www.gefnlow.co.uk) to produce the RPShygdel recipient vector. The pre-ordered oligonucleotides were cloned into the pGEM TA cloning vector (Promega, www.promega.com) according to the manufacturer’s instructions. Each modified RPS stem loop region was cut out of purified plasmids using SnaBI and BsrGI. Inserts were cloned into RPShygdel to generate each RPSmod construct. These were cloned into Agrobacterium tumefaciens (GV1301) for transformation in Arabidopsis thaliana lines by floral dip [29]. Prior to plant transformation, plasmids purified from Agrobacterium were sequenced (GATC, www.gatc.com) to ensure no sequence changes had occurred.

**Bisulfite sequencing**

Bisulfite sequencing was performed using QIAGEN Epitect conversion kit (www.qiagen.com) according to manufacturer’s instructions except the cycling conditions were increased to 90°C 5 min, 60°C 25 min, 90°C 5 min, 60°C 45 min, 90°C 5 min, 60°C 55 min, 90°C 5 min, 60°C 60 min, 90°C 5 min, 60°C 60 min. The RPS region (top strand) was amplified from treated DNA using primers RPStop-F (CTTg/aTTTTTTTCTCCCTTCA) AND RPStop-R (AAGTAGAAAG-GGAAAGAGAAAGGG). PCR was carried out using myTaq polymerase (Bioline www.bioline.com) under the following condi-
tions: 94°C, 15 secs; 54°C, 20 secs; 72°C 15 secs for 45 cycles generating either a 216 bp (RPSmod1-4) or 203 bp (RPSmod5) fragment. Amplicons were separated on a 2% agarose gel and excised using Q-spin Gel Extraction kit (Geneflow, www.genellow.co.uk). Purified fragments were cloned using the pGEM TA cloning vector (Promega, www.promega.com) according to the manufacturer’s instructions and transformed into E.coli DH5α competent cells. A minimum of ten bisulfit treated clones were sequenced for each line (GATC, www.gatc.com) and analysed by exporting their sequence to BioEdit [30]. Aligned sequences were sent to Cymate [31] to calculate and illustrate DNA methylation frequencies.

**Author Contributions**

Conceived and designed the experiments: MG PM. Performed the experiments: MG. Analyzed the data: MG PM. Contributed reagents/materials/analysis tools: PM. Wrote the paper: MG PM.

**References**

1. Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. Annu Rev Biochem 74: 401–514.
2. Zhang X, Yasaki J, Sundaresan A, Cokus S, Chan SWL, et al. (2006) Genome-wide High-Resolution Mapping and Functional Analysis of DNA Methylation in Arabidopsis. Cell 126: 1189–1201.
3. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, et al. (2008) Shotgun bisulfite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature 452: 213–219.
4. Zilberman D, Cao XF, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299: 710–719.
5. Zheng X, Zhu J, Kapoor A, Zhu J-K (2007) Role of Arabidopsis AG06 in siRNA accumulation, DNA methylation and transcriptional gene silencing. EMBO J 26: 1690–1701.
6. Chan SWL, Zilberman D, Xie Z, Johansen LK, Carrington JC, et al. (2004) RNA Silencing Genes Control de Novo DNA Methylation. Science 303: 1336.
7. Zilberman D, Cao X, Johansen LK, Xie Z, Carrington JC, et al. (2004) Role of Arabidopsis ARGONAUTE4 in RNA-Directed DNA Methylation Triggered by Inverted Repeats. Current Biology 14: 1214–1220.
8. Freitag M, Lee DW, Kothe GO, Pratt RJ, Aramayo R, et al. (2004) DNA Methylation Is Independent of RNA Interference in Neurospora. Science 304: 1939.
9. Tamaru H, Selker EU (2003) Synthesis of Signals for De Novo DNA Methylation in Neurospora crassa. Molecular and Cellular Biology 23: 2379–2394.
10. Lewis ZA, Honda S, Khaldi falsehood TK, Jeffress JK, Freitag M, et al. (2008) Relics of repeat-induced point mutation direct heterochromatin formation in Neurospora crassa. GenoResearch.
11. Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. Nature Rev Genes 8: 272.
12. Hall IM, Shankaranarayana GD, Noma K-i, Ayoub N, Cohen A, et al. (2002) Regulation of DNA甲基化. adena. Cell 126: 1189–1201.
13.Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. Nature Rev Genes 8: 272.
14. Hall IM, Shankaranarayana GD, Noma K-i, Ayoub N, Cohen A, et al. (2002) Establishment and Maintenance of a Heterochromatin Domain. Science 297: 2232–2237.
15. Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, et al. (2002) Regulation of Heterochromatic Silencing and Histone H3 Lysine-9 Methylation by RNAI. Science 297: 1833–1837.
16. Jia S, Noma K-i, Grewal SIS (2004) RNA-Independent Heterochromatin Nucleation by the Stress-Activated ATF/CREB Family Proteins. Science 304: 1971–1976.
17. Yamada T, Fischle W, Sugiyama T, Allis CD, Grewal SIS (2005) The Nucleation and Maintenance of Heterochromatin by a Histone Deacetylase in Fission Yeast. Molecular Cell 20: 173–183.
18. Muller A, Marins M, Kamisugi Y, Meyer P (2002) Analysis of hypermethylation in the RPS element suggests a signal function for short inverted repeats in de novo methylation. Plant Molecular Biology 48: 393–399.
19. Singh A, Zubko E, Meyer P (2008) Co-operative activity of DNA methyltransferases for maintenance of symmetrical and non-symmetrical cytosine methylation in Arabidopsis thaliana. Plant Journal 56: 814–823.
20. Grouillard AV, Lippman Z, Voevodin C, Colot V, Martienssen RA (2002) Dependence of heterochromatic histone H3 methylation patterns on the Arabidopsis gene DDMM. Science 297: 1871–1873.
21. Horwitz M, Leeb L (1988) An E. coli promoter that regulates transcription by RNA superhelix-induced cruciform extrusion. Science 241: 703–705.
22. Garryse A-L, Pratseh D, Helaine C (1997) Identification of a triple DNA-binding protein from human cells. Journal of Molecular Biology 267: 289–298.
23. Herbert A, Rich A (1999) Left-handed Z-DNA: structure and function. Genetics 160: 37–47.
24. Yoder JA, Soman NS, Verdone GL, BESTOR TH (1997) DNA (cytosine-5)-methyltransferases in mouse cells and tissues. studies with a mechanism-based probe. Journal of Molecular Biology 270: 385–395.
25. BESTOR T (1987) Supercoiling-dependent sequence specificity of mammalian DNA methyltransferase. Nucleic Acids Research 15: 3035–3043.
26. BESTOR TH, TYCKO B (1996) Creation of genomic methylation patterns. Nature Genetics 12: 363–367.
27. Clark J, Smith SS (2002) Secondary Structure at a Hot Spot for DNA Methylation in DNA from Human Breast Cancers. Cancer Genomics – Proteomics 5: 241–251.
28. Shlyakhtenko LS, Hsieh P, Grigoriev M, Potaman VN, Sinden RR, et al. (2000) A cruciform structural transition provides a molecular switch for chromosome structure and dynamics. Journal of Molecular Biology 296: 1169–1173.
29. Zilberman D, Cao XF, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299: 710–719.
30. RPS de novo Methylation