Epigenetic reprogramming of H3K27me3 and DNA methylation during leaf-to-callus transition in peach

Beibei Zheng1,2,†, Jingjing Liu1,3,†, Anqi Gao1,3, Xiaomei Chen1,3, Lingling Gao1,3, Liao Liao1,2, Binwen Luo1,3, Collins Otieno Ogutu1,2 and Yuepeng Han1,2,*

1CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, The Innovative Academy of Seed Design of Chinese Academy of Sciences, Wuhan 430074, China
2Hubei Hongshan Laboratory, Wuhan 430070, China
3University of Chinese Academy of Sciences, 19A Yuquanlu, Beijing 100049, China
*Corresponding author. E-mail: yphan@wbgcas.cn
†Equal contribution.

Abstract

Plant tissues are capable of developing unorganized cell masses termed calluses in response to the appropriate combination of auxin and cytokinin. Revealing the potential epigenetic mechanisms involved in callus development can improve our understanding of the regeneration process of plant cells, which will be beneficial for overcoming regeneration recalcitrance in peach. In this study, we report on single-base resolution mapping of DNA methylation and reprogramming of the pattern of trimethylation of histone H3 at lysine 27 (H3K27me3) at the genome-wide level during the leaf-to-callus transition in peach. Overall, mCG and mCHH were predominant at the genome-wide level and mCG was predominant in genic regions. H3K27me3 deposition was mainly detected in the gene body and at the TSS site, and GAGA repetitive sequences were prone to recruit H3K27me3 modification. H3K27me3 methylation was negatively correlated with gene expression.

In vitro culture of leaf explants was accompanied by DNA hypomethylation and H3K27me3 demethylation, which could activate auxin- and cytokinin-related regulators to induce callus development. The DNA methylation inhibitor 5-azacytidine could significantly increase callus development, while the H3K27me3 demethylase inhibitor GSK-J4 dramatically reduced callus development. These results demonstrate the roles of DNA methylation and H3K27me3 modification in mediating chromatin status during callus development. Our study provides new insights into the epigenetic mechanisms through which differentiated cells acquire proliferative competence to induce callus development in plants.

Introduction

Peach (Prunus persica) is a member of the genus Prunus in the Rosaceae family, which consists of many economically important fruit trees, such as strawberry, pear, apple, and various stone fruit species. Peach is diploid, with a haploid genome size of ~230 million bp (Mb), and has a short juvenile phase of 2–3 years, which could make it an ideal system for genetic research in Rosaceae. However, peach is one of the most recalcitrant species with respect to in vitro regeneration. Callus formation is an important step in Agrobacterium-mediated transformation [1], and callus can be used in plant biology research and industrial applications [2, 3]. Although various somatic tissues, such as leaf, stem, and calyx, have been successfully used to induce callus in peach, the most commonly used explant is the mature embryo [4, 5]. The main disadvantage of callus induction of seed-derived material is that the seeds are genotypically different from each other and do not represent their parental genotype due to the heterogeneity of fruit crops. To date, few studies have reported on the molecular mechanism(s) underlying callus development in vegetative tissues of peach and of other fruit tree crops.

Callus development from explants relies on the dramatic cell fate transitions from the somatic state to pluripotency [6]. Auxin and cytokinin are two major hormonal regulators of callus induction from explants of somatic tissues during in vitro culture [7–9]. To date, remarkable progress has been made towards understanding the molecular mechanism underlying hormone-induced callus development in Arabidopsis. Callus development on callus-inducing medium (CIM) is actually through the lateral root-like development pathway, which is regulated by auxin and cytokinin response regulators [10, 11]. Auxin can activate expression of auxin response factors (ARFs) to stabilize the LBD16–bZIP59 complex, leading to both callus development and lateral root formation [12, 13]. The upregulation of WIND1, a wound-induced AP2/ERF transcription factor (TF), can promote callus formation under wound stress,
which induces expression of cytokinin biosynthesis and response genes, resulting in accumulation of endogenous cytokinin prior to callus formation [14]. Overexpression of type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs), which function as TFs in the cytokinin signaling pathway, enhances callus development, and double mutants of arr1 arr12 and of arr1 arr10 display defects in callus development [15, 16]. The CYCD gene, encoding cyclin D, is a potential target of ARRs and its loss-of-function mutation impairs callus development [15].

Callus induction in vitro is associated with wound stress and hormone treatment, which activate many reprogramming regulators that are epigenetically silenced during normal growth [9]. Therefore, much attention has been paid to investigation of the epigenetic regulatory mechanisms underlying wound-/hormone-induced callus development in Arabidopsis [17]. The epigenetic mechanisms consist of DNA methylation, histone modification, differential incorporation of histone variants, and non-coding RNAs, all of which influence genome stability and gene expression [18]. DNA methylation is a form of conserved epigenetic modification that is critical for chromatin stability and gene expression [19].

In plants, DNA methylation occurs in three contexts: CG, CHG, and CHH (where H is A, C or T) [20]. Various DNA methyltransferases have been identified in Arabidopsis. METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) are able to maintain CG and CHG methylation, respectively, while CHROMOMETHYLASE 2 (CMT2) and DOMAINS REARRANGED METHYLASE 2 (DRM2) contribute to CHH maintenance [21, 22]. DNA methylation and demethylation vary during developmental stages [23]. Under conditions of tissue culture, changes in DNA methylation are required for re-activation of cell division, leading to callus development [24]. Moreover, Arabidopsis cmt3 chromomethylase mutations cause significant reduction in CHG context, resulting in a dramatic increase in callus formation capacity [25, 26].

Polycomb repressive complexes (PRCs) are the evolutionarily conserved epigenetic executors in regulating cell fate specification and developmental phase transitions in plants [27]. PRC2 possess the capacity to catalyze the trimethylation of histone H3 at lysine 27 (H3K27me3) [28], and its mutation causes an increase in the competency of somatic embryo induction from vegetative tissues [29, 30]. H3K27me3 is a repressive epigenetic mark associated with genes that are regulated by developmental and environmental cues [31]. Distribution profiles of H3K27me3 are changed when cells convert from a multipotential state to a differentiated state, and vice versa [32, 33]. Genes in the lateral root-like development pathway, which is responsible for callus induction from leaf explants, have been found to decrease H3K27me3 during the leaf-to-callus transition in Arabidopsis [32] and rice [34]. Hence, H3K27me3 is crucial for callus development in plants.

H3K27me3 can be demethylated by the JUMONJI DOMAIN CONTAINING 5 (JMJD5) protein, which belongs to the JmjC domain-only subgroup. Removal of the H3K27me3 mark by JMJD5 has been reported at the promoters of core clock genes such as CIRCADIAN CLOCK ASSOCIATED (CCA) and LATE ELOGATED HYPOCOTYL (LHY) [35, 36] and the flowering time gene, FLOWERING LOCUS C (FLC) [37]. Moreover, JMJD5 can activate the LBD genes to establish root primordia [38], suggesting its potential roles in callus development. However, to date, our knowledge about the impact of chromatin status, in particular of H3K27me3 modifications, on callus induction and development is very limited in peach and in other fruit trees. In this study we report a genome-wide cytosine methylation map as well as genome-wide reprogramming of the H3K27me3 pattern during the leaf-to-callus transition in peach. The study aims to test whether the epigenetic regulatory mechanisms play a critical role during callus formation in peach and to gain an insight into epigenetic mechanisms underlying callus induction from vegetative tissues. The results of this study will be useful for future development of the plant regeneration system from callus induction in peach.

Results
Leaf callus induction and endogenous hormone levels in peach

To understand the process of leaf-derived callus formation in peach, fully expanded leaves were cut and placed on callus-inducing medium. After 8 days of culture, clusters of callus structures were formed (Fig. 1A). To investigate the cellular process of callus induction, longitudinal sections of leaf explants at early stages of callus induction were analyzed using an SEM. A few callus cells were induced in the proximal part of the midvein after 4 days of culture, while numerous cells had proliferated 8 days after culture (Fig. 1B). Consistently, callus tissues covered the proximal part of the midvein 8 days after culture, but no calluses were produced around the cut leaf edges. This suggested that peach callus induction is relatively difficult compared with model plants such as Arabidopsis, which can easily and rapidly produce callus tissues around cut leaf edges [32].

Exogenous hormones determine the developmental fate of explants by regulating the distribution of endogenous hormone levels and triggering hormonal response signaling for cell dedifferentiation [9]. Thus, we measured endogenous hormone levels using high-performance liquid chromatography–mass spectrometry (HPLC–MS). The contents of indole-3-acetic acid (IAA), cis-zeatin (cZ)-type cytokine, and isopentenyladenine (IP)-type cytokine had significantly decreased after 4 days of culture, and then remained stable between 4 and 8 days (Fig. 2A).

The content of trans-zeatin (tZ)-type cytokine showed a decreasing trend throughout the culture period. The ratio of auxin to cytokinin was 50.78 in 0-day explants, and was
increased to 69.91 and 196.55 in 4-day and 8-day samples, respectively (Fig. 2B).

**Time-course transcriptomes of leaves during callus development in peach**

To elucidate the molecular mechanism underlying callus development, transcriptomes of leaf explants at different stages of callus development were compared. We identified 5656 and 2222 DEGs in the 4-day versus 0-day and 8-versus 4-day comparisons, respectively (Fig. 2C). These DEGs were clustered into eight groups based on their temporal expression patterns (Fig. 2D, Supplementary Data Table S1). Group 1 consisted of 203 genes displaying downregulation after 4 days of culture and upregulation after 8 days of culture, while 241 genes from group 3 showed an opposite expression pattern. Groups 2 and 4 contained 1820 and 2318 genes, and displayed down- and upregulation in explants 4 days after culture, respectively, but they both had stable expression between 4 and 8 days. Groups 5 and 6 contained 904 and 470 genes, and showed down- and upregulation, respectively, throughout the culture period. Groups 7 and 8 contained 336 and 471 genes, and they both had stable expression during the first 4 days of culture but showed increasing or decreasing expression after 8 days of culture, respectively. Notably, the majority of DEGs belonged to groups 2 and 4, accounting for 61.2% of the total DEGs. This suggested that most DEGs showed significantly changes in expression after 4 days of culture, but their expression levels were similar between 4 and 8 days of culture.

**Genome-wide DNA methylation landscape during leaf callus development in peach**

We screened the annotation of DEGs and found several methylation-related genes, such as two CMT genes *PpCMT2* (Prupe.5G024100) and *PpCMT3* (Prupe.6G011600). The expression levels of *PpCMT2* and *PpCMT3* showed downregulation by >2- and 3-fold, respectively (Supplementary Data Fig. S1). This suggested a potential role of DNA methylation in mediating callus development in leaf cultures of peach. As mentioned above, the majority of DEGs dramatically changed in expression after 4 days of culture, but had similar levels of expression between 4 and 8 days of culture. Similarly, hormone accumulation showed dramatic changes after 4 days of culture, but with little change between 4 and 8 days of culture. Therefore, leaf explants at the beginning (0) and after 4 days of culture were chosen to investigate genome-wide profiles of DNA methylation using whole-genome bisulfite sequencing (WGBS). The raw read numbers for the bisulfite sequencing libraries varied from 37,566,294
to 48,916,272. After filtering, 36,800,735–47,952,059 clean reads with total size ranging from 10.06 to 13.12 Gb were obtained for each library. The clean reads for each library covered the peach reference genome at ~50× depth. On average, the conversion rate of bisulfite-treated genomic DNA was 99.5%. The average percentages of methylated cytosine in the sequence contents of CG, CHG, and CHH were 49.6, 27.3, and 7.1%, respectively (Supplementary Data Table S2).

Overall, the distribution of the three different types of methylated cytosine was similar between the 0- and 4-day explants. The proportions of mCG, mCHG and mCHH to total mC sites were 37, 37, and 26%, respectively (Supplementary Data Fig. S2). The gene-rich regions showed low levels of DNA methylation, while the transposable element (TE)-rich regions had high levels of DNA methylation in 4-day samples (Fig. 3A and B), and similar distribution profiles were also observed in 0-day samples (Supplementary Data Fig. S3). Subsequently, DNA methylation levels were evaluated in gene body and flanking regions that contained 2 kb downstream and upstream of the transcription end site (TES) and transcription start site (TSS), respectively (Supplementary Data Fig. S4). The CG context had the highest frequency of methylation in the gene body, while both CHG and CHH displayed the highest frequency of methylation in the 2-kb upstream region. Within the gene body, the CG context had higher methylation frequency in introns and exons than both CHG and CHH. The lowest methylation levels at CG, CHG, and CHH sites were observed within regions adjacent to TSS and TES.

The effect of DNA methylation in the gene body and flanking regions on gene transcription was investigated (Fig. 3C). Genes were divided into four sets with no, low, medium, or high expression. As expected, highly expressed genes displayed low methylation levels of mCHG in most genic regions, while an opposite pattern was observed for the non-expressed genes. However, a complicated methylation pattern was detected for both CG and CHH. The non-expressed genes showed a high methylation level of CG in the 2-kb upstream and 2-kb downstream regions, especially at the TSS and TES sites. By contrast, the medium- and high-expressed genes
displayed high methylation levels of CG in the gene body. Notably, the methylation levels of CHH in the 2-kb upstream region were positively correlated with gene expression levels, while a negative correlation occurred in the gene body. Gene expression levels showed no association with the CHH methylation levels in the 2-kb downstream region and around the TSS and TES sites. The positive association of the mCHH methylation level in the 2-kb upstream region with gene expression is consistent with previous reports in rice [55] and apple [41].

As differentially methylated regions (DMRs) are potential functional regions involved in gene transcriptional regulation, DMRs were investigated between 0-day and 4-day explants (Supplementary Data Table S3; Supplementary Data Fig. S5A). We identified 2188 hypomethylated and 1375 hypermethylated DMRs. All DMRs could be divided into three types: mCG-DMRs, mCHG-DMRs, and mCHH-DMRs. Overall, mCHH-DMRs were most abundant, followed by mCG-DMRs and mCHG-DMRs. During leaf callus development, the number of hypomethylated CG- and CHH-DMRs was higher than that of hypermethylated CHG-DMRs, while the number of hypomethylated CHG-DMRs was lower than that of hypermethylated CHG-DMRs in TE and other regions. In the promoter sequences, the number of hypomethylated CHG-DMRs was higher than the number of hypermethylated CHG-DMRs (Supplementary Data Fig. S5B and C). All the 3563 DMRs occurred within 727 gene bodies and 1730 gene promoters. Most of the gene bodies and gene promoters contained only a single type of DMR (Supplementary Data Fig. S5D). A negative correlation between gene expression and DNA methylation was detected for these DMR-related genes, with either promoter containing hypo-mCG/mCHG or gene body containing hypo-mCHG/mCHH or hyper-mCHG (Supplementary Data Fig. S6).

Since DNA methylation in the promoter region has a negative impact on gene expression, we focused on DMRs in gene promoters. A total of 398 DMRs were identified in the promoter proximal to DEGs. Of the 398 DMRs in promoters, 80 were hypermethylated DMRs associated with downregulated DEGs, and 137 were hypomethylated DMRs associated with upregulated DEGs (Fig. 4A). Among the 80 hypermethylated DMRs in promoters, 13, 5, and 62 belonged to mCG, mCHG and mCHH, respectively.
Likewise, 46, 8, and 83 out of the 137 hypomethylated DMRs in promoters belonged to mCG, mCHG, and mCHH, respectively. Notably, 19 out of these 217 DMRs in promoters were identified to be proximal to TFs deposited in the PlantTFDB database (http://planttfdb.cbi.pku.edu.cn/) (Supplementary Data Table S3). Interestingly, one cytokinin response TF, PpARR3 (Prupe.1G494200), is an ARR homolog. In the PpARR3 promoter, DNA methylation level was dramatically decreased after 4 days of culture, which is consistent with the upregulation of its expression by >4-fold (Fig. 4B–D).

**Genome-wide H3K27me3 modification landscape during leaf callus development in peach**

The JMJD5 protein, a well-studied histone demethylase in *Arabidopsis*, is a member of the JMjC-domain-only subgroup that removes the H3K27me3 mark [35]. The JMJD5 gene and its repressors CCA and LHY form a feedback circuit to regulate circadian oscillation. Transcriptome analysis revealed that PpJMJD5 expression was upregulated by >2-fold after 4 days of culture, while the expression levels of PpCCA1 and PpLHY were downregulated by 2.5- and 6.3-fold, respectively (Fig. 5A). This suggested a potential role of H3K27me3 modification in mediating leaf callus development in peach. Hence, ChIP-seq was conducted to investigate the genome-wide H3K27me3 profile during leaf callus development. Overall, the peaks of H3K27me3 at the whole-genome level were mainly enriched in intergenic regions (Supplementary Data Fig. S7A). For the gene regions in peach, the highest levels of H3K27me3 modification were mainly observed either in the gene body or around the TSS, while few peaks were found in flanking regions 3 kb upstream and downstream of the TSS and TES, respectively (Supplementary Data Fig. S7B and C). In addition, TA, GA, and GAGA repetitive sequences were prone to recruit H3K27me3 modification (Supplementary Data Fig. S7D).

A total of 5748 and 2430 genes containing H3K27me3 marks were identified for the 0-day and 4-day explants, respectively. A total of 5114 genes displayed a difference in H3K27me3 enrichment. Among these genes, 4861 showed a decrease in H3K27me3 modification (Supplementary Data Table S4), while 253 genes displayed increased enrichment of H3K27me3. This suggested a decrease in H3K27me3 modification during leaf callus development. Of the 253 H3K27me3-increased
Figure 5. Genome-wide profiling of H3K27me3 methylation and gene expression during peach callus development. (A) Expression levels of PpCCA1, PpLHY, and PpMJD5 at three stages of callus formation. (B) Hierarchical clustering of altered H3K27me3 deposition regions and their corresponding DEGs. (C) Expression profiles (left) and H3K27me3 depositions (right) of PpLBD1, PpSRS1, and PpWOX4. Arrows indicate the transcription direction.

genes, 62 showed a significant difference in expression after 4 days of culture (Supplementary Data Table S4). However, these 62 genes showed no association with callus formation based on the peach reference genome annotation, and they were not included in further analyses. Of the 4861 H3K27me3-decreased genes, 642 belonged to TFs, accounting for 36.2% of total TFs in the peach genome that were deposited in the PlantTFDB database. These H3K27me3-decreased TFs mainly belonged to the AP2, bHLH, MYB, NAC, WRKY, WOX, TCP MADS, GRAS, Orphans, and LOB families (Supplementary Data Table S5).

GO annotation revealed that the 4861 H3K27me3-decreased genes were mainly involved in cytokinin metabolic process, receptor binding, and regulation of gene expression (Supplementary Data Fig. S8). Of these 4861 genes, 1188 showed a significant difference in expression after 4 days of culture (Supplementary Data Table S4). Since the enrichment levels of H3K27me3 have a negative impact on gene expression in peach [56], we focused on a set of 591 genes that exhibited decreased enrichment of H3K27me3 and upregulation after 4 days of culture (Fig. 5B and C). Screening of the 591 genes revealed two relevant to auxin, PpPIN6 (Pruple.8G018200) encoding an auxin efflux carrier (PIN) and PpIAA13 (Pruple.7G247500) encoding a putative auxin-responsive Aux/IAA protein (Fig. 6A). Moreover, a cytokinin oxidase/dehydrogenase (CKX) genes, PpCKX3 (Pruple.7G208400) as well as a cytokinin-induced gene PpCYCD4 (Pruple.6G196300), were found (Fig. 6B). All these auxin- and cytokinin-related genes showed decreased enrichment of H3K27me3 and upregulation after 4 days of culture.

Of the 591 genes, 66 belong to TFs, and some of them are associated with meristematic differentiation. For example, as shown in Fig. 5 and Supplementary Data Fig. S9, three TFs, PpLBD1 (Pruple.6G093900), PpLBD38 (Pruple.8G142100), and PpLOB (Pruple.3G290800), belong to the Lateral Organ Boundaries (LOB) family controlling lateral organ fate. Two TFs, PpSRS1 (Pruple.7G163600) and PpLRP1 (Pruple.2G036500), are members of the Shi-related sequence (SRS) family involved in lateral root primordium. One WUSCHEL (WUS)-related TF, PpWOX4 (Pruple.1G432100), belongs to the HB family related to callus development. Five NAC TFs, PpNAC39 (Pruple.1G454900), PpNAC58 (Pruple.5G221600), PpNAC43 (Pruple.8G074800), PpNAC30 (Pruple.8G058100), and PpNAC25 (Pruple.4G040900), are homologs of Arabidopsis CUP-SHAPED COTYLEDON (CUC) genes regulating shoot apical meristem (SAM) formation and auxin-mediated lateral root formation [57]. These results indicated that variation in H3K27me3 deposition could contribute to changes in the expression patterns of TFs involved in callus development.

Decreased methylation has a positive effect on leaf callus induction in peach

To validate the impact of DNA methylation on leaf callus development, the DNA methylation inhibitor 5-azacytidine was added to callus-inducing medium. After
Figure 6. Expression and methylation changes in auxin- and cytokinin-related genes during callus development in peach.
(A) Expression profiles (top) and H3K27me3 depositions (bottom) of PpPIN6 and PpIAA13 involved in auxin transport and response.
(B) Expression profiles (top) and H3K27me3 deposition (bottom) of PpCKX3 and PpCYCD4 involved in cytokinin degradation and response.

Likewise, to validate the impact of histone methylation on leaf callus development, the H3K27me3 demethylase inhibitor GSK-J4 was added to CIM. After 8 days of culture, no callus development was observed around either the proximal part of the midvein or the cut edges of leaf explants incubated on CIM supplied with GSK-J4 (Fig. 7A). The size of total callus areas in GSK-J4 treated leaf explants was significantly smaller compared with that in mock-treated leaf explants (Fig. 7B). Expression of PpMJD5 was significantly downregulated, while its repressors PpLHY and PpCCA1 showed increased expression upon treatment with GSK-J4 (Fig. 7E). Significant downregulation was also detected for PpPIN6. These results demonstrated a potential role of H3K27me3 demethylation in leaf callus induction in peach.

Discussion

Callus can be induced in vitro by exogenous application of plant hormones, and its developmental fate is determined by the balance between auxin and cytokinin. A high ratio of auxin to cytokinin promotes root formation, while a low ratio facilitates shoot development [8]. In this study, the ratio of auxin to cytokine increased during the process of callus development. The lateral root-related LBD TFs showed a significant decrease in the H3K27me3 level and were thus upregulated during the leaf-to-callus transition. However, Prupe.7G146900, a homolog of the Arabidopsis SAW1 gene controlling leaf development, showed no significant change in either H3K27me3 or DNA methylation levels, but its expression was significantly downregulated after 4 days of culture. These results clearly indicate that CIM-induced peach leaf callus occurs through a lateral root development pathway, in agreement with previous studies [17, 58]. Additionally, callus induction was only observed in the proximal part of the midvein after 8 days of culture. This finding is consistent with a previous report that callus induction on CIM is prone to occur in the pericycle or pericycle-like cells adjacent to the xylem poles through formative or asymmetric divisions [10, 59].

Cell fate transition during callus development requires global reprogramming of DNA methylation. Here, our results showed that the DNA methylation level in the peach genome is lower than those in crops with relatively larger genome sizes, such as tomato [60], apple [41], and cotton [61], but higher than that in Arabidopsis, with a relatively smaller genome size [54]. This confirms the previous finding that DNA methylation levels are positively correlated with genome size [62]. Notably, DNA methylation at the genome-wide level preferentially occurred in the CG and CHH contexts in peach, which is in contrast to the reported predominant mC in the CG context in Arabidopsis [55] and in the CHH context in apple [41], tomato [60], and cotton [61]. In terms of the gene level, DNA methylation predominantly occurred in the CG context in the promoter and gene body regions in peach, which is consistent with the recent finding of CG methylation...
instead of the CHG or CHH methylation predominant in genic regions in peach fruit [63]. Additionally, DNA methylation levels were positively correlated with TE density, but had a negative correlation with gene density in peach, which supports previous reports in other plant species [62, 64, 65].

Besides maintaining genome stability, DNA methylation has an effect on gene expression. In this study, gene expression levels negatively correlated with the frequency of DNA methylation in the CG context of promoter regions, but showed a positive correlation with the frequency of DNA methylation within the gene body, in agreement with a previous report in rice [66]. Interestingly, our study revealed that the DNA methylation inhibitor 5'-azacytidine could significantly increase callus development. This suggests an important role of DNA hypomethylation in callus development. It is well known that the regulatory role of 5'-azacytidine in DNA methylation is attributed to its inhibitory effect on DNA methyltransferases [67]. Our results showed that exogenous hormone application resulted in a decrease in the expression of genes encoding DNA methyltransferases during the leaf-to-callus transition in peach. Hence, the influence of exogenous hormones on epigenetic modifications could be partially due to changes in the expression of DNA methyltransferase genes. The regulatory role of genes encoding DNA methyltransferases in callus formation has also been reported in Arabidopsis, in which mutants of cmt3 [26] and dmr1/dmr2 [69] show a positive effect on callus formation. However, the DNA methyltransferase
Figure 8. A proposed model for epigenetic modification during callus development. DNA methylation-dependent and H3K27me3-dependent callus induction pathways are indicated by dark green and purple arrows, respectively, whereas the DNA methylation- and H3K27me3-independent pathway is indicated by dark orange arrows. Auxin- and cytokinin-related genes are marked with rectangular and hexagonal background shapes, respectively. Significantly up- or downregulated genes are highlighted in red and green colors, respectively, while their original expression at the beginning of culture is shown in black.

genes CMT2 and CMT3, homologs of PpCMT2/3 in this study, are upregulated during the process of callus formation in strawberry [68]. Thus, the role of DNA methyltransferase genes in callus formation is not conserved across plant species. Additionally, 5-azacytidine showed a negative impact on leaf culture in the micromolar concentration range (data not shown). The inhibitory effect of 5-azacytidine on callus formation in the micromolar concentration range has also been reported in wheat [67]. 5-Azacytidine in the nanomolar concentration range shows a positive effect on callus formation in wheat, which is consistent with our finding in this study.

Unlike DNA methylation, H3K27me3 modification was mainly detected in genic regions, including the gene body and the TSS site, but seldom detected in flanking regions during the process of callus induction in peach. This is in agreement with findings previously reported in Arabidopsis [32]. The GAGA factor-binding motif was enriched in H3K27me3 ChIP-seq data in the present study, which is consistent with previous reports in Arabidopsis [70], rice [71], and peach [56]. The genome-scale H3K27me3 pattern is determined by the balance between histone methylation and histone demethylation. Overexpression of JMJD5 regulating diurnal histone modification of clock genes results in a reduction of H3K27me3 deposition [37]. In our study, the expression level of JMJD5 was significantly upregulated after 4 days of culture, and was enhanced by simultaneous downregulation of its repressors CCA1 and LHY, leading to a dramatic reduction in H3K27me3 deposition. This indicates that JMJD5 could serve as an important node for mediating callus development. Expectedly, callus was dramatically reduced by exogenous application of the H3K27me3 histone demethylase inhibitor GSK-J4, which significantly repressed the expression of PpJMJD5. This finding is consistent with the report that GSK-J4 can affect the proliferation and apoptosis of various cancer cells through repressing the expression of JMJD3 (a histone H3K27 demethylase) in animals and humans [72–74]. It is worth noting that H3K27me3 deposition may be transiently diluted during cell division, but the H3K27me3 level can be quickly restored to normal levels [69]. Thus, this dilution by DNA replication is unlikely to be responsible for H3K27me3 demethylation during peach callus formation.

Our results showed that decreased H3K27me3 deposition occurred in TFs associated with plant regeneration, such as SAM-related NACs and LBD/WOX TFs, leading to an increase in their expression. This negative correlation indicated that H3K27me3 methylation participates in the regulation of callus development at the transcriptional level in peach. Additionally, H3K27me3 has been reported to participate in chromatin condensation and transcription silencing [75, 76]. Our study shows that
peach callus development is accompanied by H3K27me3 demethylation at the genome-wide level. This finding is consistent with the fact that cellular dedifferentiation and proliferation during plant tissue culture is associated with large-scale chromatin decondensation [77, 78].

Antagonistic function between H3K27me3 and DNA methylation has been reported in imprinted loci in Arabidopsis [79]. However, DNA and H3K27me3 methylation are likely to be mutually reinforced in a set of functional genes in rice [71]. Here, 153 DEGs were identified to display significant changes in both DNA methylation and H3K27me3 deposition (Supplementary Data Table S4). Among the 153 DEGs, one is PpARR3, which is homologous to Arabidopsis AtARRs involved in callus formation. PpARR3 displayed a decrease in both DNA methylation and H3K27me3 modification during the process of callus formation. Thus, reduced H3K27me3 and DNA hypomethylation seem to reinforce the transcription of PpARR3, suggesting cooperation between H3K27me3 and DNA methylation during peach callus formation.

Although auxin and cytokinin are commonly used to induce callus from explants in vitro, our knowledge of the molecular mechanisms underlying callus development is limited in plants. Comparative transcriptome analysis revealed that the auxin response genes PpARF7 and PpIAA13 and their downstream genes PpLBD16, PpLBD1, and PpSRS1 were upregulated during callus development. Likewise, upregulation was also observed for the cytokinin response gene PpARR3 and its downstream gene PpCYCD4. As mentioned above, the DNA methyltransferase genes CMT2 and CMT3 were downregulated due to exogenous hormones in CIM, and H3K27me3 and DNA methylation were reduced during the process of callus formation. This is likely responsible for the activation of PpARR3, although the concentration of endogenous cytokinin showed a decrease during the process of callus formation. Of these genes, PpCYCD4 is one of the core cell cycle regulators. By contrast, a cell cycle inhibitor, PpKRP, was downregulated during peach callus development, consistent with the previous finding that exogenous auxin and cytokinin can induce cell cycle re-entry from G1 to S phase, leading to proliferation and callus development [78]. Methylation analysis further revealed that three upregulated genes, PpLBD1, PpSRS1, and PpIAA13, showed decreased H3K27me3 deposition, while two upregulated genes, PpARR3 and PpCYCD4, displayed DNA hypomethylation and H3K27me3 demethylation, respectively, during callus development. These results suggest the roles of DNA and histone methylation in mediating cell cycle development in peach. Notably, upregulation of PpLBD16 and PpARF7 showed no association with either DNA hypomethylation or H3K27me3 demethylation. A recent study demonstrated that cellular dedifferentiation is facilitated by the H3K36me3 deposition that is catalyzed by ARABIDOPSIS TRITHORAX-RELATED 2 (ATXR2) in the promoters of LBD genes in Arabidopsis [80]. Therefore, it cannot be excluded that upregulation of PpLBD16 and PpARF7 might be attributed to other types of histone modifications.

Based on all the above results, we propose a model for epigenetic mechanisms underlying leaf callus development in peach (Fig. 8). Downregulation of PpCMTs causes a decrease in DNA methylation, while upregulation of PpIMDS as well as downregulation of its repressors PpCCA1 and PpLHY results in decreased H3K27me3 deposition. H3K27me3 demethylation results in activation of auxin-related regulators, while both DNA hypomethylation and H3K27me3 demethylation could activate cytokinin-related regulators, resulting in callus induction. However, two callus development regulators, PpLBD16 and PpARF7, show no association with either DNA hypomethylation or H3K27me3 demethylation, which may correspond to genetic regulation of callus formation. These results will improve our knowledge of reprogramming of cellular differentiation to regain the proliferative competence required for callus development in plants.

Materials and methods
Plant materials and callus induction
Leaf samples were collected from in vitro-grown proliferating shoot cultures of peach cv. ‘Shengli’ (P. persica), which were maintained on Murashige and Skoog (MS) medium with 1% sucrose at 25°C and humidity of 60–70% under a 16-hours light/8-hours dark cycle. For callus induction, fully expanded leaves were cut into small sections (1 cm × 1 cm) using a disposable knife, and cultured in CIM in darkness at 25°C and 60–70% humidity. The CIM was MS basal medium with vitamins (MS19, PhytoTech, USA) (4.43 g/L), 2,4-dichlorophenoxyacetic acid (2 mg/L), sucrose (30 g/L), agar (8 g/L), and 6-Benzylaminopurine (BA) (0.5 mg/L), pH 5.8.

Measurement of endogenous plant hormones
Approximately 120 mg of fresh callus was frozen and ground into powder in liquid nitrogen, and extracted with a mixture of 80:20 methanol/water (v/v) at 4°C. Endogenous hormones were extracted and quantified by Metware Biotechnology Co., Ltd (Wuhan, China) following a previous report [39]. Each treatment consisted of three biological replicates.

Analysis by scanning electron microscopy
Callus formation from leaf explants was examined using a scanning electron microscope (SEM). The leaf explants were collected and dried with a vacuum freeze-dryer. All samples were prepared and viewed with an SEM (Hitachi TM3030) according to a previous study [40].

Construction of bisulfite sequencing libraries and analysis of differentially methylated regions
Leaf explants at different periods of culture were collected and ground into powder in liquid nitrogen.
Approximately 100 mg of powder was subjected to DNA extraction using the EasyPure Plant Genomic DNA Kit (EE111–01). Bisulfite sequencing libraries were prepared with the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) following a previous report [41]. Library sequencing was conducted on the Illumina Hiseq 2500 platform with sequencing depths >50x. Differentially methylated regions (DMRs) were analyzed with DSS software (version DSS_2.12.0) [42], and regions with at least four methylated cytosines were considered as DMRs [41]. The Circos plot was drawn using R software (version 3.5.3). Each treatment contained three biological replicates.

McrBC–PCR analysis was performed according to a previous study [43] and primer sequences were listed in Supplementary Data Table S6. Briefly, ~1 μg of genomic DNA was digested with 20 units of restriction endonuclease (NEB, cat. no. M0272S). The McrBC-digested template along with a mock digested template was subjected to PCR analysis.

Analysis of chromatin immunoprecipitation sequencing
Chromatin immunoprecipitation (ChIP) was carried out with the Universal Plant ChIP-seq kit (Diagenode, stock number C01010152) following a previous study [44]. Briefly, ~5 g of leaf explant was vacuum-infiltrated five times for 15 minutes in cross-linking solution at room temperature. After stopping treatment, the explants were ground to fine powder in liquid nitrogen. The extracted chromatin was sheared by sonication into fragments ranging from 200 to 500 bp. The sonication was carried out with 10 cycles of 30 seconds on/off using Bioruptor™ UCD 200 (Diagenode, Sparta, NJ). Chromatin-associated DNA was immunoprecipitated using mouse H3K27me3 monoclonal antibody (Abcam, stock number ab6002).

DNA sequencing was carried out using an Illumina HiSeq2000 platform at Beijing Genomics Institute (BGI, Shenzhen, China). Clean reads with a minimum quality score of Phred20 or Q20 were aligned against the reference genome of peach using TopHat v2.0.12 [47]. The numbers of reads matched to single genes were counted using the HTSeq v0.6.1 program [48]. The level of gene expression was estimated based on the FPKM (fragments per kilobase of transcript per million mapped reads) values. Genes were defined to be differentially expressed when they had a fold change of >2 or <0.5 as well as an adjusted P-value of <0.05. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment and Gene Ontology (GO) for differentially expressed genes (DEGs) were analyzed using the GOseq R package [49] and KOBAS software [50], respectively. The UPGMA method was selected to analyze hierarchical clustering.

Real-time PCR analysis was performed using Hieff® qPCR SYBR Green Master Mix (Yeasen, stock number 11203ES03). The relative level of gene expression was normalized according to the value of the internal control gene PpTEF2 [51]. The sequences of primers used are listed in Supplementary Data Table S6.

GSK-J4 and 5-azacytidine treatments during peach leaf callus induction in vitro
DNA methylation alterations were achieved using DNA methyltransferase inhibitor 5-azacytidine [52] (Sigma–Aldrich, CAS number 320-67-2). To test the effect of 5-azacytidine on callus induction, leaves were incubated on CIM with 10 nM 5-azacytidine. Changes in histone modification patterns were achieved using the histone lysine demethylase inhibitor GSK-J4 [53]. To test the impact of GSK-J4 on callus induction, CIM was supplemented with 24 μM GSK-J4 (Targetmol, CAS number 1797983-09-5), in the micromolar concentration range commonly used in previous studies [54]. CIM containing DMSO was used as control. For these drug treatments and control, the same batches of fully expanded leaves were collected from the above-mentioned in vitro clonal propagation shoots, cut into small pieces and randomly used for callus induction at the same time. Three biological replicates were conducted for both 5-azacytidine and GSK-J4 treatments.

Acknowledgements
This project was supported by funds received from the National Key R&D Program of China (2019YFD1000800), the National Natural Science Foundation of China (3190200 and 31872087), and the Special Fund for Strategic Pilot Technology of the Chinese Academy of Sciences (XDA24030404-4).

Author contributions
Y.H. and B.Z. planned and designed the experiments. J.L., B.L., A.G., X.C., and B.Z. performed the experiments. L.L.
and L.G. performed the sequence analysis. B.Z., C.O.O., and Y.H. wrote the paper.

Data availability
The raw sequencing reads have been deposited in the Sequence Read Archive (SRA) database of NCBI under accession number PRJNA819103.

Conflict of interest
The authors declare no competing interests.

Supplementary data
Supplementary data is available at Horticulture Research online.

References
1. Ricci A, Sabbadini S, Prieto H et al. Genetic transformation in peach (Prunus persica L.): challenges and ways forward. Plants (Basel). 2020;9:971.
2. Effrth T. Biotechnology applications of plant callus cultures. Engineering. 2019;5:50–9.
3. Feher A. Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front Plant Sci. 2019;10:536.
4. Xu S, Lai E, Zhao L et al. Development of a fast and efficient root transgenic system for functional genomics and genetic engineering in peach. Sci Rep. 2020;10:2836.
5. Pérez-Jiménez M, López-Soto MB, Cos-Terrer J. In vitro callus induction from adult tissues of peach (Prunus persica L. Batsch). In Vitro Cellular & Developmental Biology - Plant. 2013;49:79–84.
6. Ikeuchi M, Ogawa Y, Iwase A et al. Plant regeneration: cellular origins and molecular mechanisms. Development. 2016;143:1442–51.
7. Ikeuchi M, Sugimoto K, Iwase A. Plant callus: mechanisms of induction and repression. Plant Cell. 2013;25:3159–73.
8. Su YH, Liu YB, Zhang XS. Auxin-cytokinin interaction regulates meristem development. Mol Plant. 2011;4:616–25.
9. Ikeuchi M, Favero DS, Sakamoto Y et al. Molecular mechanisms of plant regeneration. Annu Rev Plant Biol. 2019;70:377–406.
10. Atta R, Laurens L, Boucheron-Dubuisson E et al. Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. Plant J. 2009;57:626–44.
11. Sugimoto K, Jiao Y, Meyerowitz EM. Arabidopsis regeneration from multiple tissues occurs via a root development pathway. Dev Cell. 2010;18:463–71.
12. Fan M, Xu C, Xu K et al. LATERAL ORGAN BOUNDARIES DOMAIN transcription factors direct callus formation in Arabidopsis regeneration. Cell Res. 2012;22:1169–80.
13. Xu L. De novo root regeneration from leaf explants: wounding, auxin, and cell fate transition. Curr Opin Plant Biol. 2018;41:39–45.
14. Ikeuchi M, Rymen B, Sugimoto K. How do plants transduce wound signals to induce tissue repair and organ regeneration? Curr Opin Plant Biol. 2020;57:72–7.
15. Ikeuchi M, Iwase A, Rymen B et al. Wounding triggers callus formation via dynamic hormonal and transcriptional changes. Plant Physiol. 2017;175:1158–74.
16. Argyros RD, Mathews DE, Chiang YH et al. Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. Plant Cell. 2008;20:2102–16.
17. Lee K, Seo Pj. Dynamic epigenetic changes during plant regeneration. Trends Plant Sci. 2018;23:235–47.
18. Saze H, Tsugane K, Kanno T et al. DNA methylation in plants: relationship to small RNAs and histone modifications, and functions in transposon inactivation. Plant Cell Physiol. 2012;53:766–84.
19. Osakabe A, Adachi F, Arimura Y et al. Influence of DNA methylation on positioning and DNA flexibility of nucleosomes with pericentric satellite DNA. Open Biol. 2015;5:150128.
20. Stroud H, Do T, Du J et al. Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. Nat Struct Mol Biol. 2018;21:64–72.
21. Stroud H, Greenberg MVC, Feng S et al. Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. Cell. 2013;152:352–64.
22. Zemach A, Kim MY, Hsieh PH et al. The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell. 2013;153:193–205.
23. Teysier E, Bernachia G, Maury S et al. Tissue dependent variations of DNA methylation and endoreduplication levels during tomato fruit development and ripening. Planta. 2008;228:391–9.
24. Stelpflug SC, Eichten SR, Hermanson PJ. LATERAL ORGAN BOUNDARIES DOMAIN H3K27me3 and H3K4me3 deposition in callus and seedling reveal the epigenetic regulatory mechanisms involved in callus formation. Front Genet. 2020;11:766.
25. Jones MA, Covington MF, DiTacciio L et al. Jumonji domain protein JMD5 functions in both the plant and human circadian systems. Proc Natl Acad Sci USA. 2010;107:21623–8.
36. Lu SX, Knowles SM, Webb CJ et al. The Jumonji C domain-containing protein jmjC30 regulates period length in the Arabidopsis circadian clock. Plant Physiol. 2011;155:906–15.

37. Gan ES, Xu Y, Wong JY et al. Jumonji demethylases moderate precocious flowering at elevated temperature via regulation of FLC in Arabidopsis. Nat Commun. 2014;5:5098.

38. Okushima Y, Fukaki H, Onoda M et al. ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. Plant Cell. 2007;19:118–30.

39. Wang J, Wu B, Lu K et al. The amino acid permease 5 (OsAAP5) regulates tiller number and grain yield in rice. Plant Physiol. 2019;180:1033–45.

40. Xu L, Xu Y, Dong A et al. Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMME-TIC LEAVES1 and 2 and RLECTA functions in specifying leaf adaxial identity. Development. 2003;130:4097–107.

41. Xu J, Zhou S, Gong X et al. Single-base methylome analysis reveals dynamic epigenomic differences associated with water deficit in apple. Plant Biotechnol J. 2018;16:672–87.

42. Park Y, Wu H. Differential methylation analysis for BS-seq data under general experimental design. Bioinformatics. 2016;32:1446–53.

43. Quadrana L, Almeida J, Asís R et al. Natural occurring epialleles determine vitamin E accumulation in tomato fruits. Nat Commun. 2014;5:3027.

44. Goslin K, Zheng B, Serrano-Mislata A et al. Transcription factor interplay between LEAFY and APETALA1/CAULIFLOWER during floral initiation. Plant Physiol. 2017;174:1097–109.

45. Li H, Durbin R. Fast and accurate long-read alignment with burrows-wheeler transform. Bioinformatics. 2010;26:589–95.

46. Robinson JT, Thorvaldsdottir H, Winckler W et al. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–6.

47. Kim D, Pertea G, Trapnell C et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013;14:R36.

48. Anders S, Pyl PT, Huber W. HTSeq – a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166–9.

49. Young MD, Wakefield MJ, Smyth GK et al. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 2010;11:R14.

50. Mao XZ, Cai T, Olyarchuk JG et al. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics. 2005;21:3787–93.

51. Tong Z, Gao Z, Wang F et al. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol. 2009;10:71.

52. Schapira M, Arrowsmith CH. Methyltransferase inhibitors for modulation of the epigenome and beyond. Curr Opin Chem Biol. 2016;33:81–7.

53. Heinemann B, Nielsen JM, Hudlebusch HR et al. Inhibition of demethylases by GSK-J1/J4. Nature. 2014;514:E1–2.

54. Hofstetter C, Kampka JM, Huppertz S et al. Inhibition of KDM6 activity during murine ESC differentiation induces DNA damage. J Cell Sci. 2016;129:788–803.

55. Wang GQ, Li H, Meng S et al. Analysis of global methylation and gene expression during carbon reserve mobilization in stems under soil drying. Plant Physiol. 2020;183:1809–24.

56. Fuente I, Conesa A, Lloret A et al. Genome-wide changes in histone H3 lysine 27 trimethylation associated with bud dormancy release in peach. Tree Genet Genomes. 2015;11:45.
76. Ay N, Irmler K, Fischer A et al. Epigenetic programming via histone methylation at WRKY53 controls leaf senescence in Arabidopsis thaliana. Plant J. 2009;58:333–46.
77. Dominguez M, Berger F. Chromatin and the cell cycle meet in Madrid. Development. 2008;135:3475–80.
78. Grafi G, Florentin A, Ransbotyn V et al. The stem cell state in plant development and in response to stress. Front Plant Sci. 2011;2:53.
79. Weinhofer I, Hehenberger E, Roszak P et al. H3K27me3 profiling of the endosperm implies exclusion of polycomb group protein targeting by DNA methylation. PLoS Genet. 2010;6:e1001152.
80. Lee K, Park OS, Seo PJ. Arabidopsis ATXR2 deposits H3K36me3 at the promoters of LBD genes to facilitate cellular dedifferentiation. Sci Signal. 2017;10:eaan0316.