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A novel insight into CREs, TF binding, CRE–CRE interactions, and the potential driving force of *de novo* methylation in plants

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Abstract

Cis-regulatory elements (CREs) regulate the amplitude of gene expression, specificity, and dynamics through transcription factor (TF) binding. Many CREs, especially in the transgene state, exhibit epigenetic instability and are frequently susceptible to *de novo* DNA methylation by RNA-directed DNA methylation (RdDM), a process initiated by Pol V transcript-derived 24-nucleotide small interfering RNAs (siRNAs). The mechanisms that control transgene methylation and the associated regulatory factors remain unresolved. Recent studies have advanced understanding of the regulatory mechanisms underlying transgene-state-dependent *de novo* methylation. This review traces the progression of research on transgene methylation, with particular focus on recent evidence indicating that enhancer–promoter interactions act as a regulatory force driving *de novo* methylation of transgenes. The implications of these findings are discussed in the context of the hypermethylation commonly observed in duplicated genes, tandem-arranged transgenes, repetitive genomic regions, and repetitive enhancers or promoters in paramutagenic alleles, where such interactions are inevitable. Furthermore, the potential role of CRE interaction-induced CRE methylation as an epigenetic defense mechanism against aberrant transcription is also discussed.

Keywords: *Cis*-regulatory elements (CREs), RNA-directed DNA methylation (RdDM), Enhancer–enhancer interactions, *De novo* methylation, Paramutation

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Cis-regulatory elements (CREs), such as promoters and enhancers, primarily serve as key elements controlling the amplitude of expression, tissue- and cell-specificity, and the environment-responsive dynamics of cognate genes[1,2]. This regulation contributes to phenotypic variation and functional evolution and is mainly dependent on bound protein factors or transcription factors (TFs) that directly or indirectly drive transcription activity, as disruptions or alterations of TF binding activities or specificities often leads to erasure/distortion of the trajectory or activity of expression, resulting in serious biological consequences (e.g., disease, failed development, fatality, etc.), which have been well documented in humans[3,4] and plants[5,6].

Plant CREs introduced as transgenes are vulnerable to *de novo* DNA methylation

CREs are generally epigenetically stable, yet some undergo spontaneous DNA methylation in response to specific biotic or abiotic stresses. This methylation can modify TF binding, thereby directly impacting gene expression and resulting phenotypes[7,8]. Over evolutionary timescales, many *de novo* methylation events become fixed, allowing them to be inherited through mitosis and meiosis across generations. Such fixation can lead to permanent genomic modifications and the formation of stable epialleles in both plants[9] and mammals[10]. In plants, methylation is particularly common in transgenic CREs, especially when these elements are arranged in tandem within composite transgenes[11–14]. For example, the enhancer (*AGe*) of the *Arabidopsis thaliana AGAMOUS* (*AG*) gene, which specifies the identity of male and female floral organs and directs carpel- and stamen-specific expression[15,16], is highly susceptible to *de novo* methylation in transgenic contexts. This occurs in over 40% of cases, leading to co-silencing of endogenous *AGe* and the appearance of mutant floral phenotypes[17]. Transgene state-dependent *de novo* methylation is not exclusive to *AGe* but can also affect other enhancers and promoters[17].

The RNA-directed DNA methylation (RdDM) pathway facilitates *de novo* methylation within transgenic CREs

Detailed analyses reveal that *de novo* methylation is associated with ectopic transcription of noncoding RNAs (ncRNAs) in the transgenic *AGe*, a process suppressed in the endogenous *AGe* genomic region[17]. These ncRNAs serve as precursors or substrates to produce 24-nucleotide small interfering RNAs (siRNAs)[17], which direct RNA-directed DNA methylation (RdDM) to cytosine residues[18–20]. Collectively, these findings indicate that transgene-specific *de novo* methylation likely depends on the RdDM pathway. Unlike the *de novo* methylation mechanism in mammalian cells, which primarily depends on DNA methyltransferase 3A and 3B (DNMT3A and DNMT3B) and potentially unmethylated H3K4[18,21], *de novo* methylation in plants is mediated by a complex RdDM pathway. This pathway is initiated by 24-nucleotide siRNAs produced by the plant-specific RNA polymerase IV from RNA transcripts[22–24]. The 24-nt siRNAs associate with ARGONAUTE (AGO) 4 and AGO6 complexes. Through sequence homology, these AGO complexes interact with long noncoding RNAs (lncRNAs) transcribed by RNA polymerase V[25]. This interaction facilitates the recruitment of additional protein factors, including

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REARRANGED METHYLTRANSFERASE 2 (DRM2), a homolog of mammalian DNMT3, to RdDM target sites[26]. Guided by the 24-nt siRNAs, the AGO–DRM2 complex methylates cytosines in CG, CHG, and CHH sequence contexts, where H denotes A, T, or C[27]. Genetic analyses indicate that all components essential for the RdDM pathway are also required for *de novo* methylation of the *AGe* transgene[17]. These findings confirm that the RdDM pathway mediates the methylation observed in CRE transgenes.

CRE–CRE interactions serve as a key regulatory force driving ectopic transcription of ncRNAs

A central question concerns the mechanisms by which the transcription of RdDM-potent ncRNAs is ectopically activated outside their typical contexts or within a transgene context. Specifically, it is important to determine which regulatory mechanisms drive ncRNA transcription in transgenic CREs inserted into the genome. One hypothesis is that isolated or transgenic CREs may lack the genomic context that normally represses ncRNA transcription at gene loci, thereby permitting transcriptional activation. However, the observation that the mutant flower phenotype occurs in only 40% of *AGe* lines does not support this hypothesis. Previous studies have demonstrated that transgenic or transposon CREs can interact robustly with nearby CREs in the genome, as evidenced by the altered expression levels of reporter or target genes[28–35]. Such CRE–CRE interactions may contribute to the ectopic activation of ncRNA transcription from transgenic CREs, adjacent CREs, or both. Supporting this hypothesis, the placement of a highly interactive *CaMV35S* enhancer (*35Se*) adjacent to *AGe* within the transgene cassette led to a 50% increase in mutant flower production[17]. This increase correlated with at least a twofold rise in ncRNA transcription levels[17], indicating that enhanced ncRNA transcription likely underlies the increased production of mutant flowers. Further evidence is provided by the insertion of the same *35Se* element 3–5 kb upstream of *AGe* in the genome, which activates siRNA/ncRNA production and induces *de novo* methylation, along with the production of mutant flowers[17], phenomena typically suppressed in plant genomes. Collectively, these findings indicate that the interaction between *35Se* and *AGe* in the genome drives ectopic activation of ncRNA transcription and ultimately RdDM at *AGe*.

Epigenetic implications of CRE–CRE interaction-induced *de novo* methylation in transgenes

Identifying the enhancer–enhancer interactions involved in the activation of RdDM-potent ncRNAs may provide new insights into hypermethylation, which preferentially occurs in tandemly oriented transgenes, duplicate genes, enhancers, and repetitive genomes enriched with transposable elements[7,12,36–40]. These genomic features commonly share duplicated CREs. One of the examples is paramutation in maize (*Zea mays*), which constitutes a distinct silencing mechanism that targets specific loci, such as the *b1* locus. The underlying cause of this paramutation has remained unresolved for decades. The *b1* locus regulates red pigment production, and pigment intensity is strongly influenced by the number of copies of the enhancers. For example, tandemly duplicated enhancers (up to six copies) result in intense dark red pigmentation, whereas a single enhancer does not[37,40,41]. Interestingly, maize plants with tandemly duplicated enhancers frequently undergo paramutation, characterized by methylation of the *b1* gene and a marked reduction in color intensity. In contrast, plants with only one enhancer copy do not exhibit this effect[41]. The dependence of paramutation on enhancer duplication is linked to the ectopic activation of ncRNA transcription, the production of siRNAs, and *de novo* methylation within these tandemly duplicated enhancers[40,41]. This process requires components of the canonical RdDM pathway[40–44]. Therefore, the induction of RdDM and paramutation at the *b1* locus, which depends on the arrangement of duplicate enhancers, appears to share a mechanistic basis with RdDM activation mediated by the *35Se–AGe* interaction (Fig. 1). These findings suggest that the methylation susceptibility of duplicated genes and repetitive transposons in genomes may arise from similar mechanisms, specifically the interaction between CREs.

The mechanisms by which interactions between CREs trigger ectopic transcription of RdDM-potent ncRNAs remain unresolved. Both long-range and short-range interactions between enhancers, as well as between enhancers and promoters, coordinate gene regulatory activity in mammalian cells[45] and plants[46,47]. These interactions depend on TFs that mediate communication between regulatory elements. In plants, the *35Se* enhancer, which is bound by multiple TFs, promotes robust and constitutive expression in both vegetative and floral tissues. This enhancer can interact with diverse promoters, resulting in broad enhancement of constitutive promoters or activation of tissue-specific promoters in otherwise inactive tissues. For example, the *AGe* enhancer is specific to male and female organ tissues, and its floral organ-specific expression depends on the binding of flower-regulated TFs such as AP1, AP2, AP3, and LFY[14,17,48,49]. When the *35Se* element is present adjacent to *AGe*, *AGe*'s regulatory activity in floral tissue is ectopically activated in vegetative tissues, including leaves, stems, and roots[50]. This observation suggests that TFs binding to *35Se* may interact with those binding to *AGe* and ectopically activate *AGe*'s regulatory functions in these tissues. However, the observation that activation or enhancement of ncRNA transcription in *AGe* by *35Se* occurs exclusively in flowers and not in leaves[17], indicates that *35Se*-mediated activation of ncRNA transcription in *AGe* is mechanistically distinct from *35Se*-mediated alteration of *AGe*'s regulatory activity. This finding suggests that *AGe*-bound TFs are required for ncRNA transcription. Although a comprehensive analysis is lacking, it is plausible that these TFs, when interacting with *35Se*-bound TFs, alter the configuration of their complex in a way that genome defense systems recognize as a compromised regulatory signal. This recognition may trigger ectopic activation or aberrant ncRNA transcription, leading to the production of 24-nucleotide siRNAs and ultimately *de novo* methylation and suppression of multifunctional gene transcription. Alternatively, such TF–TF interactions may induce conformational changes that increase the accessibility of *AGe* chromatin to Polymerase IV (Pol IV), thereby priming the RdDM pathway. It is also possible that these interactions could override anti-RdDM mechanisms present in native genomic contexts or within TF complexes in *AGe*. Further investigation is required to elucidate the precise mechanisms underlying these phenomena.

Fundamental and practical implications of CRE–CRE interaction-induced *de novo* methylation

The discovery that interactions between *CREs* can activate RdDM uncovers a previously unknown regulatory mechanism responsible for *de novo* methylation of transgenes. This finding clarifies why

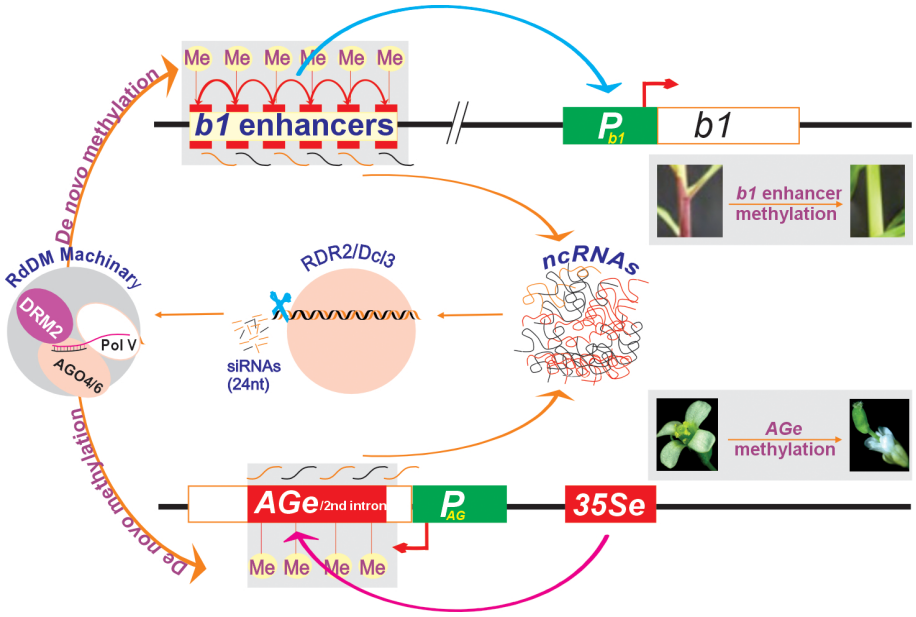


Fig. 1 The proposed CRE–CRE interaction-driven *de novo* methylation mechanism involves either the insertion of the *35S* enhancer (*35Se*) near the *AGAMOUS* (*AG*) locus in *A. thaliana*[17] or the spontaneous duplication of six 841-bp enhancer copies approximately 100 kilobases downstream of the *booster 1* (*b1*) locus in maize (*Zea mays*), which results in a dark red sheath phenotype[37,40–43]. In *A. thaliana*, *35Se* interacts with the distal *AG* enhancer (*AGe*), while in maize, the duplicated *b1* enhancers interact with each other. These interactions, through mechanisms that remain unclear, initiate ectopic transcription of ncRNAs. These RNAs are subsequently processed via the 24-nucleotide siRNA biogenesis pathway. The RDR2–DCL3 complex converts single-stranded ncRNAs into double-stranded RNAs, which are then cleaved into 24-nucleotide siRNAs. These siRNAs promote RNA-directed DNA methylation (RdDM), resulting in *de novo* methylation at the *AGe* and tandem *b1* enhancers. This methylation event silences transcription of *AG* and *b1*, leading to mutant flowers in *A. thaliana* and a shift from dark red to green coloration in maize stems.

hypermethylation often occurs in duplicated genes, enhancers, transgenes, and transposon-rich genomic regions, where CRE interactions are common. The result has practical implications. Alterations in CRE activity caused by CRE–CRE interactions can be controlled by inserting an enhancer-blocking insulator between CREs in the plant genome[50–54]. Introducing such insulators may reduce ectopic *de novo* methylation in transgenic enhancer or promoter regions. Therefore, identifying CRE–CRE interaction-activated ncRNA transcription and *de novo* methylation offers a strategy to prevent transcriptional gene silencing in transgenic plants and to maintain engineered traits.

Ethical statements

The study did not involve human participants or animal subjects, so no ethical approval was required.

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Liu Z, Zhang D; manuscript drafting: Liu Z, Zhang D, Liu H; literature curation: Liu H, Ahmad B. All authors reviewed and approved the final version of the manuscript.

Data availability

Data sharing is not applicable to this article because no datasets were generated or analyzed during the research. All data cited in this paper were reported in previously published articles.

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Conflict of interest

The authors state that there are no conflicts of interest.

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