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Mobile small RNAs and their role in regulating cytosine methylation of DNA

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ABSTRACT

Small (s)RNAs of 21 to 24 nucleotides are associated with RNA silencing and methylation of DNA cytosine residues. All sizes can move from cell-to-cell and long distance in plants, directing RNA silencing in destination cells. Twenty-four nucleotide sRNAs are the predominant long-distance mobile species. Thousands move from shoot to root, where they target methylation of transposable elements both directly and indirectly. We describe here our analyses of the different classes of shoot-root mobile sRNAs, their influence on cytosine methylation and effects on gene expression.

Introduction

Small (s)RNAs of 21 to 24 nucleotides (nt) are involved in multiple gene silencing pathways. They direct both transcriptional and post-transcriptional gene silencing mechanisms to target loci[1–3] by sequence homology between the sRNAs and their targets.[3] Gene silencing is involved in several biological processes of plants, including defending the genome from transposable elements, development, imprinting, environmental responses and stress memory.[3–11]

Cytosine residues of DNA can be modified by the addition of a methyl group. This is associated with transcriptional gene silencing and formation of repressive chromatin, influencing potentially both structure and function of target loci.[12–15] Methylation can be directed to specific cytosine residues by the RNA-directed DNA methylation (RdDM) gene silencing pathway.[16] The mechanisms of RdDM are described well in several reviews.[4,17] In brief, small (s)RNAs of 21 to 24 nucleotides (nt) sharing sequence homology with target loci are generated by DICER-LIKE (DCL) family enzymes DCL2, DCL3 and DCL4.[18] The sRNAs guide ARGONAUTE family proteins to target loci during establishment of RdDM.[2,3,19] The DOMAINS REARRANGED METHYLTRANSFERASES DRM1 and DRM2 are then recruited to these loci, methylating cytosines in non-CG contexts (CHG and CHH, where H is A, T or C).[4,20] RdDM proceeds to a self-reinforcing maintenance phase that includes methylation of CG context cytosines and deposition of repressive marks on histones at the loci.[4,21] This can result in transcriptional gene silencing.

Patterns of DNA cytosine methylation (termed methylation henceforth) may be inherited both mitotically and meiotically in *Arabidopsis thaliana*.[22–25] They also vary between accessions from different locations[26,27] and spontaneously between generations.[22,23] Striking examples of epiallelles have been observed, where variation in methylation causes macroscopic phenotype changes.[28,29] For example, the tomato (*Solanum lycopersicum*) Colorless non-ripening mutation is caused by an increase in methylation that represses expression of a key transcription factor involved in fruit ripening.[29] The result of this epimutuation is that fruit are prevented from ripening.

sRNA mobility

Plant sRNAs are mobile both cell-to-cell and over long distances.[19,30–36] It is thought likely that cell-to-cell movement is via plasmodesmata, though this mechanism lacks details currently.[34,37] sRNAs generated from transgenes move long distance, root-to-shoot, by a repeating cell-to-cell mechanism.[33] Thousands of endogenously-derived sRNAs move shoot-to-root, but via the phloem and following source-sink relationships.[38] Twenty-four nt sRNAs are the predominant shoot-root mobile species, though other sizes move also.[38,39] These direct methylation at transposable elements (TEs) from families that are typically short and located near genes as well as at transgenes.[38,40] They are able to enter root meristem tissues and direct silencing of transgenes within.[38,39] They also transmit methylation variants from shoots of one *A. thaliana* accession to roots of another[40]

The mechanism by which sRNAs are transmitted within the phloem is not fully described, but likely involves binding by proteins found in the photosynthate.[41,42] There is no evidence of selectivity over which sRNAs move from shoot-to-root; the

mobile pool transmitted to the root is composed apparently of the same sRNAs as in the source shoots.[40] This is comparable to long-distance transport of messenger RNAs, which move from shoot-to-root in large numbers via the phloem, dependent predominantly on their abundance.[43] Whether movement of sRNAs is related to their abundance remains to be discovered.

Mobile sRNAs and methylation

In Lewsey & Hardcastle *et al*[40] we demonstrated the capacity of DCL-dependent mobile sRNAs originating in the shoots to regulate thousands of methylation loci in the roots. Shoot/root grafts of *A. thaliana* were created from various combinations of two wild-type accessions, Col-0 (Col) and C24 and a *dicer-like 2 dicer-like 3 dicer-like 4* triple mutant in Col-0 background (*dcl234*). High-throughput sequencing of small RNAs and bisulphite-treated (**THIS REF? doi:10.1038/nprot.2014.114**) DNA from roots allowed the identification and quantification of small RNA[44] and methylation loci[45] respectively. We constructed various models of differential behaviour between graft types, in which we see sRNAs and/or methylation enriched or depleted across samples. These were used to define classes of interaction between sRNAs and the DNA methylome by identifying those locations where particular types of sRNA behaviour overlapped with particular types of methylation behaviour (Figure 1).

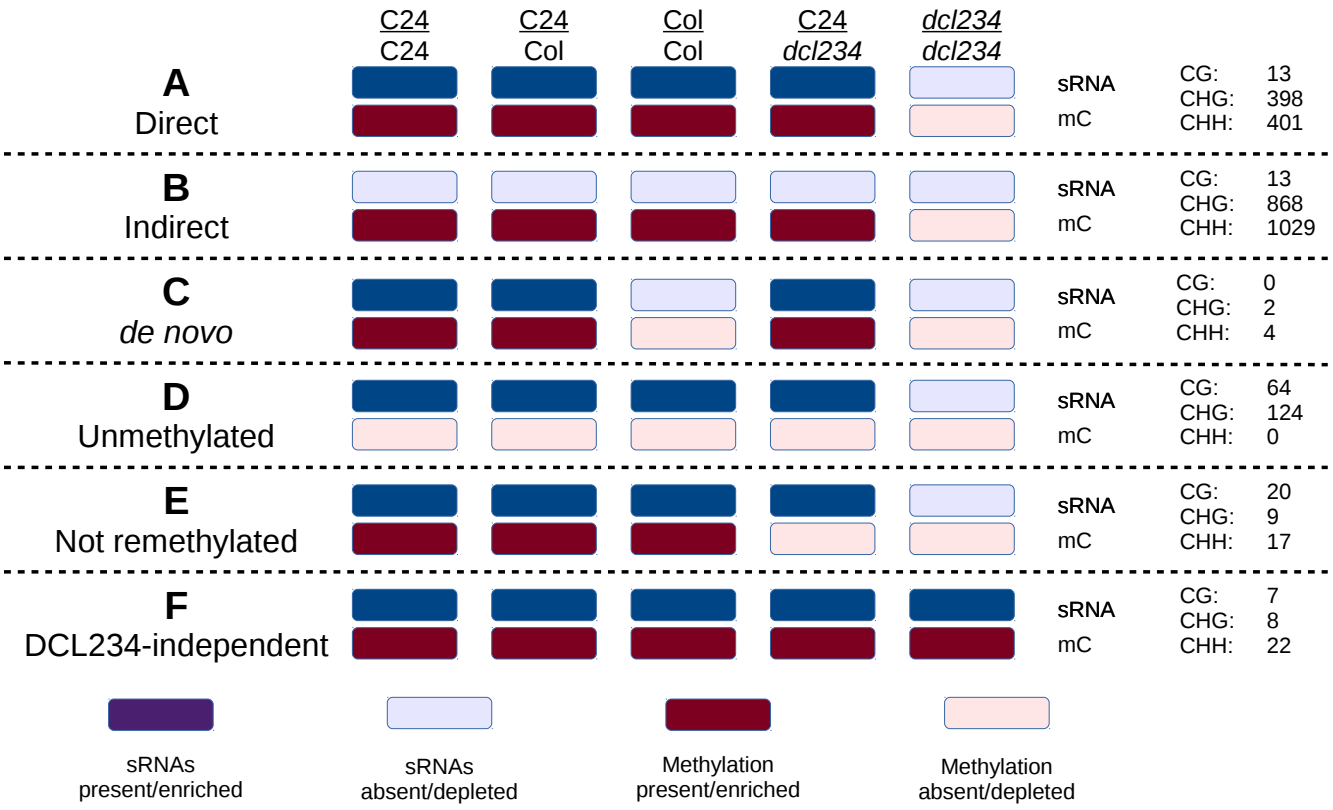

Figure 1. The six models of sRNA-methylome interaction investigated by grafting between shoots and roots of different *Arabidopsis thaliana* genotypes. The diagram represents for each model the defined combination of sRNA and cytosine DNA methylation status across roots. Graft combinations are indicated at the top (shoot above root) and model identities on the left. Loci fitting each model were identified by analyzing genome-wide sRNA-sequencing and methylC-sequencing data from roots of each graft combination.

Class A, the primary interaction of interest, is an overlap observed between mobile sRNAs and a mobile-signal associated methylation locus. These are considered direct effects of mobile sRNAs. Class B loci are considered indirect effects of mobile sRNAs. They exhibit mobile-signal dependency in that they show increased cytosine methylation when mobile sRNAs are produced by the shoot, but the methylation does not overlap with specific mobile sRNA loci. This might occur either due to low abundances of mobile sRNAs targeting these methylation loci, through secondary effects of the mobile sRNAs, or from methylation induced by non-perfect matching of the sRNAs to the genomic sequence.

Class C loci describe cytosine methylation induced in *A. thaliana* accession Col-0 by mobile sRNAs specific to accession C24. This occurs only in those grafts with accession C24 shoots and accession Col-0 roots, including *dcl234*. Class C loci are relatively rare, but demonstrate the possibility of inducing methylation at particular loci using non-endogenous sRNAs.

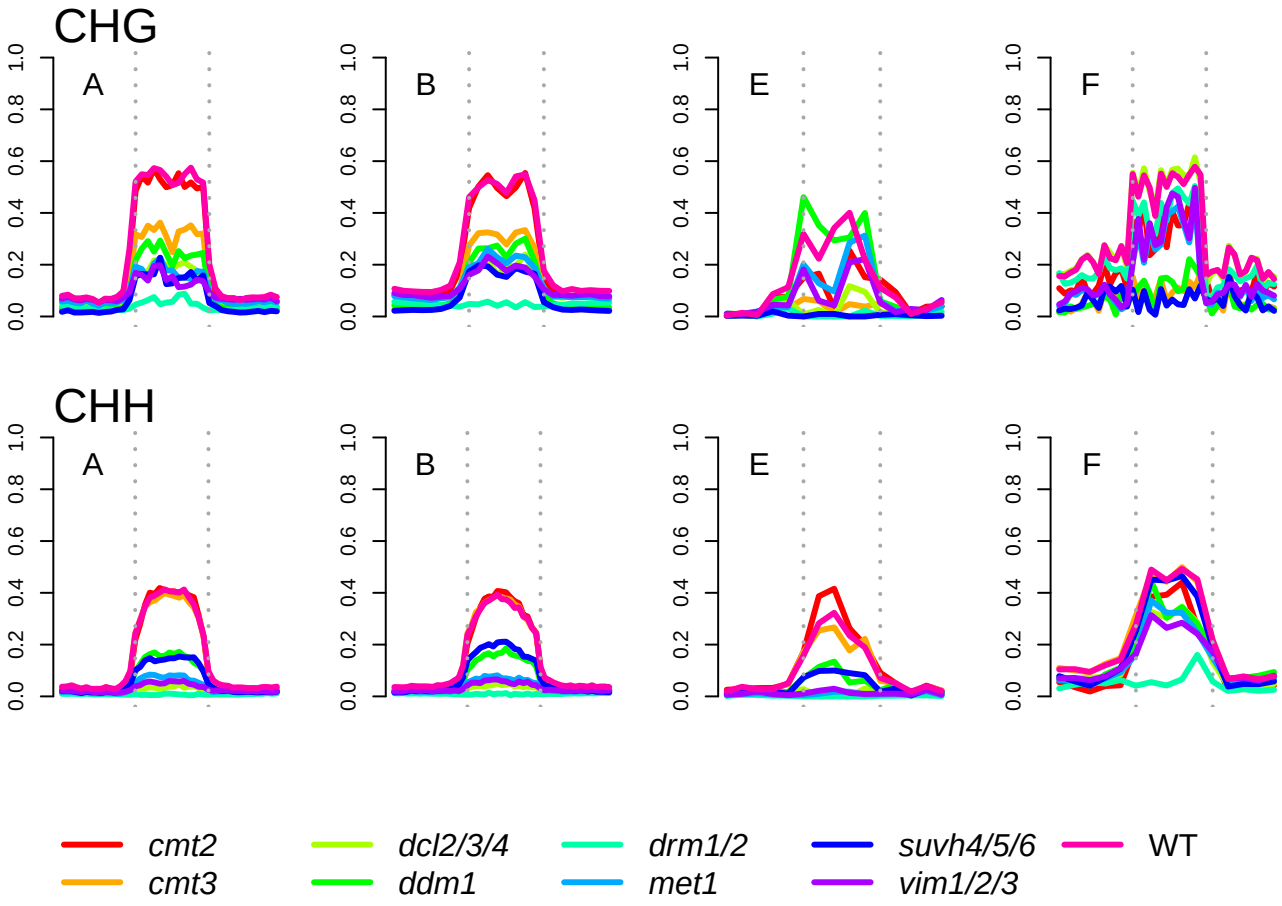

Figure 2. Specific RNA-directed DNA methylation mechanism components are required for mobile methylation. Methylation levels were assessed at class A, B, E and F loci in a mutants of RNA silencing components. Loci are size-normalized and plotted between dotted vertical lines, with methylation of the flanking 4000 nt plotted on either side.

We also include for comparison three further classes of interaction in which there appears to be no or limited interaction between methylation and the mobile signal. Class D loci have a mobile sRNA locus but no associated methylation, while class E loci show DCL2,3,4-dependent methylation that is not restored by mobile sRNAs. Class F loci show levels of methylation and total sRNA abundance unaffected by DCLs.

Comparisons between class types

We have shown previously[40] marked differences between characteristics of the class types. These include differences in the superfamilies of transposable elements with which loci are associated and in the RNA silencing mechanism components that methylation at the loci depends upon.

Classes of interaction and mechanisms of methylation

To investigate further the mechanisms by which the classes of sRNA-methylation interaction are differentiated we compare the methylation profiles of the class A & B loci in 86 mutants of RNA silencing components using the data of Stroud *et al*[46] to those observed at the E & F class loci and relate these profiles to models of methylation establishment and maintenance. Following Bond & Baulcombe,[47] we take a four stage process as a basic model for RNA-directed DNA methylation in *Arabidopsis*. An initiation stage mediated by 21/22nt small RNAs leads to an establishment phase in which DRM2 is recruited. Following this, 24nt sRNAs act through the PolIV pathway to maintain and amplify methylation in all contexts. For CG and CHG methylation, a second RNA-independent maintenance stage may then begin to operate, mediated by MET1 or the KYP-CMT2/CMT3 feedback loop that involves SUVH4 and histone methylation at H3K9me2.[48]

Examining the behaviour of the identified loci in the Stroud *et al* knockout mutants (Figure 2) suggests that type F loci, whose methylation is unaffected by the absence of DCL2,3,4, show strong reductions in CHG methylation in *cmt3* and *suvh4/5/6* but not *drm1/drm2* mutants, suggesting the importance of RNA-independent maintenance at these loci. However, the loci also show strongly reduced methylation in *ddm1* mutants, suggesting that establishment of these loci through chromatin remodelling is required.[49] Methylation of type F loci in the CHH context is largely unaffected in the knockout mutants save for *drm1/2*, **suggesting that these loci are able to exhibit wild-type levels of methylation through the establishment phase alone**. The ability of these loci to achieve this may be accounted for by examining the sRNA abundance profiles of the type F loci (Figure **??**); these are the only class of loci for which there is a substantial gain in expression of 21/22nt and >24nt small RNAs in the *dcl2,3,4/dcl2,3,4* graft, suggesting that DRM2 may be recruited by these sRNA populations.

Class E loci, in which methylation is lost in the *dcl2,3,4* and not regained through the action of sRNAs show a strong depletion in CHG context methylation in the *suvh4/5/6*, *cmt3* and *drm1/2* mutants but, in contrast to the F loci, not in the *ddm1* mutants. We hypothesise that the methylation of these loci is initiated through the action of sRNAs rather than chromatin remodelling and hence DDM1 is unnecessary; however, in contrast to type A & B loci (see below), the RNA-independent 'maintenance' stage is crucial to methylation in the E type loci. The CHH context class E loci also show substantial loss of methylation in the same mutants as in the CHG context, as well as *met1* and *vim1/2/3*. It seems likely that this is due to the substantial overlap between CHH E class loci with D and E type loci in CG and CHG contexts previously shown in Lewsey & Hardcastle *et al*;[40] we hypothesise that loss of methylation in the CHG and CG contexts caused by disruption to the RNA-independent maintenance pathways has a knock-on effect on CHH methylation for these loci, consistent with the findings of Stroud *et al*.[46]

Class A & B loci, which represent direct and indirect methylation by the mobile signal behave identically for the examined mutants in the Stroud *et al* set. For CHG context methylation, while there is some loss of methylation in the mutants involved in the RNA-independent maintenance pathway this is clearly much reduced compared to that observed in E & F type loci, demonstrating that methylation at these loci is predominantly driven by 23/24nt sRNAs. CHH methylation behaves similarly for these locus classes, in that the RNA-independent maintenance pathways appear less important than RNA-dependent methylation, although *ddm1* shows a stronger effect on CHH than CHG methylation. Curiously, the effect of DDM1 seems to be inverted between CHG and CHH context methylation; for CHG methylation DDM1 is required strongly only for type F loci, while CHH methylation is independent of DDM1 at type F loci but required for A, B and E type loci.

Classes of interaction and histone modification

We further investigated differentiation between sRNA-methylome interaction classes through comparisons with the histone modification data of Luo *et al*.[50] Peaks for nine histone modifications were identified using the MACS2 pipeline; where data from multiple samples was available this was merged using DiffBind.

Overlaps between the locus classes and regions of histone modification were evaluated for significance using a block-bootstrap method. The Class F loci showed significant overlap with all histone modifications, with particularly large numbers of overlaps being observed with H3K27me1 and K3K9me2, in line with observations made by Yang & Zhang *et al*,[51] who also identified overlap between these histone marks and Dicer-independent methylation.

Notably, class A & B loci show different overlaps between histone marks. Class A loci in all methylation contexts show significant enrichment of overlap with H3K27me3, a mark that associates with repression of targets, while class B loci show significant enrichment of overlap with H3K36me2 and H3K36me3, which are typically associated with activation of targets. Class A and B loci target similar TE superfamilies for methylation in the same, non-CG, contexts. However, these analyses demonstrate they differ in the histone marks with which they associate and may offer support for the hypothesis that class A loci are genomic locations in which sRNAs are produced while class B loci are loci targeted by, but not producing, sRNAs.

The association between the total cellular population of sRNAs and H3K9me2 has been established clearly,[52–54] whereas the associations observed here between mobile methylation loci and H3K27me3, H3K36me2 an H3K36me3 histone marks are unusual. Consequently, we examined the overlap (see XXXXXX) between histone marks and all sRNA loci in our dataset, rather than mobile sRNA loci specifically. We found that most classes of sRNA loci are enriched for overlap with H3K9me2, as previously reported[52–54] and H3K27me1, recently identified as enriched within subclasses of RdDM loci.[51] The C24 specific sRNAs do not show this enrichment, likely due to differences between loci of histone modifications between ecotypes.

We next assessed differences between the histone marks associated with mobile sRNA loci and with total sRNA loci. Differential enrichment was examined by comparing mobile sRNA loci to block-bootstrapped samplings (see Methods) of the sRNA loci (Figure 4). This showed a clear enrichment in overlap of H3K27me3 and depletion in overlaps of H3K36me3 for mobile sRNA loci, relative to the baseline number of overlaps for sRNA loci. The antagonistic relationship between H3K36me3 and H3K27me3 has been previously noted within genes,[50] together with the hypothesis that natural antisense transcript (NAT) production is repressed by a combination of cytosine methylation and H3K36me3. In combination with our data, this may suggest that either NATs have an increased tendency to generate mobile sRNAs, or that this is a more general result applying to

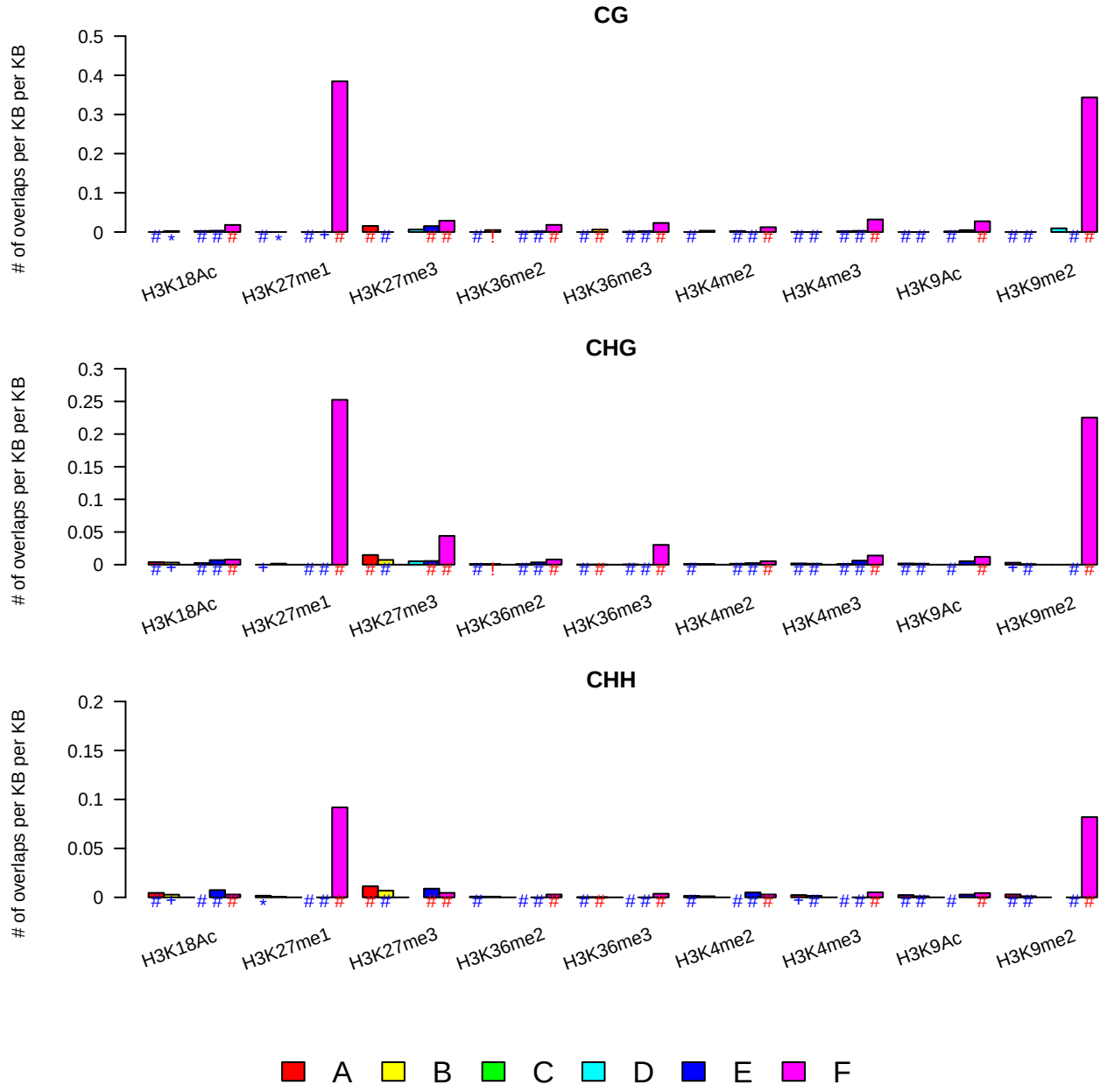

Figure 3. Number of overlaps per MB between locus class types and histone peaks. Significance levels: #, $0 < P < 10^{-5}$; +, $10^{-4} < P < 10^{-5}$; *, $10^{-3} < P < 10^{-4}$; !, $10^{-2} < P < 10^{-3}$; blue, underrepresented; red, overrepresented (relative to background).

some subset of mobile sRNA loci.

Gene expression associations

Very few genes show differential expression in the grafts in a manner consistent with regulation through the mobile signal. We previously conjectured that this might be due to the relatively low density of transposable elements in *A. thaliana*. However, an alternative possibility is that the mobile methylation signal has only a weak regulatory effect on gene expression in *A. thaliana*, and we are unable to detect this signal within individual genes given the depth of data currently available.

Using a generalised linear model similar to that described in Walters & Hardcastle[55] (see Supplemental Methods) we are

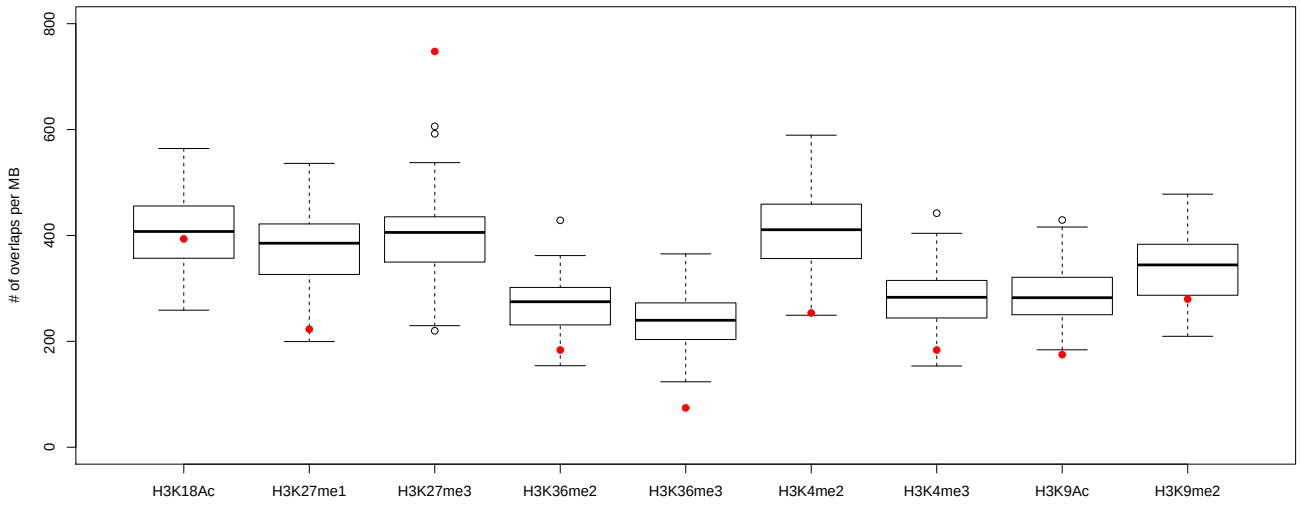

Figure 4. Number of overlaps per MB of mobile sRNA loci (filled red circles) between peaks of histone modification. Overlaps per MB observed in repeated block-bootstrap samplings of sRNA loci are shown as black box-and-whisker plots. Locus classes indicated by colours shown in boxes.

able to explore the average effect of sRNAs and methylation on gene expression. The model simultaneously examines possible effects of overlap with CG, CHG and CHH context methylation loci, sRNA loci, mobile-associated CG, CHG and CHH context methylation, mobile sRNA loci, and the interaction between mobile features and those samples which contain a mobile signal (i.e., all samples other than *dcl2,3,4/dcl2,3,4*). Figure 5 shows the magnitude of the effects obtained by fitting this model and categorizes their significance.

The model suggests that genes overlapping sRNA loci have on average substantially and significantly more expression than those not overlapping sRNA loci. We hypothesise that this effect may be caused by an increased production of sRNAs from highly expressed genes. However, this effect is more than reversed for the mobile sRNA; genes overlapping loci of this type have significantly less expression than those that do not. This effect is not associated with the presence of mobile sRNAs as the effect is independent of the mobile-sRNA associated samples. This suggests that the effect is associated with but not caused by the presence of sRNAs at these genes. It seems likely that this is due to the repressive action of H3K27me3 associated with mobile sRNA locations.

Conclusions

We have described how the epigenomes of shoots and roots use mobile sRNAs to communicate with one-another and control methylation at thousands of loci. Cohorts of directly and indirectly targeted loci exist, predominantly methylated in the non-CG context and associated with the same small, gene-proximal retrotransposon superfamilies. However, we demonstrate here that these loci differ in the histone marks which which they are associated. Direct loci associate with H3K27me3, a mark found typically in active regions of the genome, whilst indirect loci associate with H3K36me2 and H3K36me3, both of which are found typically in repressed regions of the genome. In contrast, the global sRNA population is associated with the H3K9me2 mark. Future investigations might focus on functional differences between direct and indirect loci, as well as how the indirect loci are regulated. The manner of mobile sRNA transport and their infuence upon phenotypes, molecular or morphological, also remain to be elucidated.

Acknowledgements

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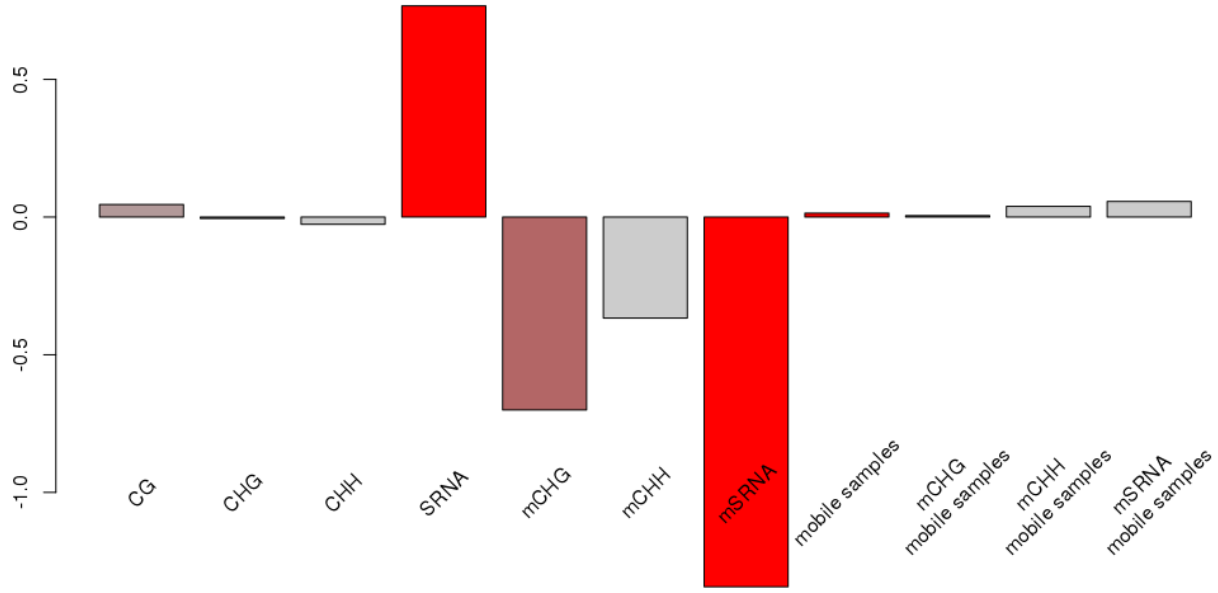

Figure 5. Effect size

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