REVIEW

An emerging role beyond genetics: DNA methylation in horticultural quality shaping

Yanpei Chen,[1] Dong Li,[1] Zhaobo Lang,[2,*] Yanqun Xu,[1,3,4,*] and Zisheng Luo[1,3,*]

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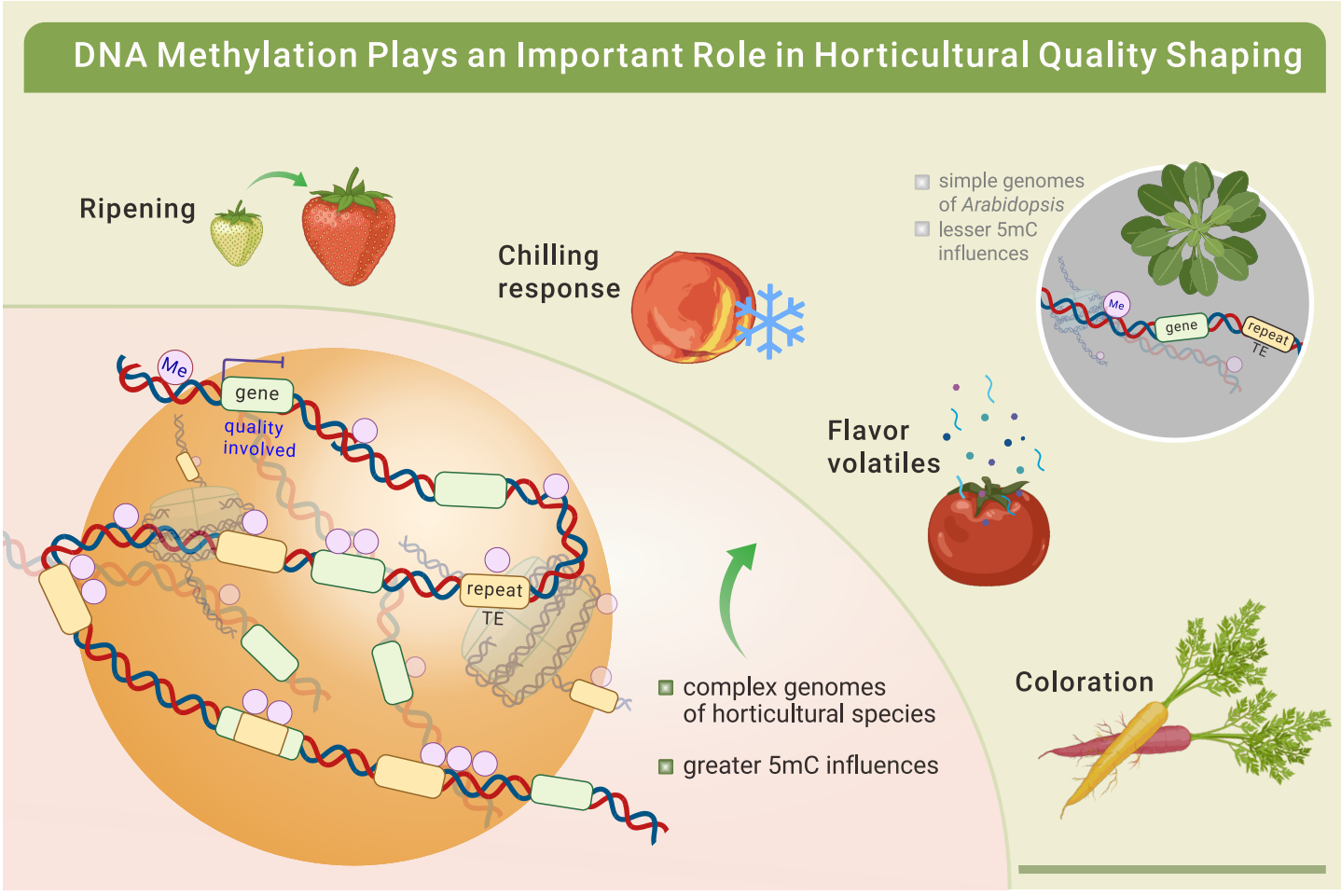
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- DNA methylation of 5-methyl-cytosine is found important for horticultural quality in this starting decade.
- Promoter methylation and spreading of transposable element methylation are important function patterns.
- Ripening, coloration, flavor volatiles, and cold action are highly methylation-affected.
- The locus-specific methylation editing has been preliminarily accomplished and is developing.
- The methylation-editing-mediated horticultural improvement may help address food challenges.

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An emerging role beyond genetics: DNA methylation in horticultural quality shaping

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Horticultural products play an increasingly vital role in addressing the nutritional needs of the world's expanding population, which has surpassed 8 billion. The global trend towards health-oriented diets has motivated consumers to seek high-quality natural horticultural food consumption. This highlights the pressing requirement for updated guidance and strategies for sustainable horticultural quality upgrading. Meanwhile, DNA methylation, an epigenetic modification having transcriptional-regulation potential, is emerging as a crucial quality dominator of horticultural food. In this current investigation, we integrate valuable methylation loci regulating quality traits in fruit and vegetable, elucidating the underlying mechanisms and emphasizing the impressive species-specificity. At this early stage, the most extensively studied aspects of DNA methylation include promoter methylation and transposable elements. Additionally, we delve into locus-specific methylation-editing techniques, whose achievable genetic-modification-free advantages are promising to alleviate consumer concerns regarding genetic-modification products. Overall, this review is devoted to providing insights into the sustainable development of horticultural produce and food quality design strategies in response to global food quality and security challenges.

INTRODUCTION

Compared with animal husbandry and aquaculture, plant-based food production in agriculture offers a more environmentally sustainable approach.[1,2] Nevertheless, our food system's current supply of high-quality plant-based nutrition lags behind population growth, even if the global shift toward healthy diets is a priority step to feed the rapidly growing population.[3] Within this context, horticultural food assumes a significant role as an integral component of modern diets, where consumption of high-quality fruit and vegetable not only enhances physical health[4] but also contributes to overall happiness and life satisfaction,[5] and various aspects of mental well-being.[6]

Horticulture, one of the oldest forms of agriculture, produces a wide range of food including fruit, vegetables, edible flowers, teas, and derivative products. They remain highly popular worldwide today. In contrast to field crops, horticultural food is valued more for quality than yield.[7] Morphology, size, firmness, texture, palatability, color, flavor, aroma, and nutrition properties determine consumer acceptance and commercial value. Formation and variation of these quality properties are intricate processes involving the development, ripening, and senescence of the edible parts, whether pre-harvest or post-harvest.

Most horticultural quality traits result from combinations of one or more phenotypes, which are determined endogenously and genetically.[7–12] Accumulated evidence substantiates the critical roles of transcriptional factors (TFs), a protein type that regulates gene transcription by DNA binding.[9,10,13] Representatively, the R2R3-MYB TF family participates in multiple secondary metabolisms to control pigmentation, flavor, aroma, and texture. MYB-related control leads to pathway regulation providing sources of novelty in fruits and vegetables, including the consumer-liking traits shaping such as the roughness of peach skins (*Prunus persica*), cuticle touch of tomato skins (*Solanum lycopersicum*), and lignification in loquat (*Eriobotrya japonica*).[8] Additionally, phytohormones are another crucial type of non-standalone contributor to quality. These include common ones like ethylene, abscisic acid, auxin, gibberellin, and cytokinin, as well as emerging ones like brassinosteroid, methyl jasmonate, salicylic acid, and melatonin. They tend to modulate global ripening and comprehensive or specific trait formation through various signaling pathways and interacting machineries.[11,12,14] The importance of microRNA (miRNA) has also been revealed in horticultural quality.[7,15] MiRNA is a 21-nt small RNA type that regulates gene expression post-transcriptionally, primarily through mRNA cleavage or translation inhibition. Significant quality involvement of miRNAs has been reported in 17 horticultural species, including the apple (*Malus domestica*), grape (*Vitis vinifera*), kiwifruit (*Actinidia chinensis*), melon (*Cucumis melo*), and pear (*Pyrus bretschneideri*).[7]

On the other hand, DNA methylation (shortened as methylation hereafter), specifically 5-methyl-cytosine (5mC) (Figure 1), is an emerging epigenetic determinant of horticultural quality. The first report of 5mC from wheat germ early in 1950 marked the beginning of the plant methylation era,[16] where the horticultural food research initiated its place within this decade. Plant 5mC occurs in three contexts, mCG, mCHG, and mCHH (H stands for A, T, or C).

The methyl group of the donor, *S*-adenosyl-methionine (SAM), can undergo addition to cytosine through *de novo* establishment and maintenance pathways. *De novo* methylation is mediated by a machinery of RNA-directed DNA methylation (RdDM). During methylation, interfering RNAs (siRNAs) loaded by Argonaute 4/6 (AGO4/6) determine the target specificity of Domains Rearranged Methylase 2 (DRM2) for methyl transfer (Figure 2A).[17] Methylation maintenance mainly contributes to recovering 5mC information in newly formed DNA strands during duplication, or it will be lost, leading to passive demethylation (Figure 2B). The maintenance pathway is also important since cell division is universal during the growth of horticultural edible organs. Methyltransferase 1 (MET1) and Chromomethylase 3 (CMT3) (partially CMT2) are responsible for mCG and mCHG maintenance, respectively. DRM2 (partially CMT2) is responsible for mCHH maintenance (Figure 2C) and DRM2-mediated maintenance is still via RdDM.[18] Moreover, methylation can be actively removed by DNA glycosylase/lyases, including Repressor of Silencing 1 (ROS1), Demeter (DME), and Demeter-like Proteins (DML) (Figure 2D).[19] Plant 5mC can be recognized and directly erased followed by the base excision repair pathway to reinstall a normal C.[19] A related milestone is the vital role of DML2 and demethylation during tomato fruit ripening and quality shaping.[20,21] Overall, these canonical pathways coordinately shape the landscape of methylation genome-wide or at specific regions/sites, regulating gene expression ubiquitously. The regulation balance with updating mechanisms is increasingly reported.[22–35] Upon endogenous or exogenous triggers, the methylation dynamics are sophisticatedly modulated via the pathways above, forming the differentially methylated regions (DMRs) to participate in horticultural quality shaping with multiple function patterns (Figure 3).

Here we integrate progress during this initial decade, intending to determine how methylation shapes ripening/senescence-related or specific quality traits. Commencing with an exploration of biological function patterns of methylation, this review introduces current research workflows of methylation in fruit and vegetable. Abundant valuable methylation loci and mechanisms are described to stress the crucial role of methylation in horticultural quality shaping. Furthermore, the next-generation epigenetic engineering, presented with a forward-looking perspective and thorough discussion, is also incorporated.

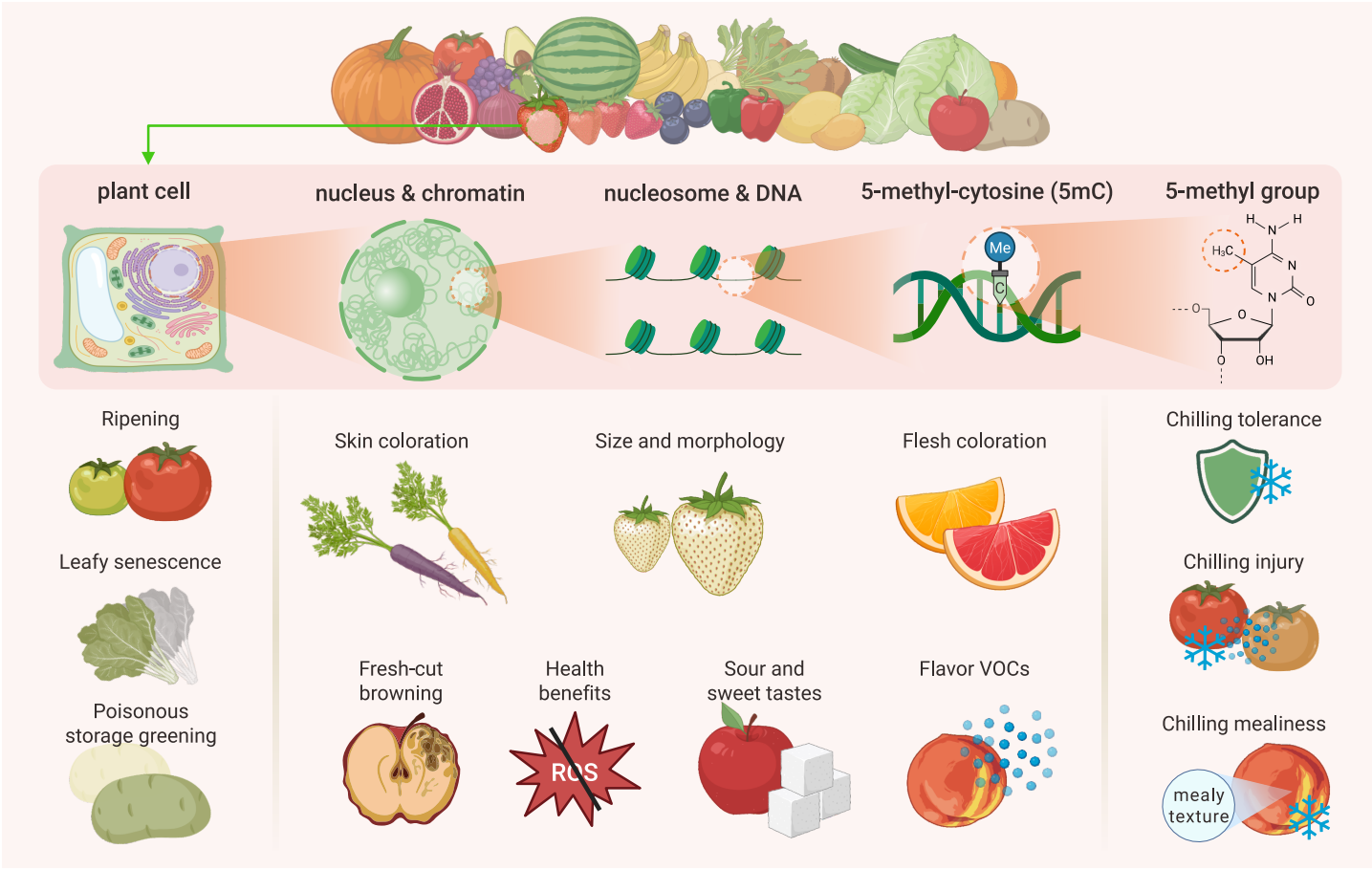

Figure 1. The visualization of 5-methyl-cytosine (5mC) location and its roles in multifaceted specific quality traits of horticultural food.

HOW DOES DNA METHYLATION ACT IN HORTICULTURAL QUALITY SHAPING?

Methylation can regulate the expression of quality-related genes when it occurs in the promoter, gene body, or downstream flanking region. Among different types of DNA methylation associated with genes, the negative impact of promoter methylation on gene expression is the initial and most widely reported principle that determines the downstream transcripts, proteins, metabolites, and related phenotypes (Figure 3A).[36,37] It can also promote expression, as in the case of hundreds of genes that inhibit tomato fruit ripening with disturbed quality formation.[19,21] The *ROS1* gene for active demethylation in *Arabidopsis* is another typical instance.[38,39] Promoter-methylation may directly inhibit transcription by alleviating transcription activator binding or promoting transcription repressor binding, which involves chromatin structure changes, histone interaction, and genome stability alteration. Recent *in vitro* assays have provided evidence that promoter-methylation massively reduces the *in vitro* binding activity to given TFs, regardless of methylation contexts.[40–42] Methylating a single cytosine in a functional core W-box motif repels AtWRKY40's binding *in vitro*. This cytosine was found to interact with the conserved tyrosine of WRKY-DNA binding domains via van der Waals interactions.[43] When a 5-methyl group is present on this specific cytosine, steric hindrance can be predicted, presumably inhibiting the tight binding of WRKY-DNA interacting domains.[43] But actually, there remains a gap in understanding promoter methylation's detailed transcriptional regulation,[18] as well as gene body methylation's action on gene expression.[44]

Gene body methylation demonstrates a limited association with gene expression and phenotype in contrast to promoter methylation.[44] This is also shared by gene downstream methylation, with scarce related research. Within gene body methylation, exons and introns (the first and the other) were found subjected to different methylation patterns against various contexts in different species, following various patterns against gene expressions.[45–50] For example, the exons are methylated mainly in the CG context in *Arabidopsis*[49] whereas in moso bamboo (*Phyllostachys edulis*), mCGs are highly enriched in introns rather than exons, with clear exon-intron boundaries. mCHGs and mCHHs in intron or exon regions decreased with increasing gene expressions in moso bamboo.[50] This significant species-specificity, as well as other multifaceted species- or tissues-specificity of methylation patterns,[51] highlights the importance of investigating a wide range of crops to explore systematic methylation mechanisms. Additionally, the evolution of gene body methylation with its selective pressures and whether it has the phenotype-affecting potential, are hotly debated topics. Aside from limited effects on gene expression, potential functions of gene body methylation include stabilizing gene expression, repressing aberrant transcription, decreasing aberrant intron retention, and resisting transposable element (TE) insertions.[44]

The spread of methylation from neighboring repeats, especially TEs, largely leads to gene-associated methylation.[18] Methylation effects on gene expression vary depending on the genome size. In *Arabidopsis*, genes with methylated promoters constitute only approximately 5 %.[52] In this case, methylation's role is limited, which is consistent with the finding that most methylation-related mutants escape from heavily impaired growth or a lethal phenotype.[17,26] Even an *Arabidopsis* mutant generated with a methylation-free genome can survive despite many development defects.[26] In comparison, more repeats and TEs neighboring the genes lead to far more methylated promoters in larger-genome species, including most horticultural crops.[19,21] As a result, methylation in horticultural species plays an increasingly important role in key gene regulation, and more quality involvements are naturally expected.

The most prominent function of DNA methylation is to silence TEs and other repeats, determining cell fate associated with quality shaping (Figure 3B). Methylation is highly enriched in the body and flanking sequences of TEs. TE is a type of unstable element translocating within a genome; however, this translocation is inhibited by TE methylation; otherwise, TE can destabilize the genome by the manner of "cut-paste", or "copy-paste"

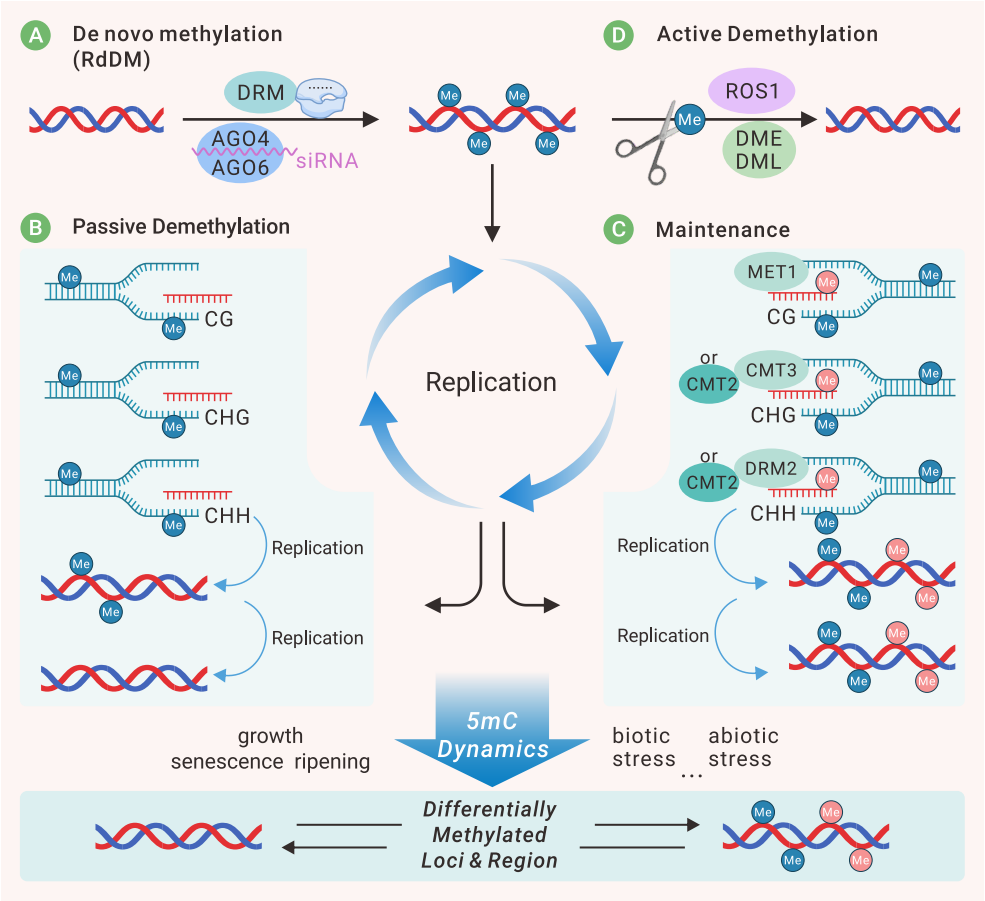

Figure 2. The brief scheme for dynamics of DNA methylation The RNA-directed DNA methylation (RdDM) with small interfering RNA (siRNA), Argonaute 4/6, (AGO4/AGO6), and Domains Rearranged Methyltransferase (DRM) (A).[17] The passive demethylation (B). The methylation maintenance with Methyltransferase 1 (MET1), Chromomethylase 2 and 3 (CMT2 and CMT3), and DRM (B).[18] The active demethylation with Repressor of Silencing 1 (ROS1), Demeter (DME), and Demeter-like (DML) (D).[19] The pathways in red are most focused on considering methylation dynamics research.

(retrotransposon, a TE subspecies that reverse transcribes for relocation). A recent study found that methylation has a strong dose-dependency on the repression of both gene and TE expressions regarding the major mCG effects in *Arabidopsis*.[26] Active genes and inactive TEs can be interspersed, leading to downstream alterations.[53] For example, other than TE methylation's repression effect, *MYB1*'s promoter-located TE surprisingly activates transcription involving the color decision in apple skins.[54] Evolutionarily, TE bursts in tea (*Camellia sinensis*) accompany genome expansion affecting theanine, caffeine, and flavonoid metabolism genes.[55] Additionally, methylation functions in RNA processing (mostly gene body methylation) and histone interactions are also found related to quality shaping, although corresponding clues are limited (Figures 3C-D).[18,56–59]

BUILDING RESEARCH PATTERNS OF DNA METHYLATION IN HORTICULTURAL FOOD

Setting methylation-related changes in research materials is a key step to start an investigation. However, within horticultural crops, access to stable transformation materials is limited. There are only a few representative horticultural species whose methylation-related research models have been established through RNAi- or CRISPR-based mutant generation, such as tomato,[21,60,61] wild strawberry (*Fragaria vesca*),[62] melon,[63] brassica (*Brassica rapa*),[64] and cotton (*Gossypium hirsutum*).[65] Additionally, the ethyl-methanesulfonate (EMS)-induced mutants,[62] somatic mutant varieties,[66] and natural mutants[67] are also acceptable, although trait acquisition is uncontrollable. Considering methylation-related changes, transient transformation is more applicable to non-model horticultural crops due to their specific characteristics and fewer technical barriers. For instance, we previously conducted the transient silencing of *AGO4*, a component in the RdDM machinery. This led to accelerated strawberry ripening (*Fragaria* × *ananassa*) and modulated quality formation.[68] These mature genetic manipulations alter specific methylation-related targets, most of which are components of pathways for methylation dynamic building or closely related, to investigate the downstream changes in horticultural quality shaping.

When it comes to setting non-selective methylation changes, the methylation regulator is commonly used in horticulture to reprogram the genome methylation status. Effective demethylators are used in a growing body of methylation-related studies, particularly the 5-azacytidine (5-azaC). It was approved for curing myelodysplastic syndrome and acute myelogenous leukemia decades ago for its significant demethylation effects via limiting DNA methyltransferase.[69,70] Indeed, by inducing hypomethylation and associated changes with influences on quality traits, the 5-azaC has been shown useful across an array of horticultural species, including the onion (*Allium cepa*),[71] tuber-bearing wild potato (*Solanum* section *Petota*),[72] spinach (*Spinacia oleracea*),[73] pumpkin (*Cucurbita moschata*),[74] tomato,[75] grape,[76] strawberry,[77] and watermelon (*Citrullus lanatus*).[78] Interestingly, Kim et al. (2016) reported that ammonium can be another methylation blocker. Their findings showed that it inhibited CMT3-mediated mCHG of target genes without affecting CMT3 expressions.[79] But the underlying mechanism remains unclear. In contrast, few horticultural cases involve methylation enhancers like methyl trifluoromethane-sulfonate (MTFMS) and SAM, which are always secondary choices after 5-azaC as a supplementary for methylation assay.[80,81]

Another focus revolves around studying horticultural material without preset methylation changes. These materials are investigated under a variety of biological conditions such as different varieties, different development, ripening, and senescence stages,[51,82] abiotic or biotic stress.[83–85] Here the objective is to identify resultant endogenous alterations of locus-specific or genome-wide methylation status, find the changed downstream traits, and explore the behind mechanisms. In this case, selecting the appropriate detection method is crucial because the methylation variation to determine may be slighter than materials with pre-set methylation changes. Abundant methods with different throughputs and precisions are available to meet the demands of epigenetic research and published reviews of related progress can provide further guidance.[86–88] Collectively, methylation-altered materials are generally studied to explore the differences caused by preset methylation-related changes considering resultant methylation patterns and downstream quality traits. On the other hand, non-methylation-altered materials are investigated more closely to better understand the effects of biological properties or biological processes on their methylation status and downstream quality traits (Figure 4).

DNA METHYLATION SHAPES OVERALL QUALITY ASPECTS

Ripening and associated quality performance

The fruit, as a specialized organ, is a distinctive feature of angiosperms, with ideal edibleness for humans. In contrast to dry fruit such as *Arabidopsis*, fleshy fruit needs certain development and ripening processes for final quality formation upon harvest. They are distinguished by a variety of cellular, metabolic, and molecular alterations, such as cell growth that guarantees fruit expansion, softening, and build-up of pigments, soluble sugars, and aromatic volatiles.[51] Further, fleshy fruit can be classified as climacteric (with bursts of ethylene production and respiration rate as ripening onset) and non-

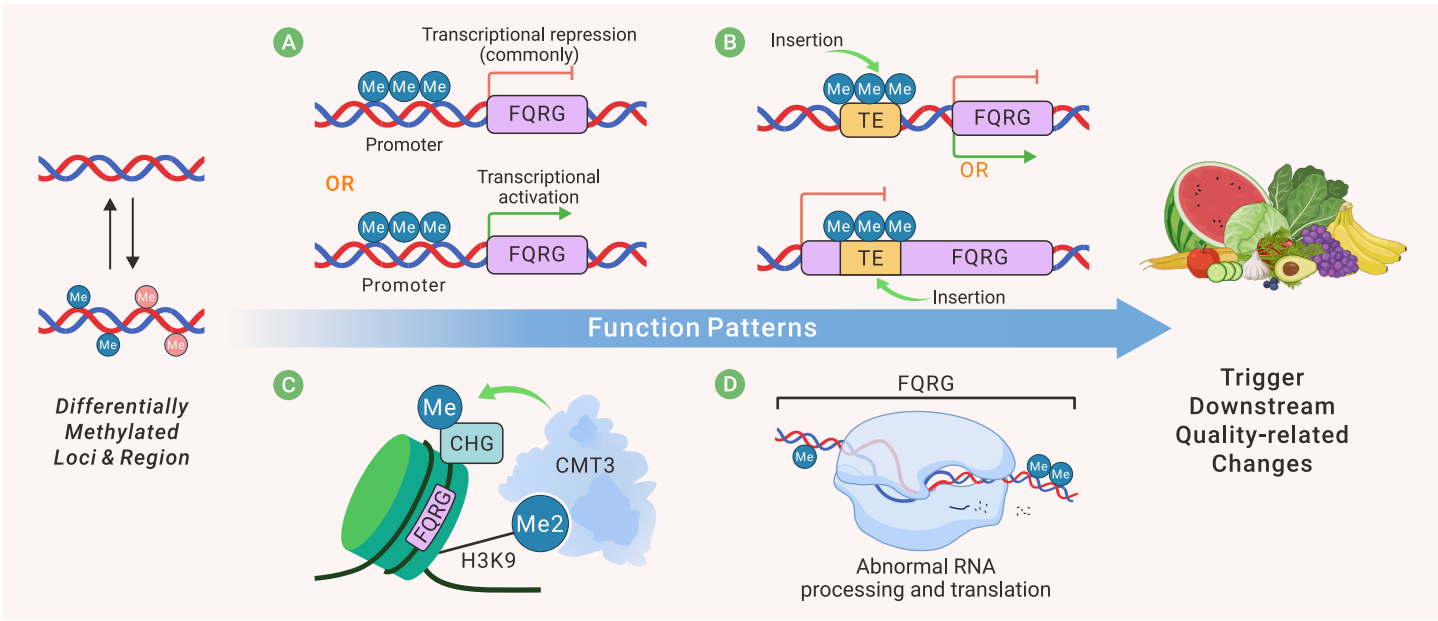

Figure 3. Potential function patterns of DNA methylation in the horticultural food quality formation and variation Methylated promoters regulate the expression of food-quality-related genes (FQRGs) (A). Transposon elements (TEs) insertion affects the FQRG expression (B). Methylation and histone modification's interaction acts on the regulation of the FQRG expression, taking CMT3 and histone 3 lysine 9 dimethylation (H3K9me2) to methylate mCHG as an instance (C). Methylation induces abnormal RNA processing and translation of FQRGs (D). Differentially methylated Loci (DMLs) and differentially methylated regions (DMRs). The patterns in red are most focused on considering methylation function studies.

climacteric (without such burst).[89] Initial attention was directed toward climacteric tomato fruit. Its ripening was linked to methylation as early as 1991, with evidence showing a tissue-specific pattern of tomato methylation as well as a reduction of methylation level in highly-expressed fruit genes coinciding with ripening onset.[90] Further clues of the methylation role in fruit ripening and quality formation emerged from the naturally occurring mutant named *Colorless non-ripening* (*Cnr*). Its fruit remains hard and yellow while normal fruit softens and turns red. The observed difference was attributed to promoter DMRs of an SBP-box TF in the CNR-contained genomic interval.[67] At the epigenome-wide level, the methylation landscape of tomato ripening was subsequently profiled with the rising era of whole genome bisulfite sequencing (WGBS) reaching a single-base resolution.[75,91] It was the first attempt to decipher the methylome of the sophisticatedly-regulated fruit ripening process. Moreover, 5-azaC-induced ripening acceleration supported the close connection between methylation and ripening with quality formation (Figure 5A).[75] DML2-mediated active demethylation contributes to the global methylation loss, as well as the activation of ripening-induced genes and the inhibition of ripening-repressed genes.[20,21] The *dml2* mutant exhibited an impressive ripening delay, indicating the key role of tomato DML2. Even if methylation alterations during ripening are not rare, such a tight methylation-ripening connection in tomato fruit is unprecedented. Tomato DML2 is also controlled by RNA N^6-methyladenosine (m^6A) modification. The m^6A demethylation post-transcriptionally regulates the stability and translatability of *DML2* transcripts through RNA demethylase binding, which therefore, reprograms the methylome and regulates the ripening and quality formation.[92]

In addition to tomato's typical climacteric ripening, we have previously profiled the methylome of strawberry fruits (*Fragaria × ananassa*) for insights into non-climacteric ripening. Strawberry fruit also undergoes global loss of genomic methylation during fruit ripening[68] (Figure 5B). Unlike tomato, this hypomethylation is caused by attenuated RdDM. Silencing *AGO4* in strawberry led to early ripening. Further investigation revealed that hypomethylation is important for ripening and involved in the abscisic acid (ABA) biosynthesis and signaling for key ripening regulation.[68] ABA generation and signaling control the ripening of strawberry and other non-climacteric fleshy fruit.[93–95] In contrast, non-climacteric sweet oranges (*Citrus sinensis*) show hypermethylation during ripening, especially mCHH, due to a weakened active demethylation[96] (Figure 5C). This stresses the ripening-dependent species-specificity regarding methylation pattern. In grape berries, the 5-azaC-induced hypomethylation accelerated ripening accompanying bigger diameter, higher firmness, more sucrose and soluble solids, as well as more anthocyanins for coloration.[97] 5-azaC can also influence the grape aroma by disrupting pre-mRNA alternative splicing due to methylation imbalance, impairing the translation process.[57] The methylation dynamics with quality alteration during ripening in papaya (*Carica papaya*)[98] and blueberry (*Vaccinium corymbosum*)[99] are also included in Figures 5E-F. Taken together, to date, our current evidence fails to reveal a conserved pattern that methylation acts in climacteric or non-climacteric ripening, excluding a few cases involving TE-related quality regulation across close varieties or cultivars.[54,100,101]

During the ripening of sweet pepper (*Capsicum annuum*), botanically a fruit whereas horticulturally a vegetable, hypomethylated promoters are observed in ripening-related genes. The typical ripening TF, *RIN*, and the rate-limiting enzyme (9-*cis*-epoxycarotenoid Dioxygenase 1, NCED1) generating ABA, are included. This may be due to the induction of *DML2-like* and repression of *MET1-like1*, *MET1-like2*, *CMT2-like*, and *CMT4-like*, affecting quality formation (Figure 5D).[102] ABA's role is highlighted based on the non-climacteric ripening of pepper. Further, transient silencing of *MET1-like1* in pepper fruit led to hypomethylation and accelerated ripening associated with enhanced levels of soluble solids content, phytoene, β-carotene, zeaxanthin, antheraxanthin, capsanthin, capsorubin, and α-carotene. These were accompanied by expression changes of genes involved in cell wall degradation, capsanthin/capsorubin biosynthesis, and phytohormone metabolism and signaling. The down-regulation of genes encoding NCED1 was also noted,[102] which suggested the vital methylation role in pepper ripening and quality formation. Besides, clues of leaf ripening and senescence were also provided: methylation-related *Arabidopsis* mutants (*ros1* and triple *drm1/2 cmt3*) demonstrated altered leaf-to-seed ratios and accelerated leaf senescence.[41] This finding is expected to inspire further exploration of methylation's involvement in leafy vegetables where leaf senescence significantly affects quality.

Postharvest senescence and associated quality performance

Even though quality traits have been preliminarily shaped at harvest, postharvest duration is still crucial to quality variation until consumption. Postharvest senescence frequently results in undesired alterations such as leaf yellowing and browning. In pak choi (*Brassica rapa*), it was found that genes encoding methyltransferases, such as *MET1* and *DRM2*, were down-

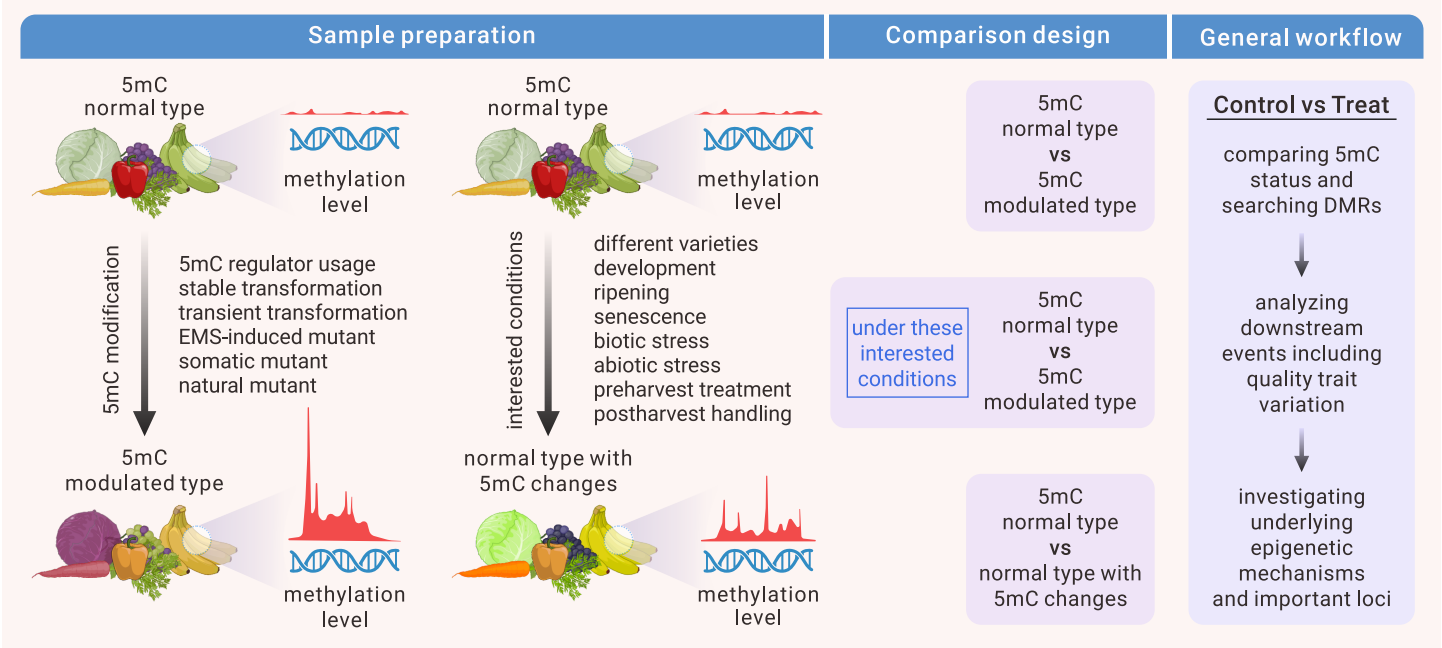

Figure 4. Current research patterns of 5mC DNA methylation in horticultural species The red peaks simulate the methylation level in a certain genome region. The "DMRs" indicates differentially methylated regions. The second comparison design indicates the goal of investigating the role of pre-modulated 5mC during a certain process or under interested conditions.

regulated during storage along with methylation changes.[103] The 5-azaC-induced hypomethylation accelerated the senescence and yellowing of pak choi edible leaves by (1) degrading chlorophyll and derivatives, (2) activating Mg-dechelatase (MDCase), Pheophytinase (PPH) and Pheophorbide-a Oxygenase (PAO), (3) inducing the senescence-related gene expressions with hypomethylated promoters (Figure S1a).

Similarly, for fresh-cut apples, senescence with rapid browning is an unavoidable issue that deteriorates the quality (Figure S1b). The browning index (BI) was found positively correlated to global methylation level as well as *CMT3* expressions involving methylation maintenance, but negatively correlated to *ROS1* and *DME* expressions involving demethylation.[104] This was based on the comparison between two "Fuji" apple somatic varieties with different browning performances. Further, the 5-azaC-induced demethylation can decelerate browning. The hypermethylated promoter of *No Catalase Activity 1* (*NCA1*) reduced its expression and reduced catalase (CAT) activity, causing impaired antioxidant capacity and enhanced browning. NCA1 serves as a molecular chaperone for CAT activation,[105] bridging the methylation processes and the development of browning in fresh-cut apples.

During storage of potato tuber (*Solanum tuberosum*), surface greening is usually seen in commercial or domestic practices, damaging quality and safety. This greening is due to light irradiation with the toxic alkaloid solanine forming, which mainly involves the transition of starch-storing amyloplasts into photo-synthetically active chloroplasts and chlorophyll synthesis.[106] Matching the light-induced demethylation in maize,[107] findings in potato tuber indicated a light-induced larger drop in methylation during storage versus the control that held a minor drop.[108] A negative correlation was also built between greening degree and methylation level. Light also demethylated the CpG island (regions rich in mCG) in *Chlorophyllide-a Oxygenase 1* (*CAO1*) promoter. This uncovered the direct role of methylation in light response and quality control, as the CAO1 exerts critical effects on chlorophyll metabolism and greening. Overall, the postharvest evidence provided an epigenetic perspective on the mechanism of horticultural quality, laying a basis for future quality maintenance efforts.

DNA METHYLATION GOVERNS SPECIFIC QUALITY TRAITS

Coloration with health-benefiting components

In the modern berry market, the "Manicure Finger" grape is popular for its unique appearance. When ripe, its berry is red-purple on top and yellow-green on the bottom. However, the underlying mechanism remains unknown until recent evidence attributed this trait to methylation (Figure S1c).[109] The differentially accumulated anthocyanins at the two ends of the berry were found as the direct cause. The related anthocyanin biosynthesis enzyme, UDP-glycose Flavonoid Glycosyltransferase (UFGT), and a TF, MYBA1 were focused on due to their expression variations at two ends. MYBA1 binds to the *UFGT* promoter to enhance anthocyanin production, however, MYBA1 coding sequences are highly similar to those of normal grape cultivars, which fails to interpret the expression variations. In terms of epigenetic analysis, findings demonstrated that the bottom skin had significantly higher methylation levels than the top skin of the *MYBA1* promoter. This corresponded to the top skin's stronger active demethylation as well as weaker RdDM and maintenance. Briefly, the DMRs in *MYBA1* promoters found in the berry's two ends led to the expression difference and ultimately shape the anthocyanin-based distinct trait.[109] Another report discovered that a retrotransposon in *MYBA1* promoter was identified in the color-skinned and color-fleshed grape variety. This TE's hypomethylation could activate *MYBA1* expression and enhance anthocyanin accumulation to pigment berries, distinguishing them from the white uncolored variety.[110]

Regarding the coloration of apple skin, early studies have revealed that the methylated promoters of *MYB1* and *MYB10* are decisive for anthocyanin biosynthesis and color patterns.[111–113] Further evidence specified that *MYB10*' s promoter-located mCHG functions well in expression regulation to pigment the apple skin.[114] mCG hypermethylation on the promoter of an anthocyanin-related TF, *Helix–loop–helix 74* (*bHLH74*), also ultimately reduces apple anthocyanin accumulation. Additionally, the mCG- and mCHG-hypomethylated promoters of several genes governing flavonoid and anthocyanin biosynthesis are also significant in coloration.[114]

However, even within intraspecies comparison within the apple, the methylation effects on anthocyanin-related gene expression may vary. Among red- and lighter-skinned apple somatic mutants, irregular correlation patterns were found between promoter-methylation and expression of genes regarding anthocyanin biosynthesis.[115] *Anthocyanidin Synthase* (*ANS*) expression was higher with a hypomethylated promoter, but *Flavanone-3-hydroxylase* (*F3H*) expression was higher with a hypermethylated promoter, and the expression of a potential anthocyanin-related TF *MYB114* positively correlated to gene body methylation. To interpret, there is a belief that altered methylation of structural genes may conditionally or synergistically determine expressions with other mechanisms such as TF binding.[115] As stated previously, *in vitro* evidence demonstrated that promoter methylation can

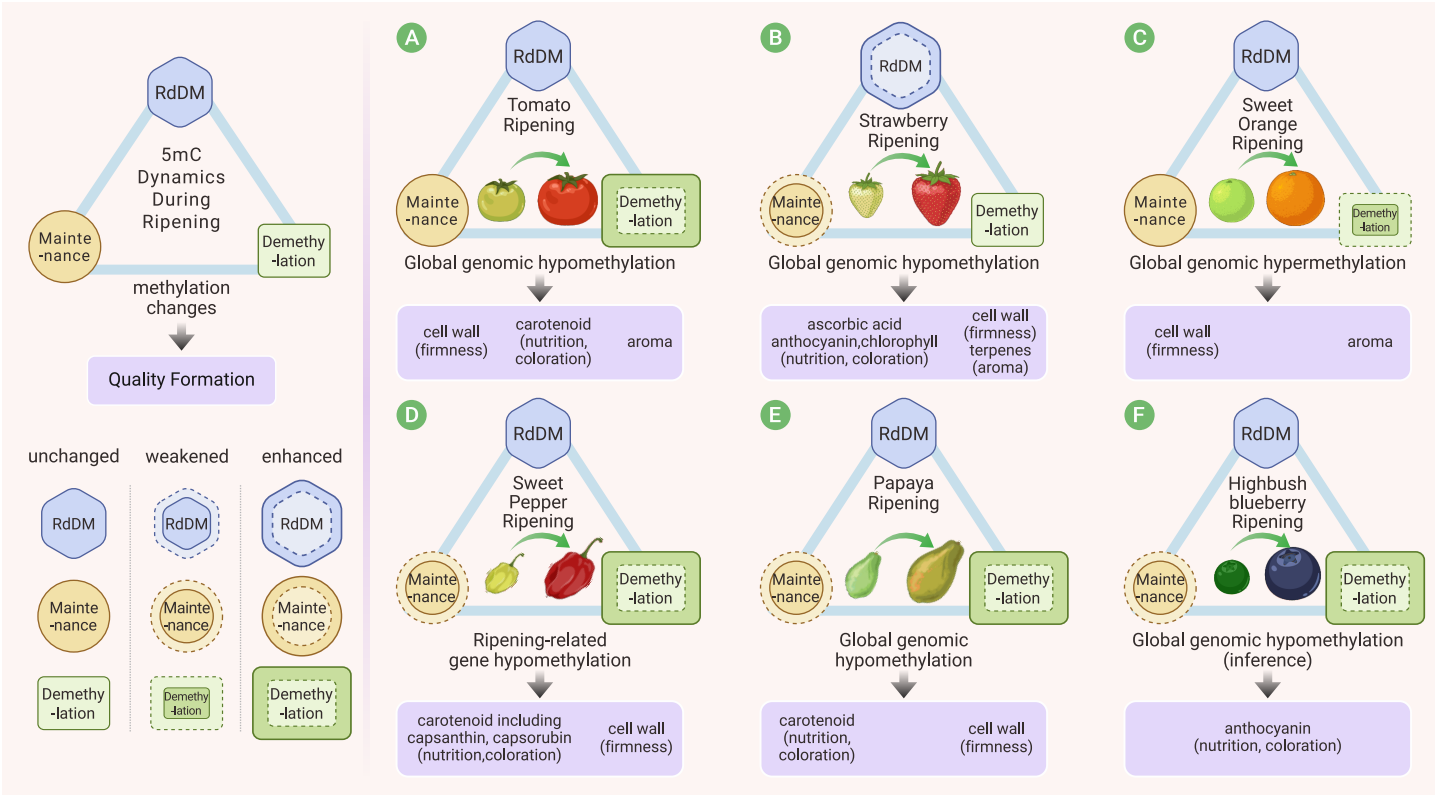

Figure 5. The 5mC methylation roles in the ripening process and related overall quality aspects The RdDM, maintenance, and demethylation pathways synergistically shape the methylation status, which affects quality formation. The enhanced demethylation during tomato ripening with influences on firmness, nutrition, coloration, and aroma (A).[21,75] The weakened methylation pathways during strawberry ripening with influences on nutrition, coloration, and aroma (B).[68] The weakened demethylation during orange ripening with influences on firmness and aroma (C).[98] The potentially enhanced demethylation and weakened maintenance during sweet pepper ripening with influences on soluble solids, nutrition, coloration, and firmness (D).[102] The enhanced demethylation during papaya fruit ripening with influences on firmness, nutrition, and coloration (E).[98] The potentially enhanced demethylation and weakened maintenance during highbush blueberry ripening with influences on coloration (F).[99]

inhibit TF binding.[40–42] However, further investigations are warranted to ascertain whether this inhibition applies universally to all structural genes, especially considering studies in peach fruit showing effective expression regulation by promoter methylation of structural genes.[116] In *Arabidopsis*, it was discovered that 75% of TFs are sensitive to methylation, implying that methylation inhibits their *in vivo* DNA binding potential.[117] Further exploration is also needed for the case of methylation-sensitive TF binding across different species. Actually, three gene clusters exist naturally considering the positive/negative/non-correlation between promoter methylation and gene expression, but their quantity and proportion vary across species or tissues.[96] These gene clusters' origins, evolution, and shared characteristics are currently unknown. Thus, it remains an ongoing challenge to identify and understand horticulturally significant genes of tight promoter-methylation-expression correlations.

How methylated *MYB1* regulates anthocyanin accumulation was also investigated.[118] Briefly, to transcriptionally control its expression, the *MYB1* promoter is mCHH-methylated through the binding of AGO4s with the methyltransfer of recruited DRM2 by RdDM to modulate the anthocyanin accumulation. Additionally, the latest high-quality genome assembly has linked an *MYB1*-neighboring TE to the red color of apple skin.[54] This unique inserted retrotransposon (named "redTE") upstream of *MYB1* in the red-skinned apple genome surprisingly enhances *MYB1* expression, whereas the counterpart of shorter TE fragments in the yellow-skinned apple genome fails (Figure S1d). In the examination of 12 well-known apple varieties, redTE is present in all 6 red-skinned apples but absent in all 6 yellow-skinned apples, revealing a seldom-seen shared methylation pattern.

Blood flesh color, a distinguishing quality trait of blood orange (*Citrus sinensis* Osbeck) based on anthocyanin presence, intensifies during cold storage at 4°C.[83] Within the promoters of anthocyanin-related *Dihydroflavonol 4-reductase* (*DFR*) and a core TF *Ruby*, methylation levels decreased in the colored-flesh areas but increased in the uncolored-flesh areas of the same single fruit. This indicated a strong block by methylation on gene expressions. Here the methylation alterations were found attributed to *DML1* changes with modulated demethylation.[83] Another report showed that there was a full or solo Copia-like retrotransposon upstream of *Ruby*, involving the temperature-dependent coloration of both skin and pulp during cold storage. The *Ruby* locus mapping in 9 *Citrus* varieties showed its presence or absence determined the blood-red red or normal-orange appearance of the orange fruit (Figure S1e).[101]

Research of red-fleshed radishes (*Raphanus sativus*) revealed that a hypermethylated "CACTA" TE in *MYB1*'s upstream region spread methylation to the promoter, suppressing *MYB1* expression and anthocyanin accumulation. This led to a white-fleshed mutant (Figure S2a).[119] Similarly, in a unique purple cauliflower (*Brassica oleracea* var. botrytis), the insertion of a Harbinger TE into the *Purple* (*Pr*) promoter caused color misregulation. This *Pr* gene encodes an MYB TF that activates a bHLH TF and a subset of the anthocyanin pathway.[120] These cases exemplify instances where TE-induced methylation disrupts secondary metabolism with influences on quality attributes, suggesting the importance of TE location.

Methylation has also been linked to cucumber fruit spine color, which significantly determines the quality and commercial value.[121] Black spines contain mostly flavonoids, such as flavonols and proanthocyanidins, whose biosynthesis is regulated by TF MYB60. Apart from the aberrant intron influencing expression, the *MYB60* promoter-methylation level was found much lower in black than in white spines (Figure S2b). Conclusively, methylation largely regulates flavonoids and anthocyanin accumulation, affecting color quality. On the other hand, natural pigments, such as anthocyanins, carotenoids, and chlorophyll, typically contribute nutritional value by providing antioxidant effects and other health advantages.[122–125]

In pepper fruit, the silenced *MET1-like1* with impaired methylation maintenance can degrade carotenoids, including pepper-unique capsanthins/capsorubins.[102] Carotenoids are important phytochemicals from horticultural supplements and provide extensive health benefits,[126,127] such as antitumor[128] and anti-cardiovascular effects of capsanthin/capsorubin.[129] In Pink

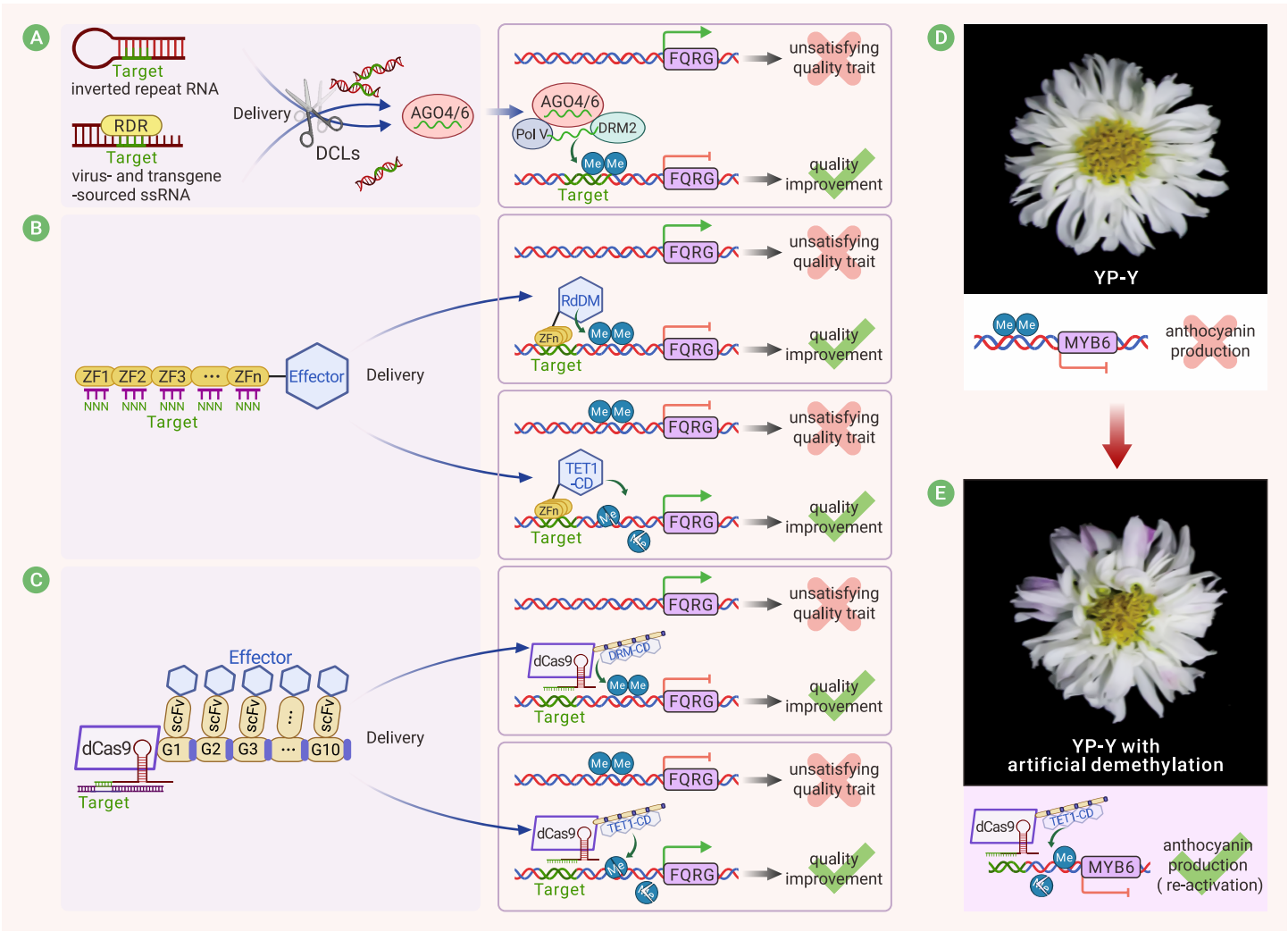

Figure 6. Mainstream systems available for locus-specific manipulation of DNA methylation/demethylation targeted to FQRGs for horticultural quality design and a case of color control in *Chrysanthemum morifolium* flowers Double-stranded RNAs (dsRNAs) from various origins have been used to produce target-specific siRNAs. The target sequence is present in each of these RNAs (represented by green lines). These dsRNAs are cut by Dicer-likes (DCLs) into siRNAs, which can be incorporated into AGO4/AGO6 and target RNA polymerase V (POL V)-dependent and DRM2-dependent methylation or transcriptional gene silencing (TGS) when directed against regulatory regions (A). A translational fusion between an effector protein and an artificial zinc finger protein (ZF) is designed to bind to a promoter. This ZF contains several different artificial zinc finger domains, each of them binding to a target DNA triplet (N=A/T/C/G). As effectors, various RdDM components or the catalytic domain of ten-eleven translocation 1 (TET1-CD) can be fused to a ZF to target locus-specific methylation and demethylation, respectively (B). A CRISPR SunTag system design. Up to ten single-chain variable fragments (scFv) GCN4 antibody-effector fusion proteins can be recruited by a modified deactivated Cas9 (dCas9) protein with a C-terminal tail containing 10 GCN4 repeats (G1 to G10 yellow blocks) separated by linker regions (purple sticks). This machinery will be directed to the target locus by dCas9-bound guide RNA (gRNA), which is shown as a purple hairpin with a green line pairing with the target sequence. CRISPR SunTag systems can be used together with the catalytic domains of tobacco DRM (DRM-CD) and TET1-CD to manipulate DNA methylation in plants for horticultural quality improvement (C).[146] The appearance of partial-pale YP-Y flowers and its *MYB6*-related methylation pattern identified (D).[100] The Suntag system was used with TET1-CD (1300-3×sgRNA-CRISPRdCas9-TET1CD vector) to target the *MYB6* promoter through transient agrobacterium infection. This artificial manipulation successfully induced part of the methylation loss of the *MYB6* promoter with the re-activation of anthocyanin biosynthesis (E).[100]

Marsh grapefruit (*Citrus paradisi* Macf.), 5-azaC-induced hypomethylation resulted in carotenoid degradation and transcriptional activation of *Carotenoid Cleavage Cioxygenases 1* (*CCD1*), which catalyzed zeaxanthin, β-carotene, and lycopene.[130] Further, promoter-methylation changes of *Tomato Agamous-like 1* (*TAGL1*) are tightly linked to tomato carotenoid and chlorophyll accumulation and strip-colored skin (Figure S2c).[131] The tomato fruit from a demethylation-impaired *dml2* mutant has fewer carotenoids with substantial hypermethylated-promoters regarding carotenoid biosynthesis. Considering this fruit, aside from coloration, metabolic activities involving organic acids, carbohydrates, flavonoids, uronic acids, and glucuronates are impacted, which jointly determine taste and health benefits.[21]

Morphology and taste

Size and morphology are also critical visible quality traits that are modulated by cell division and cell expansion. According to the results of a comparison between two apple varieties with almost identical genomes, methylation may regulate fruit size formation.[132] These two varieties' fruits were highly distinct. The bigger fruit is 5 cm in diameter while the smaller one is only 2 cm. Histology analysis revealed that reduced cell division led to a smaller size. Promoter-DMRs were identified in genes regarding cell division, including *Squamosa Promoter Binding Protein-like 13* (*SPL13*). It has an ortholog in *Arabidopsis* modulated by miR156 that closely interacts with miR172, whose overexpression lowers the apple size.[133] This implies that methylation may regulate fruit size via miR156-miR172 interactions, adding a dimension of miRNA considering the methylation action manners. Besides, another DMR-related gene, *1-aminocyclopropane-1-carboxylate Synthase 8* (*ACS8*) functions in the synthesis of ethylene that promotes cell division,[134] which also partially explains the differed apple sizes.

Another example was found in the abnormality known as "mantled" in African oil palm fruit (*Elaeis guineensis*), reducing oil yield and changing fruit shape with many developmental defects.[58] Methylation alterations of a LINE retrotransposon related to rice *Karma* (in the intron of the homeotic gene *DEFICIENS*) were noticed. It is associated with alternative splicing and premature termination. The hypermethylation-nearby *Karma* splice site (termed the *Good Karma* epiallele) predicts a normal fruit set whereas the hypomethylation-nearby site (the *Bad Karma* epiallele) predicts abnormality.[58]

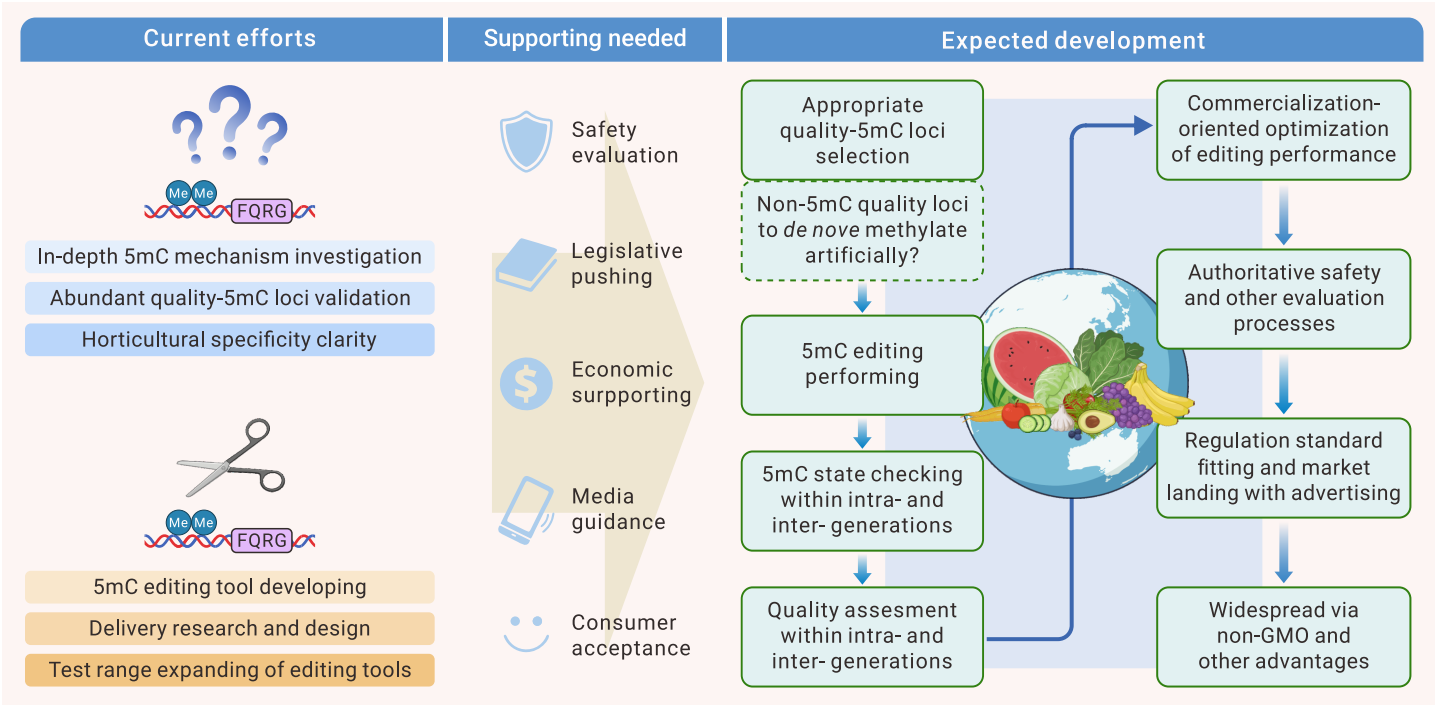

Figure 7. Current efforts, supporting needed, and expected development of next-generation epigenetic modification strategy based on 5mC DNA methylation editing.

Overall, the loss of *Karma* methylation is the key event that contributes to the origin of the oil palm fruit "mantled" abnormality.

Reduced sizes of leaf, flower, and fruit in an EMS-induced *fdm1* mutant of *Factor of DNA Methylation 1* (*FDM1*, an RdDM component) in wild strawberry were recently reported.[62] Notably, the fruit underwent a transformation from a wedge shape to a shorter and subglobular shape (Figure S2d). Subsequent validation using the CRISPR-generated fdm1 mutant confirmed the consistent occurrence of smaller organs. In *fdm1*, global hypomethylation (especially mCHH) was observed and many genes negatively regulating cell division were highly expressed with hypomethylated promoters compared with wild types. Overexpression of *FDM1* can restore methylation at the RdDM target loci. Further, the FDM1-mediated RdDM also interacted with gibberellin and auxin to affect organ sizes, demonstrating the potential crosstalk with phytohormones.

Accumulated evidence underscores the role of methylation in flavor regulation. Citric acid has a strong and pleasing sour flavor. Between a low citric acid pummelo (*Citrus maxima* LCA) and the corresponding high citric acid variety (HCA), the highly-expressed gene *Anthocyanin 1* (*AN1*) in pummelo benefits producing citric acid with a sour flavor. In LCA, hypermethylated *AN1* promoter resulted in downstream low-acid variety, while 5-azaC-induced hypomethylation was able to reaccumulate the citric acid.[135]

Ascorbic acid (AsA), an important antioxidant and nutrient, also partially contributes to tomato flavor. 5-azaC-induced hypomethylation occurred in the promoter of *D-galacturonic Acid Reductase 5* (*GalUR5*, encoding a key enzyme in AsA biosynthesis), associated with its expression and enzyme activity increased. This enhanced AsA accumulation, as seen in tomato fruits from *met1* mutant but not *dml2* mutant, which indicates that *GalUR5* is a target of methylation maintenance and tomato AsA pool keeping requires methylation maintenance.[136]

Apple flavor comes mainly from malic acid. Among certain apple varieties, the lower mCHH level on the *P3A-ATPase (Ma11)* promoter, the higher level of malic acid accumulation.[137] Another apple flavor regulator controlling soluble sugar is the Tonoplast Sugar Transporter (TSTa). Its heterologous overexpression in tomato fruit increased fructose, glucose, sucrose, and soluble sugar content. However, higher mCHH levels on the *TSTa* promoter corresponded to higher expression in the high-sugar apple variety. Here, typical methylation–expression negative correlations were just observed in mCG- and mCHG-DMRs in promoters and gene bodies, as well as mCHH-DMRs in gene bodies. Despite mCG's dominance, mCHH type was found to preliminarily regulate acid and sugar accumulation determining apple flavor.[137] Even if apple flavor control was evidenced to involve methylation, the detailed mechanisms require further investigation.

Volatile organic compounds (VOCs) and cold storage with chilling stress

Methylation-regulating VOC biosynthesis shapes the aroma of horticultural products. Linalool, a monoterpene, is one of the most common aroma-shaping phyto-VOCs. The *Terpene Synthases 3* (*TPS3*), encoding an enzyme that catalyzes the (*S*)-(+)-linalool synthesis, dominates the distinct aroma of ripe peach fruit.[116] According to Wei et al. (2021),[116] *TPS3*'s hypomethylated promoter during peach fruit ripening leads to enhanced expression and resultant linalool accumulation. At a TF level, the *Ethylene Responsive Factor 61* (ERF61) was identified, interacting with *TPS1* and *TPS3* to positively regulate linalool synthesis, and the hypomethylated promoter of *ERF61* was also linked to peach fruit linalool increase.[138]

In tomato fruit, DML2 with demethylation has been recently shown also essential for aroma VOCs.[61] There were substantial declines in fatty-acid- and carotenoid-derived VOCs (accounted for 96% of the total) in fruit from *dml2* and *nor* (the mutant of TF *Non-Ripening*, *NAC-NOR*). DML2 and NAC-NOR shared an array of VOC-related targets. Surprisingly, it was found that DML2 can demethylate the *NAC-NOR* promoter, while in turn, NAC-NOR can regulate *DML2* by promoter-binding. Here a sophisticated regulatory loop was demonstrated between DML2-mediated demethylation and NAC-NOR-mediated transcriptional network during tomato aroma formation.

Cold storage and cold-chain logistics are usually applied to maintain quality and decrease decay, however, unexpected quality loss can also occur. For example, chilling-induced tomato aroma loss at 5°C was found associated with changed VOC synthesis and methylation alteration.[85] In cold-stored tomato fruit, chilling stress caused changes in genomic methylation. Numerous promoter methylation changes were found as well in genes involving ripening, flavor VOC synthesis, and other qualities. Interestingly, most, but not all, of the methylation fluctuations were temporary, recovering to a prechilled state after 24 h at room temperature. Here *DML2* expression behaved the same. Although the reversibility principle needs further investigation, such conditionally-transient changes support methylation's role in the stress-triggered response. It is important to understand which loci can be recovered and whether this involves specific distribution, biological functions, or methylation pathway dynamics. Combining the cold storage practice, this methylation's incomplete recovery from chilling to selling contributes to consumers'

perception that modern tomatoes are sometimes flavorless.[85]

Another typical case requiring cold storage is peach fruit, accompanying chilling injury (CI). The latest research has built a temporal map of peach CI development, coupled with volatile organic compound (VOC) and methylation profiling spanning hour-to-week timescales. The findings revealed that chilling rendered methylation in all contexts peaked at 7 d, followed by further declines.[139] The expressions of *Alcohol Acyltransferase 1* (*AAT1*) and the VOC-related TF *MADS2* were negatively correlated to promoter-methylation changes. This was consistent with the chilling-reduced alterations on contents of ester and lactone, which are also peach flavor VOCs. Furthermore, chilling triggered promoter hypermethylation of TPS3, resulting in reduced expression and a decline in linalool levels, thus supporting the previously reported regulatory pattern for linalool.[116]

Indeed, CI occurs in peach fruit stored below 12°C and strengthens at 0 and 5°C. In CI fruit, chilling-induced hypermethylation was correlated to the internal browning (IB) index.[140] In most CI-associated genes that involve softening, ethylene production and signaling, reactive oxygen species (ROS) metabolism and IB, there were negatively-correlated expressions found against chilling-induced methylation changes. This indicated that methylation changes contribute to the temperature-dependent CI in postharvest peach fruit. Additionally, methylation also functions importantly in CI mitigation of peach via methyl jasmonate[141] and nitric oxide,[142] influencing fruit softening, flesh browning, and VOC synthesis.

Considering low-temperature coloration, the white- or brown-fleshed peach below 12°C is noticeable versus the red-fleshed peach at room temperature. As reported, the difference was induced by chilling-induced methylation changes in anthocyanin-related promoter-methylation, including TFs and structural genes[143] (Figure S2e). Besides, peach mealiness is another major issue of CI, which highly reduces consumer acceptance and market value. The related epigenetic mechanisms have been recently discovered:[144] mCHH-DMRs amounted most both in normal and mealy fruit after 30-day storage at 0°C and most DMRs were not *de novo* developed during the cold storage, they just underwent changes of methylation degree. Further, the potential mealiness-regulating candidates, *Cytochrome P450 82A* (*CYP82A*) and *UDP-arabinose 4 Epimerase 1* (*UA4E1*), were expression-downregulated and promoter-hypermethylated in mealy fruit. This coincided with a TE colocalization, which suggested the important roles of TE and associated methylation in peach mealiness formation under CI.

One more chilling-related instance is the winter rapeseed (*Brassica napus*), whose quality maintenance requires overcoming freezing stress. Some of them have remarkable freezing tolerance, where the epigenetic mechanism is unclear until recently.[145] Declines of global methylation level were observed in rapeseed under freezing stress with a lighter decline in freezing-resistant cultivars. Here the methylation-preferring regions remained unchanged after freezing, indicating that RdDM and maintenance tended not to build new methylation. Based on the analysis of DMR-associated genes with varied expressions, the freezing tolerance was found achieved by strengthened signal transduction, decreased lipid peroxidation, enhanced cell stability, elevated osmolytes, and increased ROS scavenging. Current findings above highlight the multifaceted roles of methylation in chilling-response, however, genomic or locus methylation performs differently across species even if under similar stresses.[85,139,145] Overall, unpredictably and species-specifically, abundant valuable methylation loci have been identified so far, being candidates for future precise horticultural quality improvement and design (Figure 1).

MANIPULATING DNA METHYLATION FOR QUALITY IMPROVEMENT AND DESIGN

While the advancing epigenetic technologies contribute to comprehending methylation mechanisms in horticultural quality, a predominant portion of research often remains at the correlation level concerning expression and methylation. This limitation is owing in large part to the lack of accessibility of methylation manipulation to give robust evidence of causality. Today, emerging epigenome editing strategies are showing the potential for precise methylation manipulation: several locus-specific 5mC manipulation systems have been developed in plants. They are based on i) the synthesis of siRNAs complementary to the targets (siRNA-based tool, Figure 6A) and ii) the direct tethering of the methylation machinery to targets via programmable DNA-binding proteins (artificial zinc-finger proteins, ZF-based and SunTag-based tools, Figures 6B-C).[146]

Inverted repeat (IR) transgenes based on siRNA mechanisms can rapidly methylate and silence both transgenes and some endogenous genes within hours.[147,148] IR constructs were used to methylate distal enhancers of the flowering major regulator, *Flowering Locus T* (*FT*), causing its downregulation.[149] Considering the special loci such as *ROS1* promoter with positive methylation-expression correlation, the methylation by IR transgene targeting exerted a positive effect on its expression.[39] In addition, RdDM components fusing to ZFs have also been examined for epigenetic editing potential.[150,151] The unmethylated promoter of *Flowering Wageningen* (*FWA*) was successfully heritably methylated with silencing effects.[151] *FWA* is a flowering inhibitor normally silenced by its mCG-rich promoter, leading to early flowering. In the *fwa* epi-allele, the missing of this mCG methylation results in delayed flowering and a prolonged vegetative period.[152] Here the *FWA* promoter serves as a methylation indicator for artificial epigenetic attempts. The CRISPR SunTag system[153] has shown high specificity and can target methylation that is heritable with stable silencing of *FWA*.[154]

On the other hand, in terms of demethylation, thorough, highly specific, and heritable targeted demethylation has been accomplished at the *FWA* promoter, causing *FWA* re-activation with altered flowering. Both ZF and SunTag systems were used, although remethylation and resilencing occurred once the trigger construct was segregated out.[155] In previous attempts, we successfully demethylated locus that can be remethylated in *nrpd1* by the rescue of NRPD1 (a key component in RdDM).[156] This was achieved via the CRISPR-dCas9-TET1CD (the catalytic domain of Ten-eleven-translocation Protein 1) system designed for mCG and mCHG removal. The findings revealed that non-mCHH methylation determines whether the locus can be remethylated by RdDM.[156]

Simultaneously, alternative systems tailored to diverse requirements are developing as well. One such advancement is the development of a CRISPR-based near-infrared upconversion-activated methylation editing system.[157] It allows for the spatiotemporal erasure and installation of methylation, introducing the notion of light-dependent methylation editing.[157] Notably, a special 5mC-to-T editing was recently accomplished using a human APOBEC3Bctd-Cas9 (the C-terminal domain Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3B) fusion for future precise manipulation.[158]

Pioneer works are generally conducted in *Arabidopsis*. In this early stage, understandably, editing-related horticultural applications are limited, which highlights the importance of the attempt in chrysanthemum (*Chrysanthemum morifolium*),[100] bearing ornamental and edible properties with popularity worldwide. This study showed that a differentially expressed anthocyanin-related TF, *MYB6*, distinguished between the yellow (YP-Y) and pink chrysanthemum flowers (YP-P). mCHH-hypermethylated *MYB6* promoter was found in YP-Y, mitotically heritable over three generations. Further, the dCas9-TET1CD system towards the *MYB6* promoter was used via transient transformation in YP-Y partial-pale flowers. As a result, the target methylation level was successfully decreased with upregulated *MYB6* expression, and the flower color returned to partial pink accompanying the re-accumulation of anthocyanin (Figures 6D-E). These findings establish an epigenetic foundation for enhancing anthocyanin-based color quality and instill confidence in the potential of epigenetic engineering in non-model species.

CONCLUSION AND OUTLOOK

Over the past decade, there has been a considerable surge in interest regarding the methylation role in horticultural food, evidenced by a rapidly growing body of related publications. This trend holds promising potential to develop more sustainable food solutions in response to global food security and quality challenges. Based on extensive investigation, we show that methylation primarily functions at the level of transcriptional interference, for genes or TEs. Downstream events include horticultural quality formation, particularly for ripening, coloration, and flavor VOCs, as well as quality variation with chilling-response. Methylation usually functions together with TFs like MYBs, involving methylating/demethylating their encoding genes or targets. Potential crosstalks with phytohormones[21,62,68,102,141] or miRNAs[132,134] are also stressed. In addition to identifying further quality-related loci, future

efforts should prioritize investigating connections between methylation patterns, stress responses, and postharvest treatments, such as methylation's role in tea handling.[88,159] As in this case, postharvest processing manners and botanical tolerance are quality determinators, which will complement insights into methylation-mediated horticultural quality shaping.

Notably, methylation function patterns differ from genetic knowledge, which can be referenced across species and tissues. In other words, while comparable coding sequences in different species usually imply the same protein function, there seems no mature mode that can be followed considering the comparable methylation messages in different horticultural species or the same species under different conditions. This drives investigations to include as many horticultural crops as possible to reveal more mechanistic clues. Our current understanding fails to explain how such impressive species- or response-specificity arises, what role it plays in an evolutionary perspective, and why it does not share the convergent evolution pattern as reported at the genome encoding level.[160] What we know today is that the horticultural diversity and complexity of methylation patterns are involved with genome size and complexity, methylation dynamic pathways' fine-tuning, contexts, density, and levels of methylation, as well as methylation distribution at structural elements such as gene promoters, terminators, exons, introns, TE bodies, flanking regions with associated effects on expressions. Further, gene characteristics, such as TF encoding or structural gene encoding, are also considered potential factors affecting methylation's action,[115] since methylation's suppression of promoter-TF binding can partially interpret the methylation mechanism.[40–42]

Such specificity poses a challenge when it comes to future prediction of horticultural methylation behavior and quality association, which are crucial for advancing epigenetic quality design. To realize predictive goals, building big data projects of epigenetic information becomes imperative, serving as a foundational resource for deeper insights. Currently, the related databases are mostly maintained for human and disease research,[161–163] one appropriate example for the current scope is the fruitENCODE project containing 45 methylation profiles. However, these are not its core aspect[160] with suboptimal maintenance. Focusing on single horticultural species, only tomato has a user-friendly database available for single-base-solution methylation profiles.[75] While valuable, these are insufficient for capturing the desired shared patterns. Looking ahead, with the accumulation of ample epigenetic data comparable to genetic data today, machine learning may emerge as a promising solution for building horticulture models, summarizing methylation patterns from massive methylation information. We believe that this can be one of the key answers for figuring out the multifaceted specificity of methylation role in horticultural quality. Moreover, integrating big data of epigenome-included multi-omics is expected to provide a comprehensive understanding of the complex interactions and regulatory networks underlying horticultural food quality formation and variation.

In our era, locus-specific methylation editing begins to bridge the theoretical basis and future applications. The heritability of manipulated methylation/demethylation makes 5mC editing a promising approach for a potentially permanent modification of desired loci, although the lasting period requires further investigation. While relatively precise and efficient manipulations can be achieved currently, substantial research is still needed to refine these tools for intra- and inter-generation stability, off-target effect reduction, and targeting efficacy. The undesired re-activated TE through artificial demethylation to induce unpredicted epigenome changes should be considered despite its tiny probability. In this period, the field of horticultural methylation remains mostly a state of a boosting volume of research on methylation function analysis. Approaches of biochemistry, molecular biology, and epigenome sequencing are primarily employed, with rare methylation editing methods,[100] lacking causal evidence. Foreseeably, the use of methylation editing will rapidly grow, progressing from basic vitality maintenance[26] to quality formation and variation,[164] from model plants to horticultural food. All of these contribute to the transition from lab cases to future field landings.

On the other hand, there is a need to develop more epigenetic editing tools that ensure us tolerant in the face of future complex and diverse situations. Epigenome editing methods are much less than genome editing methods today,[165] owing majorly to a delay in epigenetic mechanism research. Specifically, in addition to specificity, challenges arise from the unclear mechanism of methylation influences on gene expression, as well as the detailed principles and the conservation of methylation dynamic pathway and machinery. Meanwhile, there are several intriguing questions to explore: Is it possible to modify quality-related gene expression by manipulating non-promotor regions? Is it possible to modify quality-related gene expression by artificially methylating regions totally without methylation history? Is it possible to generate directed or random mutants by demethylating quality-related TE? All of these inquiries are intertwined with the fundamental understanding of current epigenetics, aiding in reducing complexity and enhancing the adaptability of future methylation editing strategy designs.

Legislatively, regulations on epigenetically modified crops exist only in the EU and Switzerland today. The decision by the Court of Justice of the European Union of 2018 (case C-528/16) declares that all mutagens and epimutagens (chemical and molecular) that have not been regularly used before the year 2001 are strictly prohibited,[166] indicating the legislative distance to the landing of epigenetic engineering. Accordingly, persistent pushing to introduce policies, guidelines, and regulations is required. In addition, as above, some epigenome editing tools are based on the CRISPR platform, but a recent survey showed that consumers tend to equate CRISPR/Cas to traditional genetically modified organisms (GMOs).[167] This signals a future barrier, although the CRISPR technique introduces local DNA changes, whereas GMOs receive foreign DNA. Importantly, the necessity for methylation manipulation can be delivered only through RNAs and proteins,[146] thus bypassing the genetic transformation and giving access to methylation manipulation. The target food product subjected to this DNA-free delivery is expected to be considered a non-genetically modified product. With multifaceted support (Figure 7), this next-generation food upgrading strategy may pave the way to address the global food crisis, including GMO-rejecting regions.

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The authors declare no competing interests.

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SUPPLEMENTAL INFORMATION

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