Global increase in DNA methylation during orange fruit development and ripening

Huan Huang^{a,1}, Ruie Liu^{a,1}, Qingfeng Niu^a, Kai Tang^{a,b}, Bo Zhang^c, Heng Zhang^a, Kunsong Chen^c, Jian-Kang Zhu^{a,b,2}, and Zhaobo Lang^{a,2}

^aShanghai Center for Plant Stress Biology, National Key Laboratory of Plant Molecular Genetics, Center of Excellence in Molecular Plant Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200032, China; ^bDepartment of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907; and ^cLaboratory of Fruit Quality Biology, Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zhejiang University, Zijingang Campus, Hangzhou, 310058, China

Contributed by Jian-Kang Zhu, November 27, 2018 (sent for review September 7, 2018; reviewed by Etienne Bucher and Yu-Jin Hao)

DNA methylation is an important epigenetic mark involved in many biological processes. The genome of the climacteric tomato fruit undergoes a global loss of DNA methylation due to active DNA demethylation during the ripening process. It is unclear whether the ripening of other fruits is also associated with global DNA demethylation. We characterized the single-base resolution DNA methylomes of sweet orange fruits. Compared with immature orange fruits, ripe orange fruits gained DNA methylation at over 30,000 genomic regions and lost DNA methylation at about 1,000 genomic regions, suggesting a global increase in DNA methylation during orange fruit ripening. This increase in DNA methylation was correlated with decreased expression of DNA demethylase genes. The application of a DNA methylation inhibitor interfered with ripening, indicating that the DNA hypermethylation is critical for the proper ripening of orange fruits. We found that ripening-associated DNA hypermethylation was associated with the repression of several hundred genes, such as photosynthesis genes, and with the activation of hundreds of genes, including genes involved in abscisic acid responses. Our results suggest important roles of DNA methylation in orange fruit ripening.

DNA methylation | fruit | ripening | orange

DNA methylation is a major and conserved epigenetic mark that is associated with genome stability, inactive transcription, developmental regulation, and environmental responses (1, 2). In plants, DNA methylation occurs not only in the symmetric CG and CHG sequence contexts but also in asymmetric CHH sequence contexts (H represents A, T, or C). Distinct mechanisms are involved in establishing, maintaining, and removing the DNA methylation mark. CG and CHG methylation is maintained through a semiconservative mechanism that requires the DNA methyltransferases METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively (3, 4). CHH methylation can be maintained by CMT2 and domain rearranged methyltransferases (DRM1 and DRM2) through the RNA-directed DNA methylation (RdDM) pathway that is also responsible for de novo methylation (5–8). DNA methylation levels are dynamically regulated by DNA methylation and demethylation reactions. Active DNA demethylation in *Arabidopsis* is initiated by 5'-methylcytosine DNA glycosylase/lyase enzymes, including REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2), and DML3 (2).

Recent discoveries indicate that dynamic changes in DNA methylation are very important for fleshy fruit ripening. Fruit ripening is a complex developmental process that involves numerous physiological, biochemical, and structural alterations and is under strict hormonal, genetic, and epigenetic controls, including but not limited to degreening, accumulation of pigments, softening, and accumulation of sugars, acids, and volatiles (9, 10). In tomato, a naturally occurring epigenetic mutation (*Cnr*) inhibits normal ripening, and the resulting fruits develop a colorless, mealy pericarp, which is due to DNA hypermethylation in

the promoter region of *CNR* (11, 12). In apple, DNA methylation of the *MdMYB10* promoter can regulate *MdMYB10* gene expression and fruit pigmentation during ripening (13–15). During tomato fruit ripening, the fruits undergo a global DNA demethylation due to an increased expression of the DNA demethylase gene *SIDML2* (15–17). Knockout of *SIDML2* prevents the DNA demethylation and thus impairs ripening (17). Interestingly, during tomato fruit ripening, *SIDML2*-mediated DNA demethylation is not only associated with the activation of gene expression, but also with the repression of several hundred genes (17). The observation suggested a broad role of DNA methylation in gene activation in tomato. It is not known whether the global DNA demethylation observed in the tomato fruit model system may take place during the ripening of other fruits.

Citrus is one of the most important and widely grown fruit crops, with an ~327-Mb genome (18). As a long-lived woody perennial plant, its fruits are nonclimacteric. The growth and development of citrus fruits can be divided into three phases: phase I is the extremely rapid cell-division process, phase II is the rapid cell-enlargement process, and phase III is the fruit maturation process with chlorophyll degeneration and carotenoid accumulation. The application of the DNA methylation inhibitor

Significance

A global loss of DNA methylation due to active DNA demethylation is critical to the regulation of tomato fruit ripening. It is unclear whether and how DNA methylation may change during the ripening of other fruits. We found a global increase in DNA methylation during orange fruit ripening, likely because of decreases in the expression of DNA demethylase genes. The DNA hypermethylation was associated with not only the repression of several hundred genes, such as photosynthesis genes, but also the activation of hundreds of genes, including genes involved in abscisic acid responses. Our study therefore revealed a global gain in DNA methylation during an important phase of plant development, and suggested a critical role of DNA methylation during orange fruit ripening.

Author contributions: J.-K.Z. and Z.L. designed research; R.L., Q.N., B.Z., H.Z., and K.C. performed research; H.H., K.T., and Z.L. contributed new reagents/analytic tools; H.H., K.T., J.-K.Z., and Z.L. analyzed data; and H.H., J.-K.Z., and Z.L. wrote the paper.

Reviewers: E.B., Agroscope; and Y.-J.H., Shandong Agricultural University.

The authors declare no conflict of interest.

Published under the PNAS license.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession nos. GSE108930, GSE108931, and GSE120025).

¹H.H. and R.L. contributed equally to this work.

²To whom correspondence may be addressed. Email: jkzhu@sibs.ac.cn or zblang@sibs.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1815441116/-DCSupplemental.

Published online January 11, 2019.

5-azacytidine could induce carotenoid degradation in the callus of citrus, suggesting that DNA methylation might regulate ripening-related biological processes in citrus (19). However, the genome-wide DNA methylation dynamics and the role of DNA methylation in citrus fruit development and ripening have not been characterized.

In this study, single-base resolution maps of DNA methylation of sweet orange fruits at five stages—from immature to ripe stages—were generated, revealing a surprising global increase in DNA methylation during orange fruit ripening, which is opposite to the global DNA demethylation during tomato fruit ripening. Application of a DNA methylation inhibitor caused a delay in ripening, suggesting that the ripening-induced increase in DNA methylation is crucial for the normal fruit ripening process. Our analyses suggested that the ripening-induced DNA hypermethylation is mainly due to a decreased expression of DNA demethylase genes during orange fruit ripening. By comparing the transcriptomes and DNA methylomes of fruits at different stages, we found that the increased DNA methylation is not only associated with gene repression, but also occurred at the promoter regions of hundreds of activated genes during ripening. Therefore, like in tomato fruits, DNA methylation may have a broad role in activating gene expression in orange fruits.

Results

Features of the Sweet Orange Fruit DNA Methylome. To investigate DNA methylation dynamics during sweet orange fruit development, we generated single-base resolution maps of DNA methylation for Newhall navel orange (*Citrus sinensis* Osbeck cv. Newhall) fruits at five different stages: 90, 120, 150, 180, and 210 d after bloom (DAB) (hereafter referred to as Cs1 ~ Cs5) (Fig. 1A), according to the timing of orange fruit development and ripening (20). Each stage was sequenced with two biological replicates (A and B). For each sample, at least 100 M paired-end reads (read length = 150 bp) were produced. Approximately 70% of the reads were mapped to the reference genome using BSMAP (21), covering >97% of the genome, and ~14% of the reads were mapped to the unmethylated chloroplast genome (22). All of our sequenced methylomes had ~15-fold average coverage per DNA strand. The coverage and depth of our sequenced methylomes are comparable to those of the published methylomes of *Arabidopsis* (23) and tomato (17).

In Cs1 fruits, ~33% of the total cytosines are methylated, which were defined using a binomial test as previously described (12), with a global methylation level of ~13%, which is lower than that of tomato fruit (22%). Average CG, CHG, and CHH methylation levels are 41%, 23%, and 8%, respectively. An analysis of the distribution of DNA methylation and gene/transposable element (TE) density showed that DNA methylation is enriched in heterochromatin (SI Appendix, Fig. S1). The TEs are preferentially located in the pericentromeric region and are characterized by enrichment of methylation in both CG and non-CG sequence contexts (SI Appendix, Fig. S1A and B). In contrast, the genes are preferentially located in the chromosome arms and are characterized by enrichment of CG methylation and depletion of non-CG methylation (SI Appendix, Fig. S1A and C).

A recent study suggested that cytosines in different subcontexts can be differentially methylated in *Arabidopsis* and several other plants (24). In sweet orange fruits, we found that among the CG subcontexts, CGA and CGT motifs have higher densities than CGC and CGG, but all of the subcontexts have comparable methylation levels across the different chromosomes; in CHG contexts, the density and methylation level of CCG motifs are much lower than those of CAG and CTG throughout the chromosomes, which is consistent with the observations in *Arabidopsis* and several other plants (24); in the subcontexts of CHH motifs, CTA and CCC have the highest and lowest densities throughout the chromosomes, respectively, and cytosines in CCH (CCC, CCA, and CCT) are less

Fig. 1. Genome-wide increase in DNA methylation during sweet orange fruit development and ripening. (A) Picture of sweet orange fruits at 90 DAB (Cs1), 120 DAB (Cs2), 150 DAB (Cs3), 180 DAB (Cs4), and 210 DAB (Cs5). (B) DNA methylation levels of genes and TEs in Cs1~Cs5. Methylation levels of mC, mCG, mCHG, and mCHH are shown. (C) PCA showing consistency between the two biological replicates of whole-genome bisulfite-sequencing samples of Cs1~Cs5. (D) Numbers of ripening-induced DMCs in Cs5 relative to Cs1 are shown for the mCG, mCHG, and mCHH sequence contexts. (E) Numbers of hyper- and hypo-DMRs in Cs2, -3, -4, and -5 relative to Cs1.

methylated compared with cytosines in CTH and CAH (H = A, T, or C) (SI Appendix, Fig. S2).

DNA Methylation Increases During Sweet Orange Development and Ripening. We calculated the average DNA methylation levels for the samples of different stages, and found that the cytosine methylation level increased from Cs1 to Cs5 (from 13 to 14.5%) and the trend is consistent in two biological replicates (SI Appendix, Fig. S3A). To exclude the possibility that the increase was caused by differences in conversion rates due to bisulfite treatments, we examined the conversion rates for all samples, and found that all libraries had a conversion rate >99.7%, based on the unmethylated chloroplast genome. In addition, we calculated average DNA methylation levels for genes and TEs, respectively, and found that, from stage Cs1 to stage Cs5, DNA methylation is gradually increased in 5' and 3' flanking regions of genes and also increased in TE body and flanking regions (Fig. 1B). Further analysis showed that the increase of DNA methylation is mostly contributed by increase in CHH methylation (Fig. 1B).

Differences in DNA methylation between two samples can be quantitatively characterized by differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs). To investigate the difference between immature and ripe fruits, we compared the DNA methylomes of Cs1 and Cs5. As shown in Fig. 1C, principal component analysis (PCA) revealed a good consistency between the two biological replicates at each stage. So we merged the data from two biological replicates to increase coverage and thus to enhance statistical power for DMC and DMR calling. We identified a total of 1.2 million DMCs in Cs5 relative to Cs1, 88% of which are hypermethylated (hyper-DMCs); and 93% of the hyper-DMCs occur in the CHH context,

suggesting an increase mainly in CHH DNA methylation from Cs1 to Cs5 (Fig. 1D). The DNA methylation increase from Cs1 to Cs5 could be due to an increased number of total mCs or an increased methylation level of existing mCs. As shown in *SI Appendix*, Fig. S3B, we found that the numbers of mCs are increased from Cs1 to Cs5, and the biggest increase occurred from Cs1 to Cs2. Besides, the methylation level of mCs increased gradually from Cs1 to Cs5, and the increase mainly occurred in the CHH context (*SI Appendix*, Fig. S3 C and D). To further characterize the change of DNA methylation from Cs1 to Cs5, a method based on Fisher's exact test was used to identify DMRs between two methylomes (25). We compared the methylomes of Cs2, -3, -4, and -5 with that of Cs1, respectively. As shown in Fig. 1E, the numbers of hyper-DMRs identified in Cs2 (5,169), Cs3 (16,086), Cs4 (29,196), and Cs5 (31,092) relative to Cs1 gradually increased from Cs2 to Cs5. These results suggest a global increase in DNA methylation from Cs1 to Cs5.

All of the above analyses suggested that CHH is obviously hypermethylated from Cs1 to Cs5. In *Arabidopsis* and rice endosperms, CG demethylation is accompanied with local CHH hypermethylation (26, 27). Global DNA hypomethylation during tomato development is also accompanied with CHH hypermethylation in TEs (12). We wondered whether CHH hypermethylation may be accompanied with CG demethylation during orange ripening. Further examination of the methylation levels in CG, CHG, and CHH contexts revealed that at Cs5 hyper-DMRs (Cs5 vs. Cs1) (*Dataset S1*) the hypermethylation mainly occurs in CHH and CHG contexts, whereas the CG methylation level has no significant change, suggesting that CHH hypermethylation during orange fruit ripening is not accompanied with CG hypomethylation (Fig. 24). At Cs5 hypo-DMRs, hypomethylation occurs in all three contexts, showing that CG hypomethylation is not accompanied with CHH hypermethylation (*SI Appendix*, Fig. S44). Our two biological replicates (A and B) showed consistent methylation levels at each stage (Fig. 24 and *SI Appendix*, Fig. S44). We calculated a robust index for each DMR (Cs5 vs. Cs1) (*SI Appendix*, *Supplementary Information Text* and *Dataset S1*). Genomic regions with lower robust index values are more credible in terms of differential methylation. We ranked the DMRs by their robust index values, examined the top 10% and bottom 10% hyper-DMRs, and found DNA methylation increases from Cs1 to Cs5 in the two replicates for both groups of DMRs (*SI Appendix*, Fig. S4B). The methylation levels of several examples of ripening-induced hyper- and hypo-DMRs are displayed in Fig. 2B and *SI Appendix*, Fig. S4 C and D. Consistent with statistical results, the methylation levels of the displayed hyper- and hypo-DMRs gradually changed from Cs1 to Cs5, and the hyper-DMRs mainly have hypermethylation in CHH, whereas decreased methylation in the hypo-DMRs occurs in all three contexts. These results show that CHH hypermethylation is not accompanied with CG demethylation in orange.

To further characterize the DMRs, we analyzed DMR compositions and distributions along genes, TEs, and their flanking sequences. We found that hyper-DMRs are preferentially located in gene promoters ($P < 1.0 \times 10^{-5}$, Fisher's exact test) (*SI Appendix*, Fig. S5A), and are particularly enriched in the 5'- and 3'-flanking regions of genes but depleted in gene body regions (*SI Appendix*, Fig. S5B); in contrast, hypo-DMRs are relatively evenly distributed along genes with a little enrichment in the 3' end of genes (*SI Appendix*, Fig. S5B). Hyper-DMRs are also preferentially located in TEs ($P < 1.0 \times 10^{-5}$, Fisher's exact test), especially TEs shorter than 2 kb (*SI Appendix*, Fig. S5), whereas hypo-DMRs are depleted in TEs. Our data support that DNA methylation is increased globally during sweet orange fruit development and ripening, which is in sharp contrast to the global loss of DNA methylation during tomato fruit ripening.

We noticed that DNA methylation is increased stage by stage from Cs1 to Cs5. This gradual change is different from the

Fig. 2. DNA methylation levels of hyper-DMRs in all stages. (A) Boxplots showing DNA methylation levels of the 31,092 hyper-DMRs in Cs5 relative to Cs1 in all stages. Methylation levels in C, CG, CHG, and CHH contexts are shown (* $P < 3.4 \times 10^{-11}$ compared with Cs1, one-tailed Wilcoxon tests; NS, not significant, A and B are two replicates); (B) Integrated Genome Browser display of DNA methylation levels of two hyper-DMRs (boxed regions). DNA methylation levels of cytosines are indicated by the heights of the vertical bars on each track.

changes in tomato, where DNA methylation, especially in the CG and CHG contexts, is dramatically lost right after the breaker stage (12, 17). In tomato, CHH hypermethylation occurs during CG and CHG hypomethylation at TEs (12). However, the CHH hypermethylation is not accompanied with CG and CHG demethylation in orange.

Decreased Expression of DNA Demethylase Genes Is Correlated with the Increased DNA Methylation During Orange Fruit Ripening. DNA methylation status can be dynamically regulated by DNA methyltransferase and DNA demethylase activities. The increased DNA methylation during orange fruit development and ripening could be caused by enhanced DNA methyltransferase activities or by decreased DNA demethylase activities. To investigate these possibilities, we examined the expression levels of DNA methyltransferase and demethylase genes at different stages of orange fruit development and ripening. First, we de novo-annotated DNA methyltransferase and DNA demethylase genes in the orange genome. In *Arabidopsis*, methylation in CG, CHG, and CHH contexts can be catalyzed by different DNA methyltransferases, including AtMET1, AtCMTs, and AtDRMs (2). In the orange genome, we identified seven genes for methyltransferase orthologs using blastp and a phylogenetic tree was constructed using the maximum-likelihood method (*SI Appendix*, Fig. S6A). Of the seven methyltransferase genes, three (Cs3g03480, Cs3g01520, and Cs3g08350) are barely expressed [fragments per kilobase of transcript per million mapped reads (FPKM) < 1] in fruit tissues; and among the other four genes, none has increased transcript levels during ripening (Fig. 34 and *SI Appendix*, Fig. S6C). Instead, the most highly expressed methyltransferase gene in fruits, CsDRM2 (Cs2g27880), as well as another two key genes in RdDM (CsNRPE1 and CsAGO4), have decreased transcript levels during ripening (Fig. 34 and *SI Appendix*, Fig. S6D). These results suggest that the increased

DNA methylation level during ripening is not caused by changes in the expression of any of the DNA methyltransferase genes.

For DNA demethylase genes, we identified four *AtROS1* orthologs, including orange1.1t01511 (*CsDME*), Cs6g15500 (*CsDML1*), Cs5g04950 (*CsDML4*), and Cs3g07800 (*CsDML3*) (*SI Appendix, Fig. S6B*). Of the four orange DNA demethylase genes, Cs5g04950 (FPKM < 3) has lower transcript abundance in fruits than the other three genes (FPKM > 10). The expression of all these four genes is significantly reduced during orange fruit ripening (Fig. 3*B* and *SI Appendix, Fig. S6E*). The gradual decrease in the expression of DNA demethylase genes is consistent with the gradual increase in DNA methylation levels from Cs1 to Cs5. Considering the expression patterns of DNA methyltransferase and DNA demethylase genes, the increase in DNA methylation during sweet orange fruit ripening is most likely contributed by the reduced expression of DNA demethylase genes.

In *Arabidopsis*, it is known that *AtROS1* antagonizes RdDM to prevent DNA hypermethylation, and *AtROS1* target regions are enriched with 24-nt small interfering RNAs (siRNAs) that cause RdDM (28). Similarly, genomic targets of *SIDML2*, an *AtROS1* ortholog in tomato, are also enriched with 24-nt siRNAs (12, 17). We found that the ripening-induced hyper-DMRs in sweet orange fruits, the presumed genomic targets of DNA demethylases during ripening, show significant enrichments of 24-nt siRNAs compared with control regions (*SI Appendix, Supplementary Information Text and Fig. S6F*), suggesting an antagonism between DNA demethylases and RdDM in sweet orange fruits.

Correlation Between DNA Methylation and Gene Expression During Orange Fruit Ripening. To investigate whether the DNA methylation increase during orange fruit development and ripening is associated with changes in gene expression, we generated transcriptome profiles for the orange fruits, with three biological replicates for fruits at each stage. PCA analysis showed consistency among the biological replicates at each stage (*SI Appendix, Fig. S7A*). We identified differentially expressed genes (DEGs) in Cs2, -3, -4, and -5 relative to Cs1. The numbers of up- and down-regulated DEGs are increased stage by stage from Cs2 to Cs5 (Fig. 4*A*), suggesting that the global gene-expression patterns are gradually altered during development and ripening. Venn diagrams show that DEGs identified in the different stages highly overlap (Fig. 4*B*).

To investigate the association between DNA methylation changes and changes in gene expression, we focused on the

analysis of hyper-DMR-associated genes because orange fruits mainly undergo DNA hypermethylation during ripening. There are 31,092 hyper-DMRs in Cs5 compared with Cs1, and 8,985 of them are located in gene promoter regions and are thus potentially involved in gene regulation during ripening. A total of 7,007 genes were identified as hyper-DMR-associated genes in the orange genome, and 5,182 of them are expressed in at least one of our sequenced samples (FPKM > 1). Among these 5,182 genes, we identified 1,113 down-regulated DEGs (cluster 1), 950 up-regulated DEGs (cluster 2), and 3,119 non-DEGs (cluster 3) in Cs5 vs. Cs1 (Fig. 4*C* and *Dataset S2*). The expression of cluster 1 and cluster 2 genes is changed gradually during ripening, while the expression of cluster 3 genes does not show significant changes. It is possible that cluster 3 genes have smaller changes in gene expression due to weaker changes in DNA methylation compared with cluster 1 and 2 genes. To test this possibility, we examined DNA methylation changes of cluster 1, 2, and 3 genes. The increase in DNA methylation in Cs5 vs. Cs1 mainly occurs in promoter regions, and the extents of increase are comparable among cluster 1, 2, and 3 genes (Fig. 4*D*). Similar to cluster 3 genes in the orange genome, there are ~5,000 genes that show changes in DNA methylation but not expression during tomato ripening (17). So, for these genes in tomato and cluster 3 genes in orange, a change in DNA methylation is insufficient to cause gene-expression changes in fruits.

We calculated the promoter (−2 kb region) methylation levels of the three clusters of genes at all stages (*SI Appendix, Fig. S7B*). The results showed that the DNA methylation levels of cluster 1 and cluster 2 genes increased gradually from Cs1 to Cs5. The transcript levels of cluster 1 and cluster 2 genes also changed gradually from Cs1 to Cs5 (Fig. 4*C*). Thus, the changes in DNA methylation and gene expression appear coupled, even though cluster 1 genes are down-regulated, whereas cluster 2 genes are up-regulated. We examined the correlation between DNA methylation and gene expression for several individual genes. For each gene, the expression levels and promoter DNA methylation levels at five different stages (Cs1 to -5) were plotted and the correlation coefficient was calculated. For the tested cluster 1 genes, their gene-expression level and DNA methylation level have a strong negative correlation ($R < -0.95$) (Fig. 5*A* and *SI Appendix, Fig. S7C*), which is consistent with the canonical role of DNA methylation in transcriptional silencing. For the tested cluster 2 genes, their gene transcript level and DNA methylation level have a strong positive correlation ($R > 0.95$) (Fig. 5*B* and *SI Appendix, Fig. S7D*). We calculated the correlation coefficients for all of the three clusters of genes, as shown in Fig. 5*C*. Cluster 1 genes show a peak near −0.9, indicating that a strong negative correlation between DNA methylation and gene expression exists for most cluster 1 genes. In contrast, cluster 2 genes show a peak near 0.8, indicating that a strong positive correlation between DNA methylation and gene expression exists for most of the cluster 2 genes. DNA methylation is usually thought to cause transcriptional repression. However, a recent study revealed that DNA hypermethylation is associated with gene activation of several hundreds of genes during tomato fruit ripening (17). Our results here suggest that DNA methylation may also have a positive role in the regulation of many genes in orange fruits.

Analysis of Genes Associated with DNA Hypermethylation. To understand the potential role of DNA hypermethylation in orange fruit development and ripening, we performed Gene Ontology (GO) analysis of cluster 1 and cluster 2 genes. For cluster 1 (i.e., the down-regulated DEGs), genes involved in photosynthesis, cell wall organization and modification, and fruit development are highly enriched (*SI Appendix, Fig. S8A* and *Dataset S3*). The citrus fruit has three developmental phases: cell division, expansion, and ripening. During early fruit development, genes involved in cell wall organization and modification are active and

Fig. 3. Expression of DNA methyltransferase and DNA demethylase genes in fruits at different stages. Transcript levels of DNA methyltransferase (*A*) and demethylase (*B*) genes in Cs1~Cs5. Error bars indicate means $\pm$ SD, $n = 3$.

Fig. 4. Characterization of the expression and DNA methylation of hyper-DMR-associated genes. (A) Histogram showing the numbers of DEGs in Cs2, -3, -4, and -5 relative to Cs1. (B) Venn diagrams showing overlaps among the up- and down-regulated DEGs. (C) Heatmaps showing the expression changes of the three clusters of hyper-DMR-associated genes during ripening. (D) DNA methylation changes of the three clusters of genes in Cs5 vs. Cs1. The sequences flanking genes were aligned by transcription start sites (TSSs), and DNA methylation levels for each 100-bp interval were plotted. The dashed line marks transcription start sites.

are important for cell division and expansion, but these genes are not required during the ripening phase. Our GO analysis found 57 cell wall-related genes enriched in cluster 1, suggesting that the repression of these genes is regulated by ripening-induced DNA hypermethylation ([Dataset S3](#)). Photosynthesis is important for the rapid growth of young fruits and for production of starch, which can be converted to soluble sugars during ripening. As fruits ripen, they undergo an important transition: the chloroplasts, where photosynthesis actively takes place, differentiate into chromoplasts, which are sites for carotenoid accumulation for fruit color. During this transition, the expression of genes involved in photosynthesis is decreased. The result shows that ripening-induced hypermethylation is associated with the repression of five genes involved in light harvesting and four genes involved in electron transport in photosystem I ([SI Appendix, Fig. S8A](#) and [Dataset S1](#)). For cluster 2, the up-regulated DEGs, genes involved in abscisic acid (ABA) response and signaling pathway, steroid metabolic process, and aromatic compound metabolic process are enriched. It is known that ethylene is crucial for the ripening of climacteric fruits, such as tomato, whereas ABA plays a more important role in the ripening of nonclimacteric fruits (29). Sweet orange is a nonclimacteric fruit. We found that 51 hyper-DMR associated genes are in the category of “response to ABA,” and 21 genes are in the category of “ABA-activated signaling pathway” ([SI Appendix, Fig. S8B](#)). Our analysis suggests that DNA hypermethylation potentially contributes to sweet orange fruit ripening through regulation of genes in diverse pathways that are important at different stages of fruit development.

To test the significance of DNA methylation for orange development and ripening, the DNA methylation inhibitor 5-azacytidine was applied on immature orange fruits. We found that the injection of DNA methylation inhibitor into the orange fruit peel prohibited orange from degreening (Fig. 6A). Degreening is an important indicator of orange fruit ripening. To examine whether the inhibitor treatment altered DNA methylation during orange fruit development, we assayed the DNA methylation levels of two genomic regions using methylation sensitive McrBC-qPCR. We digested genomic DNA with McrBC, a restriction enzyme that cuts methylated but not unmethylated DNA, and then performed McrBC-qPCR using the digested DNA as template. As shown in Fig. 6B, our methylation-sensitive McrBC-qPCR assay showed that these two genomic regions are hypermethylated in Cs5 compared with Cs1 in mock-treated samples, which is consistent with the whole-genomic bisulfite sequencing results. In 5-azacytidine-treated samples, there was a decrease in DNA methylation levels, as expected. The two examined genomic regions are close to the 5'-ends of a cluster 1 gene (Cs3g20300) and a cluster 2 gene (Cs4g09560), respectively. To investigate the influence of 5-azacytidine treatment on gene expression, we examined the transcript levels of these two genes. As shown in Fig. 6C, the 5-azacytidine treatment increased the expression of Cs3g20300, a gene down-regulated in Cs5 compared with Cs1. The inhibitor treatment also decreased the expression of Cs4g09560, a gene up-regulated in Cs5 compared with Cs1 (Fig. 6C). These results (Fig. 6C and *SI Appendix, Fig. S9*) support an important role of DNA

Fig. 5. Correlation between DNA methylation and gene-expression levels during sweet orange fruit ripening. (A and B) Correlation between DNA methylation and gene-expression levels. Three cluster 1 (A) and three cluster 2 (B) genes are shown. The x axis is DNA methylation levels of gene promoter region (2 kbp). For each gene, the transcript level and promoter DNA methylation levels in Cs1–Cs5 are plotted, and are used to calculate the correlation coefficient (R). (C) Distribution of correlation coefficients of the three clusters of genes.

hypermethylation in citrus fruit ripening and in regulating the expression of ripening-associated genes.

Discussion

DNA methylation has been suggested to be the third important regulator of fleshy fruit ripening, besides hormones and transcription factors (12, 16, 17). During tomato fruit ripening, DNA methylation is dramatically decreased, and the application of DNA methylation inhibitor can trigger an early-ripening phenotype (12). Most of the work on epigenetic regulation of fruit ripening has been carried out in tomato, a typical climacteric fruit. However, single-base methylome analysis of nonclimacteric fruits has not been reported. Through whole-genomic bisulfite sequencing, our study here revealed that, different from tomato, the sweet orange, a nonclimacteric fruit, undergoes a global increase in DNA methylation during ripening. In 2014, using high-performance capillary electrophoresis, Xu et al. (30) found that the total genomic 5mC level was increased in sweet orange peels during ripening. However, it was not known where DNA hypermethylation occurred in the genome and which genes were affected. Using single-base resolution bisulfite sequencing, we determined the specific genomic regions where DNA hypermethylation occurs, and by comparing the DNA methylomes and transcriptomes, we identified the genes whose expression is affected by the DNA hypermethylation. In tomato, global CG and CHG hypomethylation is accompanied with CHH hypermethylation during ripening. However, CHH hypermethylation during sweet orange fruit ripening is not accompanied with CG and CHG hypomethylation. In both tomato and orange fruits, DNA methylation changes substantially during the ripening process, suggesting that dynamic regulation of DNA methylation is important for normal fruit ripening, even though DNA methylation changes in opposite directions in the two fruits.

DNA hypomethylation during tomato fruit ripening is due to an increased expression of *SIDML2*, which is orthologous to the *Arabidopsis* DNA demethylase gene *ROS1* (28). We found that the transcripts of the sweet orange DNA demethylase genes are down-regulated during ripening, while the transcripts of DNA methyltransferase genes are relatively less abundant and are not up-regulated (Fig. 3). These results suggested that the increased DNA methylation during orange fruit ripening is most likely caused by decreased expression of the DNA demethylase genes.

Therefore, in both tomato and orange fruits DNA demethylases are regulated to reshape the DNA methylation landscape.

In *Arabidopsis*, AtROS1 mainly antagonizes RdDM (28). In the RdDM pathway, 24-nt siRNAs associate with AGO4 and AGO6 to guide the DRM DNA methyltransferases to genomic targets (2). In this study, we found that ripening-induced hyper-DMRs are significantly enriched with 24-nt siRNAs, suggesting that the DNA demethylases antagonize RdDM at these genomic regions in sweet orange fruits. In addition, we noticed that *CsDRMs*, *CsNRPE1*, and *CsAGO4*, three key components in RdDM, are down-regulated during fruit ripening (Fig. 3 and *SI Appendix, Fig. S6B*). Based on these results, we propose that the hyper-DMRs are regulated by both RdDM and DNA demethylase activities, and during ripening, the effect of decreased demethylation overshadows that of a weakened RdDM, resulting in DNA hypermethylation. Besides global hypermethylation, hypomethylation occurred at 1,089 genomic regions during orange fruit ripening. Gradual changes in DNA methylation occur not only at the hyper-DMRs, but also at the hypo-DMRs during ripening (Fig. 2A and *SI Appendix, Fig. S4A*), indicating that the methylation changes in both directions are not random. It is known that in *Arabidopsis*, only a part of RdDM targets are antagonized by AtROS1. At RdDM targets that are not regulated by AtROS1, DNA hypomethylation is observed in plants defective in both RdDM and AtROS1 (28). Considering that both the RdDM and DNA demethylation genes are down-regulated during orange fruit ripening, the hypo-DMRs may correspond to the genomic regions that are regulated by RdDM but not by the DNA demethylases. Consistently, we observed enriched 24-nt siRNAs at the hypo-DMRs, supporting that the hypo-DMRs are targets of RdDM (*SI Appendix, Fig. S6D*). Our

Fig. 6. The significance of DNA methylation for orange fruit ripening. (A) Pictures of sweet oranges that were treated with DNA methylation inhibitor, 5-azacytidine (5'-Aza), or mock (ddH₂O). The treated areas are circled. (B) DNA methylation levels of promoter regions of Cs3g20300 and Cs4g09560. McrBC-qPCR analysis was performed in Cs1, Cs5, and mock and 5'-Aza-treated samples. Methylated DNA can be digested by McrBC, thus higher qPCR signals indicate lower methylation levels. (C) Gene expression of Cs3g20300 and Cs4g09560 in untreated Cs1 and Cs5 fruits, and in mock and 5'-Aza treated fruits. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars indicate means $\pm$ SD.

results suggest that the hyper- and hypo-methylation patterns during orange fruit ripening can be generated through the expression changes of genes in the DNA methylation and demethylation pathways.

DNA methylation is usually considered as a transcriptionally repressive mark. Consistently, ripening-induced hypermethylation in orange fruits is associated with the repression of hundreds of genes that are no longer needed in ripened fruits, such as genes involved in cell wall organization and photosynthesis (Fig. 4 and *SI Appendix, Fig. S8A*). However, during tomato fruit ripening, it was discovered that DNA methylation is also associated with the activation of many genes (17). Interestingly, the ripening-induced hypermethylation in orange is highly correlated with the active expression of several hundred genes that are important for orange fruit ripening, including genes in ABA response and signaling pathways (Fig. 4 and *SI Appendix, Fig. S8B*). Our results from 5-azacytidine treatments supported the importance of DNA hypermethylation in orange fruit ripening. The results also suggested an important role of DNA methylation not only in the repression of gene expression but also in the activation of some genes. The molecular mechanisms underlying the DNA methylation and gene expression changes during orange fruit development and ripening require further investigation.

Materials and Methods

Plant Materials. Fruits of Newhall sweet orange (*Citrus sinensis* Osbeck) were obtained from a commercial orchard in Songyang (Zhejiang, China). Fruits at five different developmental stages—90, 120, 150, 180 and 210 DAB—were collected. Orange fruit peels, similar to the tomato fruit pericarp tissue, which is the tissue developed from the ovary wall of the flower, were collected and were immediately frozen in liquid nitrogen and kept at -80°C until use for DNA and RNA extraction. For DNA methylation inhibitor treatment, fruits 2 wk before the breaker stage were selected. For the treatment, 1 mL of 50 mM 5'-azacytidine (Sigma) dissolved in water was

injected into the fruit peel. The injection was weekly repeated four times. The negative control (mock) fruits were injected with 1 mL of ddH₂O.

McrBC-qPCR Analysis. Genomic DNA was extracted from mock and 5-azacytidine-treated samples. For the 5-azacytidine-treated sample, the green patch of peel was used for DNA extraction. One-microgram of genomic DNA was digested overnight with McrBC (New England Biolabs), and qPCR was performed with 20 ng DNA as template; the negative control for the digestion, which was performed without GTP, was used as the standard.

Whole-Genome Bisulfite Sequencing and Analysis. Genomic DNA was extracted from fruits using a DNeasy Plant Maxi Kit (Qiagen) and then was used for library construction using Illumina's standard DNA methylation analysis protocol and the New England Biolabs Ultra II DNA Library Prep Kit. The samples were sequenced at the Genomics Core Facility of the Shanghai Centre for Plant Stress Biology, Chinese Academy of Sciences using Illumina HiSeq2500. Quality control, mapping and differential methylation analysis are described in the *SI Appendix, Supplementary Information Text*.

RNA Analysis. Total RNA was extracted with TRIzol reagent (Ambion) from peels of orange fruits. For reverse transcription, 1 μg of RNA and oligo dT primers were used to synthesize cDNA in a 20- μL reaction using the qScript cDNA SuperMix kit (Quanta). For RNA-seq, the libraries were constructed and sequenced at the Genomics Core Facility of the Shanghai Centre for Plant Stress Biology, Chinese Academy of Sciences, using Illumina HiSeq2500. Quality control, mapping, and differential methylation analysis are described in the *SI Appendix, Supplementary Information Text*.

Data Access. The sequencing data generated in this study have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through the GEO Series accession nos. GSE108930 (31), GSE108931 (32), and GSE120025 (33).

ACKNOWLEDGMENTS. This work was supported by the National Key Research and Development Program (2018YFD1000200) and Strategic Priority Research Program of the Chinese Academy of Sciences (Grant XDB27040000) (to Z.L.).

- Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11:204–220.
- Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. *Annu Rev Genet* 43:143–166.
- Finnegan EJ, Kovac KA (2000) Plant DNA methyltransferases. *Plant Mol Biol* 43:189–201.
- Lindroth AM, et al. (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* 292:2077–2080.
- Matzke MA, Mosher RA (2014) RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. *Nat Rev Genet* 15:394–408.
- Zhang H, Zhu JK (2011) RNA-directed DNA methylation. *Curr Opin Plant Biol* 14:142–147.
- Zemach A, et al. (2013) The *Arabidopsis* nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. *Cell* 153:193–205.
- Stroud H, et al. (2014) Non-CG methylation patterns shape the epigenetic landscape in *Arabidopsis*. *Nat Struct Mol Biol* 21:64–72.
- Gapper NE, McQuinn RP, Giovannoni JJ (2013) Molecular and genetic regulation of fruit ripening. *Plant Mol Biol* 82:575–591.
- Osorio S, Scossa F, Fernie AR (2013) Molecular regulation of fruit ripening. *Front Plant Sci* 4:198.
- Manning K, et al. (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat Genet* 38:948–952.
- Zhong S, et al. (2013) Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nat Biotechnol* 31:154–159.
- El-Sharkawy I, Liang D, Xu K (2015) Transcriptome analysis of an apple (*Malus × domestica*) yellow fruit somatic mutation identifies a gene network module highly associated with anthocyanin and epigenetic regulation. *J Exp Bot* 66:7359–7376.
- Telias A, et al. (2011) Apple skin patterning is associated with differential expression of MYB10. *BMC Plant Biol* 11:93.
- Teyssier E, et al. (2008) Tissue dependent variations of DNA methylation and endoreplication levels during tomato fruit development and ripening. *Planta* 228:391–399.
- Liu R, et al. (2015) A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proc Natl Acad Sci USA* 112:10804–10809.
- Lang Z, et al. (2017) Critical roles of DNA demethylation in the activation of ripening-induced genes and inhibition of ripening-repressed genes in tomato fruit. *Proc Natl Acad Sci USA* 114:E4511–E4519.
- Xu Q, et al. (2013) The draft genome of sweet orange (*Citrus sinensis*). *Nat Genet* 45:59–66.
- Xu J, et al. (2017) Dynamic changes in methylome and transcriptome patterns in response to methyltransferase inhibitor 5-azacytidine treatment in citrus. *DNA Res* 24:509–522.
- Wang J, et al. (2016) Regulation of cuticle formation during fruit development and ripening in 'Newhall' navel orange (*Citrus sinensis* Osbeck) revealed by transcriptomic and metabolomic profiling. *Plant Sci* 243:131–144.
- Xi Y, Li W (2009) BSMAP: Whole genome bisulfite sequence MAPPING program. *BMC Bioinformatics* 10:232.
- Bausher MG, Singh ND, Lee SB, Jansen RK, Daniell H (2006) The complete chloroplast genome sequence of *Citrus sinensis* (L.) Osbeck var 'Ridge Pineapple': Organization and phylogenetic relationships to other angiosperms. *BMC Plant Biol* 6:21.
- Lister R, et al. (2008) Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell* 133:523–536.
- Gouli Q, Baulcombe DC (2016) DNA methylation signatures of the plant chromomethyltransferases. *PLoS Genet* 12:e1006526.
- Ausin I, et al. (2012) INVOLVED IN DE NOVO 2-containing complex involved in RNA-directed DNA methylation in *Arabidopsis*. *Proc Natl Acad Sci USA* 109:8374–8381.
- Hsieh TF, et al. (2009) Genome-wide demethylation of *Arabidopsis* endosperm. *Science* 324:1451–1454.
- Zemach A, et al. (2010) Local DNA hypomethylation activates genes in rice endosperm. *Proc Natl Acad Sci USA* 107:18729–18734.
- Tang K, Lang Z, Zhang H, Zhu JK (2016) The DNA demethylase ROS1 targets genomic regions with distinct chromatin modifications. *Nat Plants* 2:16169.
- Li C, Jia H, Chai Y, Shen Y (2011) Absciscic acid perception and signaling transduction in strawberry: A model for non-climacteric fruit ripening. *Plant Signal Behav* 6:1950–1953.
- Xu JD, Xu HD, Xu Q, Deng XX (2014) Characterization of DNA methylation variations during fruit development and ripening of sweet orange. *Plant Mol Biol Rep* 33:1–11.
- Huang H, Liu R, Niu Q, Zhu J, Lang Z (2018) Global increase in DNA methylation during ripening of sweet orange [RNA-seq]. Gene Expression Omnibus. Available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108930>. Deposited December 28, 2018.
- Huang H, Liu R, Niu Q, Zhu J, Lang Z (2018) Global increase in DNA methylation during ripening of sweet orange [BS-seq]. Gene Expression Omnibus. Available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108931>. Deposited December 28, 2018.
- Huang H, Liu R, Zhu J, Lang Z (2018) Global increase in DNA methylation during ripening of sweet orange [ncRNA-seq]. Gene Expression Omnibus. Available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120025>. Deposited December 28, 2018.