Dynamic differential methylation facilitates pathogen stress response in *Arabidopsis*

Xiaoyu Zhang[1]
Department of Plant Biology, University of Georgia, Athens, GA 30602

In plants, DNA methylation plays important roles in silencing transposable elements (TEs) and endogenous genes. Several elegant examples of dynamic changes in DNA methylation during gamete or seed development have been recently described in *Arabidopsis* (1, 2). A considerable number of cell-to-cell variations in methylation have also been detected in vegetative tissues (3, 4); however, whether differential methylation during vegetative development is a regulated process with any biological function remained unknown. In PNAS, Dowen et al. (5) describe the finding that pathogen attacks result in dynamic changes in DNA methylation, which in turn lead to the transcriptional activation of defense-related genes and elevated resistance against pathogens.

Correlated Methylation and Transcriptional Changes in Response to Pathogen Attack

Mutations in the CG methyltransferase *MET1* and the non-CG methyltransferases *DRM1*, *DMR2*, and *CMT3* lead to genome-wide hypomethylation and pleiotropic developmental defects (6, 7). Unexpectedly, Dowen et al. discovered that the *met1* and the *drm1 drm2 cmt3* (*ddc*) mutants were more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Results from RNA-seq experiments revealed that many pathogen-responsive genes were constitutively expressed in *met1* and *ddc* in the absence of *Pst* treatment. In addition, upon *Pst* infection, some defense-related genes were induced or repressed to a larger degree compared with WT plants under the same treatment. It therefore appears that hypomethylation in *met1* and *ddc* may be responsible for the misregulation of pathogen-responsive genes and increased resistance to *Pst* infection.

However, does DNA methylation normally regulate pathogen-responsive genes in WT plants? Methylation changes following *Pst* infection have been previously detected by using methylation-sensitive amplified fragment length polymorphism (AFLP) (8). However, the nature and potential consequences of these changes remained unclear. To address these questions, Dowen et al. (5) compare the methylomes of untreated and *Pst*-treated plants by deep sequencing of bisulfite-treated DNA. Although the overall methylation pattern remained largely similar before and after *Pst* treatment, numerous cytosines at CG and CHH sites (but not CHG sites) became differentially methylated (referred to as "DmCG" and "DmCHH," respectively). DmCHHs are enriched in the 5′ and 3′ flanking regions of protein-coding genes, a distribution resembling that of total mCHH. In contrast, DmCGs are enriched in several distinct regions, including the transcription start sites, the polyadenylation sites, and a regions ∼1 kb upstream of transcription start sites. The enrichment of DmCGs at gene ends may be significant because these regions tend to be the targets of active demethylation and normally contain very little methylation (3, 9), and methylation changes in these regions seem to have the most pronounced effects on transcription (3, 10, 11). Two additional lines of evidence suggest that DNA methylation may play important roles in regulating defense-related genes: that differentially methylated regions (DMRs) are preferentially associated with genes involved in defense response, and that hypomethylation in DMRs is often accompanied by up-regulation of corresponding genes, particularly those involved in defense response.

Dowen et al. present clear evidence of dynamic changes in DNA methylation following pathogen attack.

To determine whether dynamic changes in DNA methylation were specifically induced by virulent *Pst* infection, the authors compared the methylomes of *Arabidopsis* plants treated with virulent *Pst*, an avirulent strain of *Pst* [*Pst*(*avrPphB*)], or exogenous salicylic acid (SA; a hormone involved in defense response). Interestingly, on a genomewide level, these three treatments seemed to elicit distinct DNA methylation changes. The predominant type of differential methylation following SA treatment is hypomethylation, particularly in the pericentromeric heterochromatin. In contrast, *Pst*(*avrPphB*) infection results in genomewide hypermethylation, and *Pst* infection leads to intermediate changes. Despite these differences, a subset of mCGs and mCHHs are similarly affected by all three types of treatments, indicating that DNA methylation at these sites may be modulated by a common mechanism. As in the case of *Pst* infection, SA-induced DMRs are also preferentially associated with defense-related genes, and the hypomethylation at these DMRs is accompanied by the transcriptional up-regulation of proximal genes. Notably, many genes associated with stress-induced hypomethylated DMRs are also misregulated in the *met1* and *ddc* mutants, either constitutively or after pathogen attack, indicating that the elevated *Pst* resistance in *met1* and *ddc* can at least be partially explained by the hypomethylation at or near defense-related genes.

A potential complication in interpreting these DNA methylation changes is that biotic stresses and the activation of the SA signaling pathway trigger widespread cell death. However, direct evidence linking cell death to differential DNA methylation has not been reported, and DNA degradation during cell death is not expected to grossly affect the analysis here because methylation levels are determined by normalizing methylated cytosines to total cytosines in intact DNA. In addition, methylation changes observed here preferentially take place at CG and CHH sites, with a biased distribution relative to genes, and they are often coupled with active processes such as transcriptional up-regulation. It therefore seems unlikely that the differential methylation described here can be fully accounted for by cell death.

The distribution of DMRs is similar to that of previously identified demethylation targets, indicating that stress-induced hypomethylation may involve active demethylation. However, it is also possible that transcriptional activation at some loci and the associated histone modification changes may compromise the maintenance of DNA methylation at these sites and lead to the passive loss of methylation following cell divisions. Detailed temporal

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[1]E-mail: xiaoyu@plantbio.uga.edu.

analyses of WT plants and demethylase mutants subjected to pathogen attacks in future studies should help to discern the relative contributions of these pathways. It is also of interest to determine the long-term stability of stress-induced DMRs and whether they can confer stress memories to defend against future pathogen attacks. Consistent with this possibility, methylation changes remain largely similar between 3 and 5 d after *Pst* infection.

DNA methylation can target defense-related genes in two ways: TEs or other repeats located in close proximity to genes can be methylated, or the genes themselves can be methylated (perhaps in part because many resistance genes are present as high copy number gene families). In both cases, stress-induced hypomethylation at DMRs is frequently associated with transcriptional up-regulation, whereas hypermethylation seems to have little effect. It therefore appears likely that, in the absence of pathogen attack, DNA methylation provides an additional level of transcriptional repression to prevent leaky expression of defense-related genes. A general repressive effect of TE insertions near genes has been previously proposed (12), but minimizing the expression of defense-related genes may be particularly important in alleviating the burden on normal development. In support of this notion, ectopic activation of defense-related genes through the hyperactivation of the SA signaling pathway or in the *met1* mutant can lead to developmental defects such as dwarfism.

Pathogen-Induced Overproduction of 21-nt Small RNAs from TEs

In addition to affecting the transcription of proximal genes *in cis*, stress-induced differential methylation may also regulate gene expression *in trans* through the RNAi pathway. Analysis of the small RNA (sRNA) population after SA treatment revealed an overproduction of 21-nt sRNAs at hypomethylated and transcriptionally activated TEs, whereas the abundance of 24-nt sRNAs remained unchanged. The length of these sRNAs is curious, because previous studies have shown that 21-nt sRNAs are less dependent on the RNA polymerase IV/RNA-dependent RNA polymerase 2/DICER-LIKE 3 (Pol IV/RDR2/DCL3) pathway than 24-nt sRNAs (13–15). Future studies in available RNAi pathway mutants should clarify which RNAi components are involved in producing the precursors of these 21-nt sRNAs, or whether they are generated from single-stranded Pol II transcripts with hairpin-like secondary structures. The latter scenario is consistent with the higher level of TE transcripts described here and by many previous studies on stress-induced TE activities. Regardless of the mechanisms responsible for their biogenesis, it is likely that some of these 21-nt sRNAs may target cellular genes involved in defense response. Indeed, a clear example has been described by McCue et al. (16): hypomethylation of the *Athila* LTR retrotransposon leads to the production of 21- to 22-nt sRNAs (siRNA854), which in turn regulate the *UBP1b* gene (an RNA-binding protein involved in stress response) *in trans* by targeting the 3′ UTR of its mRNA. Further analysis of SA-induced 21-nt sRNAs may turn up additional target genes involved in defense response. Finally, it is tempting to speculate that some of these sRNAs might function in a non–cell-autonomous manner to confer systematic or transgenerational resistance to bacterial pathogens.

In summary, Dowen et al. (5) present clear evidence of dynamic changes in DNA methylation following pathogen attack, as well as the functional consequences of differential methylation in regulating defense-related genes. A large fraction of these changes are facilitated by TEs. Because TE insertions are mostly stochastic, a considerable number of natural variations may exist among different *Arabidopsis* ecotypes to facilitate their defense against individual pathogens. It is also interesting to consider that TEs may play similar but more diverse roles in larger and TE-rich genomes, particularly in agriculturally important crop species.

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