An Efficient Chromatin Immunoprecipitation (ChIP) Protocol for Studying Histone Modifications in Peach Reproductive Tissues

Monica Canton

Università degli Studi di Padova: Università degli Studi di Padova

Silvia Farinati

National Research Council: Consiglio Nazionale delle Ricerche

Cristian Forestan

Università di Bologna: Università di Bologna

Justin Joseph

Università degli Studi di Padova: Università degli Studi di Padova

Claudio Bonghi (✉ claudio.bonghi@unipd.it)

Università degli Studi di Padova: Università degli Studi di Padova <https://orcid.org/0000-0002-7100-8160>

Serena Varotto

Università degli Studi di Padova: Università degli Studi di Padova

Research

Keywords: ChIP assay, Peach, Floral buds, Fruit, H3K4me3, H3K27me3

Posted Date: December 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1170021/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Plant Methods on March 31st, 2022. See the published version at <https://doi.org/10.1186/s13007-022-00876-0>.

Abstract

Background

Perennial fruit trees display a perennial growth behaviour characterized by an annual cycling between growth and dormancy, with complex physiological features. Rosaceae fruit trees represent excellent models for studying not only the fruit growth/patterning, but also the progression of the reproductive cycle depending upon the impact of climate conditions. In addition, the current development of high-throughput technologies is starting to have an important impact on Rosaceae tree research for investigating genome structure and function as well as (epi)genetic mechanisms involved in important developmental and environmental response processes during fruit tree growth. Among the epigenetic mechanisms, chromatin remodelling mediated by both histone modifications and other chromatin-related processes play a crucial role in gene modulation, controlling gene expression process. A very useful technique to investigate the chromatin states in plants and their dynamics is chromatin immunoprecipitation (ChIP), generally applied for studies on chromatin states and enrichment in post transcriptional modifications (PTMs) of histone proteins.

Results

Because peach is a model in Rosaceae family for studies in bud formation, dormancy and organ differentiation for climacteric fruits, in our work, we primarily established specific protocols for chromatin extraction and immunoprecipitation in reproductive tissues of peach *Prunus persica*. Subsequently focused our investigations on the role of two chromatin marks, namely trimethylation of histone H3 at lysine in position 4 (H3K4me3) and trimethylation of histone H3 at lysine 27 (H3K27me3) on modulating specific gene expression. Bud dormancy and fruit growth were investigated in a nectarine genotype called Fantasia as a model system.

Conclusions

Here we presented general strategies to systematically optimize ChIP protocols for buds and mesocarp tissues and analyzed the correlation between gene expression and chromatin mark enrichment/depletion. Confirming like histone modifications are implicated in regulating bud dormancy progression and the core ripening genes.

Background

The edible Rosaceae crops display an extraordinary spectrum of fruit types, including fleshy peach, apple, and strawberry, that provide unique contributions to a healthy diet for consumers and represent an important source of an array of secondary metabolites with tangible roles in reducing risks for different diseases [1]. Amongst these, Rosaceae fruit trees represent excellent models for studying not only the fruit growth/patterning, but also the progression of the reproductive cycle depending upon the impact of climate conditions. In fact, in the majority of temperate or boreal species, the reproductive cycle, from

flower bud initiation to fruit/seed maturation, is completed in two years after experiencing cold temperature to overcome the winter rest period, called dormancy. To date the reproductive cycle has been largely investigated both in physiological and molecular terms, but not in a comprehensive manner. As an initial broad observation, these studies established that the reproductive cycle resulted from coordinated changes in expression of hundreds to thousands of genes involved in the regulation of structural (cells/tissues differentiation of flower and fruit) and metabolic (fruit metabolites and hormones) traits of reproductive organs [2, 3]. However, the attention was mainly paid to the fruit and to its ripening phase: in fact, in literature, there is an impressive number of studies focused on understanding the main processes that take place during the progression from an immature to ripe fruit, whereas the earlier phases, corresponding to the pre-pollination stage are not well clarified [4]. In particular, the comprehension of bud dormancy phase is strategic when considering the impact of climate change on plant physiology. Dormancy is an evolutionary process that is entrusted to temperature resilient structures such as buds and can be interpreted as “a state of self-arrest of the shoot apical meristem (SAM) which is maintained under growth-promoting conditions” [5, 6]. Therefore, the dormancy mainly ensures survival under extremely low temperatures during winter and it can also influence developmental functions, including fruit setting and patterning, when inadequate temperature compromises overcoming dormancy [7].

The emerging progress of high-throughput methods and bioinformatics technologies for analysing genome structure and function has had an important impact on research in fruit trees and has significantly contributed towards accelerating the discovery of specific DNA regulatory elements that interact with transcription factors (TFs) responsible for plant growth and responses to plant-environment interaction [8]. For instance, whole genome sequencing projects of two fruit crop species, namely peach and apple, has fostered in-depth molecular studies in *Prunus* and *Malus* species over recent years [9–11], allowing the identification of factors which interact in a multilevel process and triggers the coordinated action of master regulators, including hormone signalling, microRNAs, and epigenetic mechanisms [12]. Among the latter, chromatin-remodelling mechanisms, mediated by both histone modifications and other chromatin-related processes, play a crucial role in gene modulation, by influencing the ability of transcription factors (TFs) to bind DNA regulatory elements and thereby controlling gene expression [13]. However, the vast majority of plant cis-elements in gene promoters are unknown [14], because optimised experimental protocols for recovering nucleic acids to be utilized for genome-wide analyses are still lacking.

A very useful technique to investigate DNA-protein interaction and chromatin states and their dynamics is chromatin immunoprecipitation (ChIP). It rely on the use of a specific antibody raised against the target transcription factor or the histone modification under investigation and it is widely used for a few model systems, including *Arabidopsis*, though the establishment of this technique is still remarkably challenging in other plant systems [15, 16]. In plants, ChIP is generally applied for studies on chromatin states and enrichment of post transcriptional modifications (PTMs) in histone during development or stress response mechanisms proteins [17]. The integration of expression data, at either single gene or genome-wide level, with histone PTM enrichments in specific gene contexts can unveil possible direct correlations between gene transcriptional variations and histone modification dynamics, during plant tissue

differentiation and development. Recent studies on fruit tissues revealed that a native chromatin immunoprecipitation protocol (N-ChIP), performed without cross-linking, is better for profiling histones and histone modifications studies for improved antibody specificity, higher pull-down efficiency, lower background and less bias when compared to an X-ChIP (cross-linked chromatin followed by immunoprecipitation) procedure [18].

In this work, we describe specific protocols for ChIP procedure in different reproductive tissues (flower buds and fruit) of *Prunus persica* with the aim to investigate histone marks distributions. Interestingly, histone modifications have been implicated in regulating both bud dormancy and fruit development progression [19, 20] and peach represents a model in the Rosaceae family for studies of biological processes like bud formation/dormancy and differentiation/development of climacteric fruits [10]. Here we describe in detail a method for chromatin extraction and immunoprecipitation to systematically optimize an X-ChIP protocol, suitable for subsequent gene target (ChIP-qPCR) and genome wide analyses (ChIP-seq). Our aim is to figure out a possible correlation between gene expression and presence of specific chromatin marks. We focused our investigations on the role of two chromatin marks (described in detail in the Results section), namely trimethylation of histone H3 at lysine in position 4 (H3K4me3) and trimethylation of histone H3 at lysine 27 (H3K27me3), in modulating specific gene expression during these fundamental biological processes in the nectarine cultivar Fantasia.

Results And Discussion

ASSESSMENT OF CHROMATIN QUALITY: EFFICIENCY CHECK OF CHROMATIN FIXATION/EXTRACTION AND FRAGMENTATION

We have developed and optimized an immunoprecipitation protocol on crosslinked chromatin (X-ChIP) suitable for analysing either genome wide distributions and/or specific distribution at target loci of single post-transcriptional histone modifications, in peach reproductive tissues. One of the main advantages of the procedure proposed here is its applicability to hard plant tissues (i.e. FB and FM) stored at -80°C after freezing in liquid nitrogen.

Previous studies have described how the success of a ChIP procedure depends upon both the nature of the starting material and the initial processing steps. In these studies, the use of fresh and unfrozen plant tissue as starting material for the chromatin extraction and following analyses has often been strongly recommended [21]. Moreover, the use of vacuum mediated infiltration has also suggested to ensure an efficient penetration of the fixative for the crosslinking reaction into the plant tissue; the crucial step which distinguishes a N-ChIP from X-ChIP procedure [16, 18]. However, in both methods preserving chromatin structure during the isolation and subsequent steps is the main aim of the procedure. For this reason, the cross reaction with the fixation agent (in our case formaldehyde) in a X-ChIP strategy was the

initial step to be evaluated and optimized in our experiments. Insufficient crosslinking will not preserve the chromatin structure, while over crosslinking will hamper the ChIP procedure as reported by [22].

In Fig. 1A, the efficiency check conducted to determine the optimal crosslinking conditions for FB and FM tissues is reported. In FB, the developed protocol for chromatin fixation and extraction was very efficient in all three stages (0, 475 and 770CU) during endodormancy. With the progression of endodormancy buds have an increase in number of scales and maturity degree while also accumulating soluble carbohydrates which hinder chromatin extraction. Bud scales act as protection for newly formed leaves and branch outgrowth. They form at the end of the growth season once the leaves have fallen off the branches. By removing the scales, the procedure was more efficient for all time points (Fig. 1A). Despite the absence of scale removal from buds in other published protocols [16], this process allowed to shorten the number of chromatin extraction steps and avoids the use of β -mercaptoethanol in our experiments.

For FM, the efficiency of chromatin fixation and following extraction steps depended on fruit developmental stage. In fruit pre-ripening phase, from the onset to the end of the S3 phase, the chromatin extraction was reasonable: the extraction was satisfactory in FM collected at 83, 104 and 111 DAFB, when the fruit expansion and endoreduplication processes occur [20]. Indeed, during these stages, the accumulation of sugars and other metabolites is reduced due to the high energy requirement for the expansion of FM cells [23]. On the contrary, in samples collected at 118 and 125 DAFB the high level of polysaccharides and other secondary metabolites stored showed an inhibitory effect on the extraction phase, causing a dramatic reduction in terms of quality and yield (data not shown) as also reported in other works [19, 24, 25]. However, considering both the cellular uniformity of the mesocarp tissue and the general technical limits of the procedure, the chromatin extraction procedure was performed after pooling three biological replicates and the results are reported in the following sections.

According to other works conducted on different plant tissues [22], our results with FB and FM indicated that the addition of formaldehyde 1% (v/v) into the fixation starting buffer is efficient for the following ChIP steps, since the chromatin is neither over- or under-crosslinked, and it allows to recover a substantial amount of DNA from the reverse crosslinking step (Fig. 1A).

The size of the chromatin fragments used as input material is the second determinant factor for the resolution and success of a ChIP procedure since a proper size distribution of DNA fragments is crucial for specific ChIP applications. Ideally, the bulk of the chromatin for the following application includes a length between 250 and 750 bp but, depending on the intended use of ChIP applications (gene target vs whole sequencing), an appropriate shearing step must be determined for each chromatin preparation. In Fig. 1B we report the best results obtained after testing different sonication conditions, in terms of number, timing and amplitude of rounds. We decided to use a sonication procedure vs an enzymatic MNase-mediated fragmentation since when using formaldehyde crosslinking the access to chromatin is restricted [26]. After testing various sonication conditions, we used 60% amplitude with a number of rounds ranging between 15 (for FM) and 25 (for FB) for 10 sec each. These conditions allowed to obtain a physical shearing of chromatin compatible with the following purposes. For our samples a smear

between 200 and 500 bp was observed for FB and between 200 and 800 bp for FM. In both cases, the fragmentation was suitable for the following molecular analyses and, in particular, the higher fragmentation of chromatin obtained from buds allowed for a successful library preparation for ChIP-Seq analysis accordingly [16].

ASSESSMENT OF CHROMATIN IMMUNOPRECIPITATION ASSAY BY QPCR AND ChIP BY SEQUENCING IN REPRODUCTIVE PEACH TISSUES

After the application of specific precautions in the chromatin fixation/extraction protocol the quality and the pull-down efficiency of the immunoprecipitation was tested. The aim was to define whether our operative changes specific for each peach plant material, affected the results of the following molecular procedures to be performed.

To monitor chromatin states and find out potential correlations between gene transcript levels and the enrichment in specific histone modification, two euchromatin regulative histone marks of interest were examined: trimethylation of lysine in position 4 of histone H3 (H3K4me3), which represents an active mark typically enriched around TSS of transcribed genes, and H3K27me3, a silencing mark generally distributed over the whole gene coding region [27–29].

For the mesocarp tissue, as target gene, we focused the attention on *PpFLESHY* (PRUPE_6G159200), also known as HECATE3 (HEC3)-like, a TF with a putative key role in fleshy fruit mesocarp tissue identity [30]. In the FAN fruits, *PpFLESHY* did not exhibit relevant variations in its expression level during S1 and S2, while its transcription increased at S3 and highly accumulated at early S4 [30]. This expression pattern was reconfirmed by our investigations on *FLESHY* transcript levels during different fruit developmental phases (Fig. S1). After chromatin immunoprecipitation with the two specific Abs against H3K4me3 and H3K27me3 modified histones, qPCR Real Time assays were performed to verify and semi-quantify the enrichment of *FLESHY* in ChIPed DNA populations, following the indication reported by [21] (Fig. 2). Based on the different typical distribution patterns of H3K4me3 and H3K27me3 histone marks along the gene sequences, we analysed the *FLESHY* sequence by considering its predicted 'TSS around-' and 'gene body-' subregions and using four couples of primers (A, B couples for 'TSS around-' and C, D couples for 'gene body-' subregions. For details see table S1). qPCR results demonstrated a significant enrichment of H3K4me3 activation mark at the level of *FLESHY* 'TSS around-', in comparison to that measured in the 'gene body-' subregion (dark vs white bars). Additionally, whereas no enrichment was observed at the 'gene body' during 83, 104, and 111 DAFB, a higher and significant increase (relative to input), ranging from 4,3% up to 7%, was measured at the 'TSS around' region (Fig. 2A). The H3K4me3 preferential enrichment at TSS level is in agreement with data reported in literature in other species and tissues [29, 31, 32].

An opposite trend was observed for the silencing mark H3K27me3: a lower enrichment, in terms of % IP of this mark was observed in all time points in comparison to that observed for the activating mark

H3K4me3 in both the investigated gene regions. However, at TSS level a small reduction of H3K27me3 was measured during the progression of fruit growth, with values ranging from 1.4–0.5%, while at gene body level a comparatively lower and constant signal was measured with a less pronounced fold change (Fig. 2B). To exclude a reduced Ab efficiency, we analysed in the same mesocarp samples, the putative enrichment of H3K27me3 into a Polygalacturonase family member (*ppePG22*, PRUPE_4G262200), known to be a representative silenced locus in these same developmental stages (Fig. S2), confirming the enrichment of this histone mark along the whole gene sequence.

In parallel, we investigated the enrichment of the same histone modification marks H3K4me3 and H3K27me3 in FB tissues by performing chromatin immunoprecipitation followed by Illumina sequencing (ChIP-Seq). In brief, The ChIP-Seq raw reads were processed for adapter clipping and quality score trimming using Trimmomatic v 0.39 [33]. Clean reads were mapped to the *P. persica* genome v.2.0 [9] obtained from Ensembl (<http://plants.ensembl.org/index.html>) using the spliced aligner HISAT2 [34]. ChIP-Seq enrichment was calculated using Model-based Analysis of ChIP-Seq (MACS) [35].

For each histone modification, we calculated the enriched peaks by comparing the time points in pairs encompassing six different comparisons (0CU vs 475CU; 475CU vs 0CU; 475CU vs 770CU; 770CU vs 475CU; 0CU vs 770CU and 770CU vs 0CU). The nearest gene was called after peak calling. Differentially expressed genes (DEGs; $p \text{ adj} < 0.05$) identified in a previous RNA-Seq analysis (see *Availability of data and materials* section) data were associated to the called genes that were H3K4me3 and H3K27me3 enriched and the putative function for each gene was deduced from the *Arabidopsis thaliana* homolog. Transcript sequences were scanned by blastx against UniProt/Swiss-Prot and UniProt/ TrEMBL to homology search. Joined biological replicates were loaded into the Integrative Genomics Viewer (IGV) genome browser to visualize both H3K4me3 and H3K27me3 peaks along with gene expression (RNA-Seq) peaks in the peach genome (Fig. 3) in which we reported the expression profile of some epigenetic regulator genes identified as marker genes [3] associated to their histone modification profile during endodormancy and endo-ecodormancy transition.

As far as it concerns genes enriched in H3K4me3 (Fig. 3A), with the progression of endodormancy, an active transcription of the CHROMOMETHYLASE 3 (CMT3-like, PRUPE_6G011600) and a HDA19-like gene (PRUPE_8G183700) was observed. The DNA methyltransferase CMT3 maintains CHG (H = A, C, or T) methylation at constitutive heterochromatin in plants and thus it is important for maintaining genome stability [36]. Histone deacetylase 1 of Arabidopsis (AtHD1 or AtHDA19), a homolog of yeast RPD3, is known to be a global regulator of many physiological and developmental processes [37].

A correlation between gene expression and H3K4me3 mark deposition was observed also for the Methyl-CPG-binding domain 8 gene (MBD8-like, PRUPE_8G062800). During bud dormancy, for this gene a depletion of H3K4me3 is associated with a decrease in MBD8-like transcript accumulation. MBDs are proteins with a putative Methyl-CpG-Binding that are considered to function as interpreters of DNA methylation signals [38].

The expression pattern and H3K4me3 distribution at loci of the reported genes indicate that bud dormancy overcoming is accompanied by the chromatin control of transcriptionally active regions through the enrichment in the H3K4me3 mark.

Regarding H3K27me3, our results indicate that there is not a good correlation between gene expression and chromatin enrichment in H3K27me3 (Fig. 3B). Although this histone mark is responsible for PCR2 mediated gene silencing in euchromatic regions, the enrichment in H3K27me3 does not always correlate with gene silencing or with a low gene expression. This is also the case of some selected chromatin modifiers, such as ATXR7-like (PRUPE_2G42400 a putative ARABIDOPSIS TRITHORAX-RELATED7 a putative Set1 class H3K4 methylase) [39], SUVR2-like (PRUPE_8G216300 a putative SU(VAR)3-9-like histone methyltransferase) [40] and JMJ24-like (PRUPE_6G322900 a jmjC histone demethylase possibly involved in gene silencing) [41]. All of them present a significant decrease in H3K27me3 mark in their chromatin, during dormancy progression; however, this does not correlate to their transcript level. In the case of flower bud, this missing correlation might be due to the strong tissue specificity of H3K27me3 chromatin mark [42] while flower buds are composed of different specialized tissues. However, this lack of correlation was observed by other authors and in other plant tissues.

Conclusions

ChIP represents a powerful tool in the study of histone modification status in plant tissues. Many examples in literature strongly support the indispensably important role of the ChIP approach in the field of chromatin research. In this report, we describe an optimized X-ChIP procedure for recalcitrant plant tissues because of their structural properties and composition, such as peach reproductive tissues. To overcome some methodological obstacles for investigating specific chromatin modifications occurring in the reproductive organs during their development, we developed an efficient and reproducible protocol for the chromatin fixation and extraction, applying changes that affect positively the following molecular investigations (Fig. 4). The general aim was to provide an affordable and repeatable procedure for studying the distribution of modified histones in buds and fleshy fruits or other plant organs/tissues with high levels of polysaccharides, secondary metabolites (like phenols), high content of water and large vacuoles.

One advantage of the developed ChIP protocol is to avoid the step of clean nuclei isolation and the possibility to use frozen tissues for the extraction of chromatin, without the addition of β -mercaptoethanol, as recommended by other procedures [16, 18, 43].

In addition, our procedure was optimized for the following molecular investigations and allowed to analyse the distribution of histone marks at single gene level by qPCR or at genome wide level by NGS sequencing. By integrating the information on gene expression and the enrichment/depletion of specific modified histone at selected loci, we obtained information on the effect of a histone modification on gene expression and correlated the expression variation with variations in chromatin marks distribution and enrichment during fruit growth/ripening and bud dormancy in peach.

Results pointed out that, similar to what was observed for other species [12, 44], in the reproductive cycle of peach, from bud to fruit, epigenetic regulation plays a fundamental role through the coordination of the response to endogenous and environmental signals.

Materials And Methods

PLANT MATERIAL

Ten-year old trees of *Prunus persica* (L. Batsch), cv. Fantasia (FAN), cultivated following the standard horticultural practices, in the experimental farm 'L. Toniolo' of the University of Padova, Italy (GPS coordinates: 45°20'48.9" 110 N 11°57'00.3" E) were used for buds and fruits sampling.

Flower buds (FB) were collected at three time points during the winter 2018 and 2019 (05/11/2018, 10/12/2018, 07/01/19), corresponding to 0, 475, and 770 chilling units (CU) respectively, calculated as described by [45]. Daily temperature readings were retrieved from Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto (ARPAV; <https://www.arpa.veneto.it/>). At each time point, buds were collected from groups of 3-4 plants each, corresponding to two (for 0CU and 770CU) and three (for 475CU) biological replicates, their scales were removed and immediately frozen in liquid nitrogen for their storage at -80° C until subsequent molecular analyses. Fruits were collected following the fruit growth by measuring the equatorial diameter. The fruit double sigmoidal growth kinetics have been divided in four phenological stages, named S1, S2 S3 and S4, as described by [46]. Phases corresponding to the first exponential growth phase due to cell division, pit hardening phase, second exponential growth phase mainly owing to cell expansion, and ripening processes respectively, were described in detail in [20]. For conducting the expression analysis, fruit samples collected at 41, 48, 55, 62, 69, 83, 90, 97, 104, 111, 118, 125 and 132 days after full bloom (DAFB) were used. For chromatin extraction and the following immunoprecipitation only samples belonging to S3 and S4 phases (83, 104, 111, 118 and 125 DAFB), were used. A mesocarp portion (FM sample), derived from at least 15 fruits (corresponding to 3 biological replicates, each composed of 5 fruits), was collected by taking, from each fruit, a radial section from the epicarp to the first cell layer of endocarp tissue, with high cellular type homogeneity [20]. This portion was immediately frozen in liquid nitrogen and stored at -80°C.

Reagent Setup For Chromatin Extraction

Nuclear Isolation Buffer (NIB)

10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 0.6% Triton X-100, 0.5 mM PMSF, 1% PVP (or 5% PVP for mesocarp tissue).

Percoll solution

15% Percoll, 10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 5% PVP.

Wash Buffer_FM (Fruit Mesocarp)

10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 5% PVP.

Wash Buffer 1_FB (Floral Buds)

10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 1% PVP.

Wash Buffer 2_FB

10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA.

Lysis Buffer

50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 10 mM Na-butyrate, and protease inhibitor cocktail (Sigma).

Reagent Setup For Chromatin Immunoprecipitation

Low Salt Buffer

20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS.

High Salt Buffer

20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS.

LNDET Buffer

20 mM Tris-HCl pH 8.0, 250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate.

TE Buffer

10 mM TRIS pH 8.0, 1mM EDTA.

TE Buffer for Proteinase K

40 mM TRIS pH 8.0, 10mM EDTA

Chromatin Extraction And Immunoprecipitation Assay

The general procedure described is valid for both FB and FM tissues. All buffers used were prepared using autoclaved stock solutions on the day of use and kept on ice until required. Phenylmethylsulfonyl fluoride (PMSF, Sigma), Na-butyrate and protease inhibitor cocktail (PI, Sigma) should be added into the

solutions just before use. The detailed explanation of all steps are reported in Supplementary M&M, with indicated “**Notes**” describing specific points for each tissue sample.

In summary, for chromatin extraction/purification the frozen FB and FM tissues were finely powdered with liquid nitrogen and the chromatin was extracted by transferring the fine powder into a 50 mL tube and fixing with 1% formaldehyde in cold NIB with a ratio 0,5 g FB or 1,5 g FM tissue in 25 ml NIB. Fixation was performed for 15' at RT and then blocked for 5' with 3.4 mL of 1M Glycine. Subsequently, the lysates were filtered through a single layer of Miracloth (Millipore) into a new tube and centrifuged at 5000 g for 15' at 4°C. The white pellet was respectively washed twice with 3 ml of cold Wash Buffer 1_FB/Wash Buffer 2_FB for Floral Buds or 5 ml Wash Buffer_FM for Fruit Mesocarp samples. The latter pellet was then additionally suspended in 5 ml of 15% Percoll solution and after centrifugation at 5000 g for 15' at 4°C. Finally, FB and FM pellets were suspended in 500 ul of Lysis Buffer. The extracted chromatin was sonicated by Microson Ultrasonic Cell Disruptor (Heat Systems, Germany) at power 30%-60%, for a variable number of pulses (10-15 s each, followed by 30s in ice) to 200–700 bp fragments and centrifuged at 16,000 g for 10' at 4°C. A fraction of the supernatant was saved and reverse cross-linking was performed with 0.2 M NaCl for 16 h at 65°C. DNA was purified by Phenol/Chloroform/isoamyl alcohol (25:24:1) as input in the following PCR reactions. Moreover, a quality check and yield estimation was conducted.

The subsequent immunoprecipitation procedure was performed using the Dynabeads protein G (ThermoFisher Scientific) adapting the steps to our tissues. The Dynabeads protein G were -pre-washed and, after their recovery, suspended with 100 ul Lysis Buffer (for each biological sample and Ab used). The suspension was subdivided into two aliquots of 50 ul and one aliquot was incubated with 5 ug or 10 ug of FB or FM chromatin sample respectively, for 4-5 hours at 4-10°C (“pre-clearing of chromatin step”), while the other one was incubated with an appropriate quantity of specific Ab and incubated on a rotating incubator at 4-10° for two hours (5 to 7 µg of antibody against H3K4me3 -Active Motif, 39159- or 10 µg of antibody H3K27me3 –Millipore, 07-449-) (“antibody preparation step”). The following immunoprecipitation step was performed by combining the pre-cleared chromatin samples with the Ab-Dynabeads protein G complexes, obtained from previous steps, and incubating them O/N at 4-10°C with gentle rotation. A chromatin aliquot processed similarly but without the addition of any antibody (No Ab sample) was used as background control.

The beads linked to histone-DNA immunoprecipitated complexes were then washed sequentially, once with Low Salt, High Salt, LNDET, and twice with TE Buffers. The histone-DNA complexes were eluted with 0.1M NaHCO₃ and 1% SDS and the cross-linking was reversed with 0.2 M NaCl for 16 h at 65°C. The DNA samples were precipitated with Glycogen (Sigma) and 2 volumes of absolute ethanol for 2h at -20°C. After pellet collection followed by washing it with 70% ethanol, DNA was suspended in TE Buffer for proteinase K and treated with RNase I for 30 min at 37°C and with proteinase K for 1 h at 42°C, extracted once with phenol-chloroform and with QIAquick PCR purification kit (QIAGEN). One microliter of this ChIPed DNA and an appropriate dilution of input were used for the following qPCR analyses.

Pull-down Efficiency Evaluation Of Immunoprecipitation

One microliter of ChIPed DNA and an appropriate dilution of input were used for the following Real time qPCR analyses. For every enrichment of histone marks in each target gene investigation, at least two pairs of primers were considered for PCR reactions at specific regions, and the sequences of best working primers (in terms of dimer formation and specificity), with relative positions compared to predict TSS, are reported in Table S1. qPCR data analyses were performed as reported in Rossi et al. 2007 and significant differences in the level of each analysed histone mark were assessed by Student t tests. In summary, at least three technical replicates were performed for each sample during the same PCR investigation, and a threshold cycle (TC) mean value with a standard error were calculated. A ΔTC value was calculated by subtracting the TC mean value for the samples to be compared. A following fold difference (FD), for a given primer combination, was then determined by raising 2 to the ΔTC power. Data were graphed as % INPUT since standardized to input chromatin, representing a whole chromatin condition specific for each biological sample. A subtraction of background signal, originating from the omission of antibody in ChIP experiment in corresponding sample, was determined.

CHIP-SEQ ANALYSIS (for FB tissues)

A total of 15 ChIP libraries (2 biological replicates X 2 antibodies (Abs) for the sample 0 and 770 CU and 3 biological replicates X 2 Abs for the sample at 475 CU) and one control library (input) representing whole chromatin (WC) were used for the ChIP-Seq assay. Libraries and sequencing were performed by IGA Technology Services (Udine, Italy) according to the standard operation. Ovation® Ultralow V2 DNA-Seq Library Preparation Kit (NuGEN, Redwood City, CA) was used for library preparation following the manufacturer's instructions. Immunoprecipitated DNA was quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Final libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Agilent Bioanalyzer DNA assay or Caliper (PerkinElmer, Waltham, MA). Libraries were then prepared for sequencing and sequenced on 20 M reads single-end 75 bp mode on NovaSeq6000 (Illumina, San Diego, CA). For the WC control library, 60 M of 75 bp single end reads were produced.

FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to assess the quality of the reads. The CHIP-Seq raw reads were processed for adapter clipping and quality score trimming using Trimmomatic v 0.39 [33]. Clean reads were mapped to the *P. persica* genome v.2.0 [9] obtained from Ensembl (<http://plants.ensembl.org/index.html>) using bowtie2 [47]. ChIP-Seq peaks calling and differential analysis between the three analysed samples were performed using Model-based Analysis of ChIP-Seq (MACS) [35].

Rna Extraction And Gene Expression Analysis

For FB, total RNA was extracted from 70-80 mg of frozen and ground sample using RNeasy Plant Mini kit (Qiagen) with minor modifications: 1.5% PVP-40 was added in the extraction buffer RLT in a total volume

of 750 µl instead 450 µl. On the contrary for FM, total RNA extraction was performed as reported in [30]. For both tissue samples, RNA concentration and quality were determined by measuring OD260/230 and OD260/280 ratio on a NanoDrop 2000c spectrophotometer (Thermo Scientific).

For FM samples, cDNA synthesis was performed with the SuperScript III reverse transcriptase kit (Invitrogen), according to manufacturer's instructions. Quantitative Real-Time PCR expression (qRT-PCR) analysis was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and the FAST SYBR® GREEN PCR Master Mix (Thermo Fisher Scientific), following manufacturer's guidelines. Three technical replicates were carried out for each primer combination in each sample and an absolute quantification of gene expression (normalized to UBIQUITIN (UBQ-PRUPE_4G204900 transcript quantities) was performed with the StepOne Software 2.3 (Thermo Fisher Scientific). The primers used are listed in Table S1. For qRT-PCR oligo nucleotides were designed respectively on primary transcripts.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

RNA-Seq and ChIP-Seq data from this article can be found in the Gene Expression Omnibus data library under accession number GSE189882 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189882>, secure token for reviewer access: qxuvqsyctbqdnsj) and GSE190586 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190586>, secure token for reviewer access: ktglcoyehjupzup), respectively.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by BIRD 2019-University of Padova to a grant to BC and SV.

Authors' Contributions

SV and CB: conceptualization; MC and SF: methodology and writing draft and final manuscript preparation; CF and JJ: review and editing. All authors read and approved the final manuscript.

Aknowledgements

We thank Dr. Vincenzo Rossi (Cereal and Industrial Crop, CREA, Italy), for his suggestions for ChIP experiments and data processing.

References

1. Lara MV, Bonghi C, Famiani F, Vizzotto G, Walker RP, Drincovich MF. Stone Fruit as Biofactories of Phytochemicals With Potential Roles in Human Nutrition and Health. *Front Plant Sci* 2020;11:1323. <https://doi.org/10.3389/fpls.2020.562252>.
2. Batista-Silva W, Nascimento VL, Medeiros DB, Nunes-Nesi A, Ribeiro DM, Zsögön A, et al. Modifications in organic acid profiles during fruit development and ripening: Correlation or causation? *Front Plant Sci* 2018;8:71. <https://doi.org/10.3389/fpls.2018.01689>.
3. Canton M, Forestan C, Bonghi C, Varotto S. Meta – analysis of RNA – Seq studies reveals genes with dominant functions during flower bud endo – to eco – dormancy transition in *Prunus* species. *Sci Rep* 2021;1–15. <https://doi.org/10.1038/s41598-021-92600-6>.
4. Osorio S, Carneiro RT, Lytovchenko A, McQuinn R, Sørensen I, Vallarino JG, et al. Genetic and metabolic effects of ripening mutations and vine detachment on tomato fruit quality. *Plant Biotechnol J* 2020;18:106–18. <https://doi.org/10.1111/pbi.13176>.
5. Paul LK, Rinne PLH, Van der Schoot C. Shoot meristems of deciduous woody perennials: Self-organization and morphogenetic transitions. *Curr Opin Plant Biol* 2014;17:86–95. <https://doi.org/10.1016/j.pbi.2013.11.009>.
6. Zhang Z, Zhuo X, Zhao K, Zheng T, Han Y, Yuan C, et al. Transcriptome Profiles Reveal the Crucial Roles of Hormone and Sugar in the Bud Dormancy of *Prunus mume*. *Sci Rep* 2018;8:1–15. <https://doi.org/10.1038/s41598-018-23108-9>.
7. Fadón E, Herrera S, Guerrero BI, Engracia Guerra M, Rodrigo J. Chilling and heat requirements of temperate stone fruit trees (*Prunus* sp.). *Agronomy* 2020;10. <https://doi.org/10.3390/agronomy10030409>.
8. Ong Q, Nguyen P, Phuong Thao N, Le L. Bioinformatics Approach in Plant Genomic Research. *Curr Genomics* 2016;17:368–78. <https://doi.org/10.2174/1389202917666160331202956>.
9. Verde I, Jenkins J, Dondini L, Micali S, Pagliarani G, Vendramin E, et al. The Peach v2.0 release: High-resolution linkage mapping and deep resequencing improve chromosome-scale assembly and contiguity. *BMC Genomics* 2017;18. <https://doi.org/10.1186/s12864-017-3606-9>.
10. Aranzana MJ, Decroocq V, Dirlewanger E, Eduardo I, Gao ZS, Gasic K, et al. *Prunus* genetics and applications after de novo genome sequencing: achievements and prospects. *Hortic Res* 2019;6:58. <https://doi.org/10.1038/s41438-019-0140-8>.
11. Daccord N, Celton JM, Linsmith G, Becker C, Choisne N, Schijlen E, et al. High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. *Nat Genet* 2017;49:1099–106. <https://doi.org/10.1038/ng.3886>.

12. Farinati S, Rasori A, Varotto S, Bonghi C. Rosaceae Fruit Development, Ripening and Post-harvest: An Epigenetic Perspective. *Front Plant Sci* 2017;8:1–14. <https://doi.org/10.3389/fpls.2017.01247>.
13. Catarino RR, Stark A. Assessing sufficiency and necessity of enhancer activities for gene expression and the mechanisms of transcription activation. *Genes Dev* 2018;32:202–23. <https://doi.org/10.1101/gad.310367.117>.
14. Priest HD, Filichkin SA, Mockler TC. cis-Regulatory elements in plant cell signaling. *Curr Opin Plant Biol* 2009;12:643–9. <https://doi.org/10.1016/j.pbi.2009.07.016>.
15. Cortijo S, Charoensawan V, Roudier F, Wigge PA. Chromatin immunoprecipitation sequencing (ChIP-seq) for transcription factors and chromatin factors in *Arabidopsis thaliana* roots: From material collection to data analysis. *Methods Mol. Biol.*, vol. 1761, Humana Press Inc.; 2018, p. 231–48. https://doi.org/10.1007/978-1-4939-7747-5_18.
16. Vimont N, Quah FX, Schöepfer DG, Roudier F, Dirlewanger E, Wigge PA, et al. ChIP-seq and RNA-seq for complex and low-abundance tree buds reveal chromatin and expression co-dynamics during sweet cherry bud dormancy. *Tree Genet Genomes* 2020;16. <https://doi.org/10.1007/s11295-019-1395-9>.
17. Li G, Jagadeeswaran G, Mort A, Sunkar R. ChIP-Seq analysis for identifying genome-wide histone modifications associated with stress-responsive genes in plants. *Methods Mol. Biol.*, vol. 1631, Methods Mol Biol; 2017, p. 139–49. https://doi.org/10.1007/978-1-4939-7136-7_8.
18. Huang X, Pan Q, Lin Y, Gu T, Li Y. A native chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in strawberry fruits 2020:1–12. <https://doi.org/10.1186/s13007-020-0556-z>.
19. Leida C, Conesa A, Llácer G, Badenes ML, Ríos G. Histone modifications and expression of DAM6 gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. *New Phytol* 2012;193:67–80. <https://doi.org/10.1111/j.1469-8137.2011.03863.x>.
20. Farinati S, Forestan C, Canton M, Galla G, Bonghi C, Varotto S. Regulation of Fruit Growth in a Peach Slow Ripening Phenotype 2021.
21. Rossi V, Locatelli S, Varotto S, Donn G, Pirona R, Henderson DA, et al. Maize histone deacetylase hda101 is involved in plant development, gene transcription, and sequence-specific modulation of histone modification of genes and repeats. *Plant Cell* 2007;19:1145–62. <https://doi.org/10.1105/tpc.106.042549>.
22. Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M. Chromatin immunoprecipitation: Optimization, quantitative analysis and data normalization. *Plant Methods* 2007;3:1–16. <https://doi.org/10.1186/1746-4811-3-11>.
23. Walker RP, Battistelli A, Bonghi C, Drincovich MF, Falchi R, Lara M V., et al. Non-structural Carbohydrate Metabolism in the Flesh of Stone Fruits of the Genus *Prunus* (Rosaceae) – A Review. *Front Plant Sci* 2020;11:1577. <https://doi.org/10.3389/fpls.2020.549921>.
24. Gambino G, Perrone I, Gribaudo I. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochem Anal* 2008;19:520–5.

<https://doi.org/10.1002/pca.1078>.

25. Ionescu IA, López-Ortega G, Burow M, Bayo-Canha A, Junge A, Gericke O, et al. Transcriptome and metabolite changes during hydrogen cyanamide-induced floral bud break in sweet cherry. *Front Plant Sci* 2017;8:1233. <https://doi.org/10.3389/fpls.2017.01233>.
26. Bellard M, Dretzen G, Giangrande A, Romain P. Nuclease digestion of transcriptionally active chromatin. *Methods Enzymol* 1989;170:317–46. [https://doi.org/10.1016/0076-6879\(89\)70054-9](https://doi.org/10.1016/0076-6879(89)70054-9).
27. Hu G, Huang B, Wang K, Frasse P, Maza E, Djari A, et al. Histone posttranslational modifications rather than DNA methylation underlie gene reprogramming in pollination-dependent and pollination-independent fruit set in tomato. *New Phytol* 2021;229:902–19. <https://doi.org/10.1111/nph.16902>.
28. Lauria M, Rossi V. Epigenetic control of gene regulation in plants. *Biochim Biophys Acta - Gene Regul Mech* 2011;1809:369–78. <https://doi.org/10.1016/j.bbagr.2011.03.002>.
29. Forestan C, Farinati S, Zambelli F, Pavesi G, Rossi V, Varotto S. Epigenetic signatures of stress adaptation and flowering regulation in response to extended drought and recovery in *Zea mays*. *Plant Cell Environ* 2020;43:55–75. <https://doi.org/10.1111/pce.13660>.
30. Botton A, Rasori A, Ziliotto F, Moing A, Maucourt M, Bernillon S, et al. The peach HECATE3-like gene FLESHY plays a double role during fruit development. *Plant Mol Biol* 2016;91:97–114. <https://doi.org/10.1007/s11103-016-0445-z>.
31. Zhang X, Bernatavichute Y V, Cokus S, Pellegrini M, Jacobsen SE. Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in *Arabidopsis thaliana*. *Genome Biol* 2009;10. <https://doi.org/10.1186/gb-2009-10-6-r62>.
32. He G, Zhu X, Elling AA, Chen L, Wang X, Guo L, et al. Global epigenetic and transcriptional trends among two rice subspecies and their reciprocal hybrids. *Plant Cell* 2010;22:17–33. <https://doi.org/10.1105/tpc.109.072041>.
33. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–20. <https://doi.org/10.1093/bioinformatics/btu170>.
34. Kim D, Langmead B, Salzberg SL. HISAT: A fast spliced aligner with low memory requirements. *Nat Methods* 2015;12:357–60. <https://doi.org/10.1038/nmeth.3317>.
35. Feng J, Liu T, Qin B, Zhang Y, Liu XS. Identifying ChIP-seq enrichment using MACS. *Nat Protoc* 2012;7:1728–40. <https://doi.org/10.1038/nprot.2012.101>.
36. Lindroth AM, Cao X, Jackson JP, Zilberman D, McCallum CM, Henikoff S, et al. Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* (80-) 2001;292:2077–80. <https://doi.org/10.1126/science.1059745>.
37. Fong PM, Tian L, Chen ZJ. *Arabidopsis thaliana* histone deacetylase 1 (AtHD1) is localized in euchromatic regions and demonstrates histone deacetylase activity in vitro. *Cell Res.*, vol. 16, NIH Public Access; 2006, p. 479–88. <https://doi.org/10.1038/sj.cr.7310059>.
38. Grafi G, Zemach A, Pitto L. Methyl-CpG-binding domain (MBD) proteins in plants. *Biochim Biophys Acta - Gene Struct Expr* 2007;1769:287–94. <https://doi.org/10.1016/j.bbaexp.2007.02.004>.

39. Tamada Y, Yun JY, Woo SC, Amasino RM. ARABIDOPSIS TRITHORAX-RELATED7 is required for methylation of lysine 4 of histone H3 and for transcriptional activation of FLOWERING LOCUS C. *Plant Cell* 2009;21:3257–69. <https://doi.org/10.1105/tpc.109.070060>.
40. Han Y-F, Dou K, Ma Z-Y, Zhang S-W, Huang H-W, Li L, et al. SUVR2 is involved in transcriptional gene silencing by associating with SNF2-related chromatin-remodeling proteins in Arabidopsis. *Cell Res* 2014;24:1445–65. <https://doi.org/10.1038/cr.2014.156>.
41. Audonnet L, Shen Y, Zhou DX. JMJ24 antagonizes histone H3K9 demethylase IBM1/JMJ25 function and interacts with RNAi pathways for gene silencing. *Gene Expr Patterns* 2017;25–26:1–7. <https://doi.org/10.1016/j.gep.2017.04.001>.
42. Zhao N, Zhang K, Wang C, Yan H, Liu Y, Xu W, et al. Systematic Analysis of Differential H3K27me3 and H3K4me3 Deposition in Callus and Seedling Reveals the Epigenetic Regulatory Mechanisms Involved in Callus Formation in Rice. *Front Genet* 2020;11:766. <https://doi.org/10.3389/fgene.2020.00766>.
43. Saleh A, Alvarez-Venegas R, Avramova Z. An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants. *Nat Protoc* 2008;3:1018–25. <https://doi.org/10.1038/nprot.2008.66>.
44. Tang D, Gallusci P, Lang Z. Fruit development and epigenetic modifications. *New Phytol* 2020;228:839–44. <https://doi.org/10.1111/nph.16724>.
45. Richardson EA, Seeley S, Walker D. A model for estimating the completion of rest for “Redhaven” and “Elberta” peach trees 1975.
46. Tonutti P, Bonghi C, Ruperti B, Tornielli GB, Ramina A. Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *J Am Soc Hortic Sci* 1997;122:642–7. <https://doi.org/10.21273/jashs.122.5.642>.
47. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012;9:357–9. <https://doi.org/10.1038/nmeth.1923>.

Figures

Figure 1

Crosslinking efficiency and physical shearing chromatin analyses.

(a) Bud and mesocarp tissues were crosslinked in buffers containing increasing amounts of formaldehyde. Samples were subjected or not to a reverse crosslinking phase (decrosslinked sample + and -, respectively), and DNA isolated using phenol/chloroform extraction as described in MM section. While DNA is efficiently isolated from samples that were not crosslinked (lanes indicated with 0%), a decrosslinking procedure is required for the isolation of DNA from cross-linked samples (with 1% indicating the relative concentration of formaldehyde used in testing analyses, which resulted in a better yield of signal).

(b) Chromatin shearing check after the application of 60% amplitude with several 10 sec shearing rounds ranging between 25 (for FB) and 15 (for FM) followed by a reverse crosslinking phase and a DNA isolated using phenol/chloroform extraction.

Figure 2

Chromatin marks analysis by X-ChIP method for peach mesocarp tissue.

Histone modification analysis on chromatin extracted from FAN mesocarp tissue at 83, 104, and 111 DAFB. The 'TSS around-' (dark bars, questioned with primer set A) and 'gene body-' (white bars, questioned with primer set C) subregions of *FLESHY* genomic locus, were investigated by real-time PCR quantification on ChIPed DNA immunoprecipitated with α -H3K4me3 (a), and α -H3K27me3 (b). Data are reported as percentage of chromatin input (% IP), normalized on background signal (No Ab serum control sample, measured by omitting antibody during ChIP procedure). Three PCR repetitions for each ChIP assay. Standard errors are reported. Asterisks indicate statistically significant changes: * = $p \leq 0.05$, ** = $p \leq 0.01$. DAFB: Days after full bloom.

Figure 3

ChIP-Seq and RNA-Seq profiles obtained in floral buds.

H3K4me3 **(a)** H3K27me3 **(b)** and RNA- Seq (green reads) profiles during dormancy in peach flower buds. Integrative Genomics Viewer (IGV) screenshot of the 0, 475 and 770CU and their corresponding input. Genes are represented by blue rectangles. TSS, Transcription Start Site.

Figure 4

Schematic workflow of ChIP protocol.

The protocol phases are reported on the left of the picture and the main relative improvements applied are described on the right.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMM.docx](#)
- [TableS1.pdf](#)
- [FigS1.tif](#)
- [FigS2.tif](#)
- [SUPPLEMENTARYREFERENCES.docx](#)