Chromatin immunoprecipitation-sequencing and RNA-sequencing for complex and low-abundance tree buds

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ABSTRACT

BACKGROUND

Chromatin immunoprecipitation-sequencing (ChIP-seq) is a robust technique to study interactions between proteins, such as histones or transcription factors, and DNA. This technique combined with RNA-sequencing (RNA-seq) is a powerful tool to better understand biological processes in eukaryotes.

RESULTS

We developed a combined ChIP-seq and RNA-seq protocol for tree buds (*Prunus avium* L., *Prunus persica*, *Malus x domestica Borkh.*) that has also been successfully tested in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Tree buds contain phenolic compounds that negatively interfere with ChIP and RNA extraction. In addition to solving this problem, our protocol is optimised to work on small amounts of material. In this protocol, samples for ChIP-seq are cross-linked after flash freezing, making it possible to work on trees growing in the field and to perform ChIP-seq and RNA-seq on the same starting material, differentiating it from previously published protocols.

CONCLUSIONS

This protocol will allow analysis of chromatin and transcriptomic dynamics in tree buds, notably during its development and response to the environment.

BACKGROUND

The term 'epigenetics' has traditionally been used to refer to heritable changes in gene expression that take place without altering DNA sequence (1), but it is also used, in a broader sense, to refer to modifications of the chromatin environment (2). Epigenetic modifications are important for a wide range of processes in plants, including seed germination (3), root growth (4), flowering time (5), disease resistance (6) and abiotic stress responses (7). Post-transcriptional modifications of histone proteins and chromatin structure regulates the ability of transcription factors (TFs) to bind DNA and thereby influences gene expression (8, 9). For example, tri-methylation of histone H3 at lysine 4 (H3K4me3) is associated with open chromatin and gene activation (10) while methylation of histone H3 at 27 (H3K27me3) defines a restrictive chromatin environment associated with gene repression (11). Analysing the dynamics of chromatin modifications and DNA-protein interactions is a critical step to fully understand how gene expression is regulated. Chromatin Immunoprecipitation (ChIP) is one of the few methods enabling the exploration of *in vivo* interactions between DNA and proteins, in particular histones and transcription factors. When followed by next generation sequencing (ChIP-seq), this method allows the detection of these interactions at a genome-wide scale. Since chromatin modifications and the regulation of gene expression are tightly linked, ChIP-seq for chromatin marks and TFs are often combined with RNA-sequencing (RNA-seq) to extract key features of the role of chromatin modification in regulating transcription.

While this method is routinely performed in plant model organisms like Arabidopsis thaliana, it is still a challenge to carry out ChIP-seq and RNA-seq on tree buds. Here we present an efficient protocol for ChIP-seq and RNA-seq on complex, low-abundance tree buds, with the possibility of studying trees growing in the field and rapid chromatin dynamics owing to an improved cross-linking method. The general process can be divided in four sections illustrated in Figure 1: [1] the material sampling, [2] the ChIP and library preparation, [3] the RNA extraction and library preparation and [4] the quantification and pool of libraries for sequencing. Analysis of ChIP-seq and RNA-seq data is out of the scope of this paper.

Over the past decade, numerous ChIP protocol in plants and mammals have been published (see (12)-(20) references for a non-exhaustive list). However, these protocols and a specific ChIP protocol for wood-forming tissue (stem-differentiating xylem) (21) cannot be directly used for plant materials with high phenolic content (alkaloids or lignified cell walls) like trees buds (22). Such chemicals need to be chelated during the chromatin extraction to prevent inhibition of downstream processes. In addition, the requirement for large amounts of starting material in previous protocols has made it a challenge to perform ChIP on low-abundance tissues such as tree buds. Moreover, the cross-linking step is conventionally conducted on fresh tissues immediately upon harvesting, and is thus impractical for samples harvested in

the field. In most cases, analyses of TFs occupancy and/or chromatin marks enrichment are complemented with a transcriptome analysis using RNA-seq in order to associate variations of gene expression with changes in the chromatin environment. Therefore, we propose a protocol that addresses these issues.

To sum up, the following points set this protocol apart from others previously published:

• Our ChIP-seq/RNA-seq protocol can be carried out on complex plant tissues that contain interfering compounds (phenolic complexes, scales, protective layers), by adding chelators of these compounds in the extraction buffers. This protocol can also be easily adapted for other systems and has been successfully tested in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*, where it has been used to study the binding of histone variant H2A.Z (Suppl. Figure 1 A) and that of heat-associated transcription factor Hsf1 (Suppl. Figure 1 B).

• The cross-linking step is performed on frozen, pulverised material, thus allowing sample collection in the field, where cross-linking equipment is not available. It also allows studying fast responses by flash freezing material immediately after a stimulus (e.g. transient temperature stress) rather than cross-linking directly on fresh tissue or cells. In previous protocols, the cross-linking step was performed using a vacuum and lasted at least 10 minutes and up to 1 hour (12, 18-20). Owing to this optimised step, it is possible to perform ChIP-seq/RNA-seq for kinetic experiment with short intervals (every minute or less) with no changes in term of metabolites or expression since the material is directly flash frozen in liquid nitrogen. Moreover, cross-linking on powder allows for a more homogenous cross-linking, as it is almost impossible to have a homogenous penetration of the formaldehyde in rigid and thick tissues like tree buds that are protected by an impermeable and rigid wall rich in lignin (22).

• By using frozen, pulverized material, ChIP-seq and RNA-seq can be performed on the same starting material for a direct and robust comparison of epigenetic regulation and gene expression.

• Our protocol can be used to perform ChIP-seq and RNA-seq on a small amount of biological material. In some cases, the amount of starting material is a limiting factor, such as for axillar buds and meristems. We optimised this protocol to start from 200 to 500 mg of buds, which is considerably lower than the usual amount of 0.8 to 5 g of starting material for ChIP protocols in plant tissues (12, 18, 19, 20, 23).

We have used this protocol to analyse histone modification profiles in several tree species. In a first instance, we performed H3K27me3 ChIP-seq in buds of *Prunus persica* (peach) and successfully compared our results with previously published data. To demonstrate the versatility of this protocol, ChIP-seq for H3K4me3 and H3K27me3 was also performed on buds of two other tree species: sweet cherry *(Prunus avium* L.) and apple (*Malus x domestica* Borkh.). Furthermore, carrying out RNA-seq on sweet cherry buds as proposed in this protocol, we demonstrated the correlation between the

above histone marks and gene expression in sweet cherry *(Prunus avium* L.) for two control genes *AGAMOUS* (*AG*) and *ELONGATION FACTOR 1* (*EF1*) known to be under control of H3K27me3 and H3K4me3, respectively. We have thus developed a combined ChIP-seq and RNA-seq protocol working on tree buds for many species, which will allow a better understanding of transcriptional regulatory events and epigenomic mechanisms in tree buds.

RESULTS

The cross-linking on frozen powder is more efficient than whole fresh buds

The procedure for conventional *in vivo* ChIP consists of cross-linking DNA and proteins on fresh material at the time of collection using the common fixing agent formaldehyde under vacuum (12, 13, 29). Here we perform cross-linking on frozen pulverised material without vacuum, using an extraction buffer containing formaldehyde.

We used *AGAMOUS* (*AG*) locus in *Arabidopsis* (30) and the *ELONGATION FACTOR 1* (*EF1*) locus in petunia (31) as ChIP-qPCR controls, which are known to be under control of H3K27me3 and H3K4me3, respectively. AG and EF1 were also used as control genes for H3K27me3 and H3K4me3 in previous studies, for example in Japanese pear (32). In order to demonstrate the feasibility of the cross-linking on frozen powder, ChIP-qPCR for H3K4me3 were carried out on whole cherry floral buds (without scales) and on frozen and pulverised cherry floral buds (Figure 2). The similar *EF1*/*AG* ratio between these two different conditions demonstrates that the cross-linking on powder works properly with no difference with a conventional cross-linking on fresh material using a vacuum (Figure 2). We also analysed H3K4me3 at the *DAM2* gene. We observe an increased ration of *DAM2*/*AG* when the cross-linking is performed on frozen and pulverised buds contrary to a conventional cross-linking performed on fresh and whole buds without scales (Figure 2).

Time saving: the ChIP-seq and RNA-seq protocol is also working in presence of scales

To facilitate the penetration of the formaldehyde solution trough the buds, scales are generally removed from buds prior to material cross-linking. However this step is time-consuming and can damage the buds resulting a potential change of epigenetic and transcriptomic responses. The cross-linking on frozen powder (with the presence of scales) allows overcoming this obstacle. To demonstrate the added value of our optimised cross-linking step, ChIP-qPCR for H3K4me3 were carried out on frozen and pulverised cherry flower buds with and without scales (Figure 3 A). No difference is observed at the *EF1*/*AG* and *DAM5*/*AG* ratio in presence or absence of scales (Figure 3 A). We also performed RT-qPCR on the same biological material and observe that *DAM5* expression is the same in presence or absence of scales (Figure 3

B). These results indicate that the presence of scales is not a problem to successfully perform ChIP and RNA extraction following our protocol.

Validation of the protocol robustness in peach

H3K27me3 profiling by ChIP-seq as been already described in non-dormant bud of *Prunus Persica* (33), as this is an important developmental stage of fruit trees. Dormancy is a period of repressed growth that allows trees to persist under low winter temperature and short photoperiod (34). A proper regulation of the timing of the onset and release of bud dormancy is crucial to insure optimal flowering and fruit production in trees. Consequently, unravelling the associated molecular mechanisms is essential and numerous studies have been conducted in trees to answer this question. De la Fuente and colleagues (33) have shown that *DORMANCY-ASSOCIATED MADS-box* (*DAM*)-related genes, which are involved in the regulation of bud dormancy under unfavourable climatic conditions in peach (33, 35, 36) , leafy spurge (37), pear (32), apple (38) and apricot (39) are up-regulated in dormant peach buds. It has been shown that DAM genes are marked with H3K27me3 in non-dormant buds, but not in dormant buds of *Prunus persica.* Using our improved ChIP-seq protocol, we also observe H3K27me3 enrichment in the gene body of a *DAM* gene cluster on Prunus Persica non-dormant buds (Figure 4). Input data is present to visualise the background signal and can be used to normalise data. A higher enrichment is present at the *DAM5* and *DAM6* loci compared with other *DAM* genes at this stage of dormancy (Figure 4) as expected from previous findings (33). The replication of these previously published results confirms that our improved ChIP-seq protocol is well working on tree buds.

Successful application of this protocol on different species

To demonstrate the versatility of this protocol, ChIP-seq for two histone marks (H3K27me3 and H3K4me3) was performed on buds of three tree species: peach (*Prunus persica*), apple (*Malus x domestica* Borkh.) and sweet cherry *(Prunus avium* L.) (Figure 5). We observed a strong H3K4me3 signal at *EF1* and enrichment for H3K27me3 at *AG* locus for the three species (Figure 5). To test the adaptability of this protocol for other biological system, ChIP-qPCR have been successfully conducted following the ChIP procedure in *Arabidopsis thaliana* to study the binding of histone variant H2A.Z at *HSP70* locus (40, 41) (Suppl. Figure 1 A) and in *Saccharomyces cerevisiae* to study the heat-associated transcription factor Hsf1 binding at the SSA4 promoter (42) (Suppl. Figure 1 B),

Direct comparison of ChIP-seq and RNA-seq data

As proposed in this protocol, by carrying out RNA-seq on the same starting material as ChIP-seq on sweet cherry floral, we can directly compare gene expression with the presence of histone marks. We observe a link between gene expression levels and the presence of active or repress histone mark in sweet cherry: H3K4me3 is observed at *EF1* locus, which is also highly expressed, while H3K27me3 is observed at *AG* locus, which is also repressed in our data (Figure 5 C).

DISCUSSION

In this study, we describe a ChIP/RNA-seq protocol for low abundance and complex plant tissues such as tree buds. This method allows a robust comparison of epigenetic regulation and gene expression as we use the same starting material. More notably, this protocol permits to perform ChIP/RNA-seq for kinetic experiment with short intervals (every minute or less) and suitable for collecting samples in the field that were previously difficult or impossible.

Several studies have led to the identification of molecular mechanisms involved in dormancy, including a cluster of *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes (43). *DAM*-related genes are up-regulated in dormant buds in peach (33, 35, 36), leafy spurge (37), pear (32), apple (38) and apricot (39). Conversely, *DAM*-related genes are down-regulated in non-dormant buds and this change in their expression is associated with the presence of the repressive histone mark H3K27me3 at these genes. In particular, H3K27me3 abundance is increased at *DAM5* and *DAM6* loci during the transition from dormancy to dormancy release (33). Using the proposed ChIP-seq protocol, we found similar results for H3K27me3 abundance in the *DAM* genes in peach in non-dormant buds (Figure 5), thus validating our ChIP-seq method.

We demonstrated the correlation between the presence of histone marks and gene expression in sweet cherry *(Prunus avium* L.) for two control genes *AGAMOUS* (*AG*) and *ELONGATION FACTOR 1* (*EF1*) known to be under control of H3K27me3 and H3K4me3, respectively (Figure 6 C). As the ChIP-seq and RNA-seq were performed on the same biological material, the level of gene expression and the presence/absence of particular histone marks can be directly compared with confidence.

In the last two decades ChIP has become the principal tool for investigating chromatin-related events at the molecular level such as transcriptional regulation. Improvements to the ChIP-seq approach are still needed and will include an expansion of available ChIP-grade antibodies and a reduction of the hands-on time required for the entire procedure. A remaining challenge is to further decrease the amount of starting material without compromising the signal-to-noise ratio.

MATERIALS AND METHODS

MATERIAL SAMPLING SECTION

Harvest tree buds in 2 ml tubes with screw cap and immediately flash-freeze in liquid nitrogen. There is no need to remove the scales after harvesting. The biological material can be conserved at -80°C up to twelve months before proceeding with ChIP-seq (six months if the tissues are ground into powder). Grind the tissues to a fine powder using mortars and pestles pre-chilled with liquid nitrogen. Add liquid nitrogen several times while grinding to facilitate cell lysis and to ensure that the material remains completely frozen to prevent degradation of tissues.

For the cross-linking and chromatin extraction, weigh out 300 to 500 mg of powder (200 to 400 mg if scales have been removed from the buds) in a 50 ml Falcon tube pre-chilled with liquid nitrogen. The same amount of powder should be used for all samples to allow a direct comparison of results. Then proceed to "ChIP and library preparation section".

For RNA extraction, weigh out 50-70 mg of powder (20-40 mg without scales) in a 2 ml tubes (screw cap) pre-chilled with liquid nitrogen. Then proceed to "RNA extraction and library preparation section". Due to the small amount of starting material, it is necessary to keep the tubes in liquid nitrogen to prevent any degradation.

ChIP AND LIBRARY PREPARATION SECTION

Cross-linking and chromatin extraction: Timing 2-3 hours

Add 25 ml of ice-cold Extraction buffer 1 [0.4 M sucrose, 10 mM HEPES pH 7.5, 10 mM MgCl2, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 % PVP-40 (polyvinylpyrrolidone), 1 tablet of complete protease inhibitor EDTA free for 50 ml of buffer from Sigma cat# 11836170001] to the powder. For each buffer, the protease inhibitor (PMSF), the tablet of complete protease inhibitor and β-mercaptoethanol should be added directly before using the buffer. When extracting chromatin in other biological systems such as *Arabidopsis thaliana* or *Saccharomyces cerevisiae*, the PVP-40 in Extraction buffer 1 may optionally be removed.

Immediately add 675 µl of 37% formaldehyde solution (1% final concentration) and invert the tube several times to resuspend the powder. Cross-link the samples by incubating at room temperature for 10 minutes and then quench the formaldehyde by adding 1.926 ml 2 M of fresh glycine solution (0.15 M final concentration). Invert the tube several times and incubate at room temperature for 5 minutes. Filter the homogenate through Miracloth (Millipore cat# 475855) in a funnel and collect in a clean 50 ml Falcon tube placed on ice. Repeat the filtration step once more. Centrifuge the filtrate at 3,200 × g for 20 minutes at 4 °C. Discard the supernatant by inverting the tube, being careful not to disturb the pellet. Gently resuspend the pellet in 1ml of Extraction buffer 2 [0.24 M sucrose, 10 mM HEPES pH 7.5, 10 mM MgCl2, 1 % Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1 tablet protease inhibitor EDTA free for 50 ml of solution], without creating bubbles, and transfer the solution to a clean 1.5 ml tube. Centrifuge at 13,500 × g for 10 minutes at 4°C.

Carefully remove the supernatant by pipetting. If the pellet is still green, repeat the resuspension in 1 ml of Extraction buffer 2 and centrifugation at 13,500 × g for 10 minutes at 4°C. In a new 1.5 ml tube, add 300 µl of Extraction buffer 3 [1.7 M sucrose, 10 mM HEPES pH 7.5, 2 mM MgCl2, 0.15 % Triton X-100, 5 mM β- mercaptoethanol, 0.1 mM PMSF, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. Slowly resuspend the pellet in 300 µl of Extraction buffer 3 to prevent the formation of bubbles. Take the 300 µl solution of resuspended pellet and carefully layer it on top of the 300 µl Extraction buffer 3. Centrifuge at 21,200 × g for 1 hour at 4°C. During this process, nuclei are pelleted through a sucrose cushion to remove cellular contaminants.

From this step, chromatin fragmentation can be performed in two different ways: (A) sonication, to shear the chromatin into 100-500 bp fragments, or (B) MNase (Micrococcal nuclease) digestion, to enrich for mono-nucleosomes (~150-200 bp).

A. Sonication: TIMING 3-4h hours (+ 8 hours of incubation)

i. Chromatin fragmentation

Carefully remove the supernatant with a pipette and resuspend the nuclei pellet in 300 µl of Sonication buffer [50 mM HEPES pH 7.5, 10 mM EDTA, 1 % SDS, 0.1 % sodium deoxycholate, 1% Triton X-100, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. To break the nuclear membrane and release the chromatin, flash-freeze the tube in liquid nitrogen and then thaw rapidly by warming the tube in your hand. Repeat once more. Centrifuge the tube at 15,800 × g for 3 minutes at 4°C to pellet debris, and carefully recover the supernatant into a new tube. Complete the tube to 300 µl with the Sonication buffer. Set aside a 10 µl aliquot of chromatin in a PCR tube to serve as the non-sonicated control when assessing sonication efficiency by gel electrophoresis and keep on ice. Shear the chromatin into ~300 bp (100–500 bp) fragments by sonication (e.g. using Diagenode Bioruptor Twin- UCD400, sonicate 300 µl chromatin in 1.5 ml microcentrifuge tubes for 14 to 16 cycles, on High setting, with 30s ON/30s OFF per cycle). The number of cycles of sonication to obtain DNA fragments of around 300 bp should be tested and optimised for different tissues and different concentrations of chromatin. Transfer 40 µl of sheared chromatin to a PCR tube, which will be used to check the sonication efficiency. The rest of the sonicated chromatin should be stored at -80°C. Sonicated chromatin can be kept frozen at –80 °C for 3 months without significant loss of chromatin quality.

ii. Analysis of sonication efficiency

Complete the sonicated (40 µl) and non-sonicated (10 µl) aliquots to 55.5 µl with TE buffer [10 mM Tris-HCl pH 8, 1 mM EDTA], add 4.5 µl of 5 M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Add 2 µl of 10 mg/ml RNase A (Fisher cat# EN0531) and incubate at 37°C for 30 minutes. Add 2 µl of 20 mg/ml proteinase

K (Fisher cat# EO0491) and incubate at 45°C for 1 hour. During this step, take out the SPRI beads (e.g AMPure beads; Beckman Coulter, cat# A63880) from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use).

To extract DNA using SPRI beads, vortex the beads until they are well dispersed, add 126 µl of beads to 60 µl of sample (2.1 × ratio) and mix well by pipetting up and down at least 10 times. Incubate 4 minutes at room temperature and then place the tubes on a magnetic rack (96 well; Fisher, cat# AM10027) for 4 minutes to capture the beads. Carefully remove and discard the supernatant without disturbing the beads. Leave the tubes on the magnetic rack and add 200 µl of freshly prepared 80 % v/v ethanol. Incubate for 30 seconds and discard the supernatant. Repeat the ethanol wash once more and then completely remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. Tubes can alternatively be placed in a fume hood for 10 minutes to accelerate drying. The beads must be completely free from ethanol as it can interfere with downstream processes. Remove the tubes from the magnetic rack and resuspend the beads in 15 µl of 10 mM Tris-HCl (pH 8) by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature and place on the magnetic rack for 4 minutes to capture the beads. Carefully transfer 14 µl of supernatant containing DNA to a new tube. Add 2.8 µl of 6× Loading dye to 14 µl of DNA. Separate the DNA by electrophoresis on a 1.5 % agarose gel for at least 1 h at 70 V. The smear should be concentrated between 100–500 bp (Figure 6 B). If necessary, perform additional sonication cycles. Otherwise, continue directly to the "Immunoprecipitation (IP)" step.

B. MNase digestion: TIMING 4-5 hours (+ 2 × 8 hours of incubation)

i. DNA quantification prior to MNase digestion

Carefully remove the supernatant with a pipette and resuspend the nuclei pellet in 500 µl of MNase buffer [20 mM HEPES pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 % NP-40, 3mM CaCl2, Triton X-100, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. To break the nuclear membrane and release the chromatin, flash-freeze the tube in liquid nitrogen and then thaw rapidly by warming the tube in your hand. Repeat once more. Transfer 40 µl of chromatin to a PCR tube to quantify DNA prior to MNase digestion and complete to 55.5 µl with MNase digestion buffer, add 4.5 µl of 5M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Keep the rest of the chromatin at –80 °C. Add 2 µl of 10 mg/ml RNase A and incubate at 37°C for 30 minutes. Add 2 µl of 20 mg/ml proteinase K and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Proceed to the DNA extraction using SPRI beads as explained before in the

sonication analysis section (ii). Use 1 µl from each sample to quantify the DNA using a Qubit fluorometer (ThermoFisher Scientific), or a Nanodrop spectrophotometer (Thermo Scientific).

ii. MNase digestion

Adjust all samples to the same concentration according to the quantification results using MNase buffer and to a final volume of 500 µl. Set aside a 20 µl aliquot of chromatin in a PCR tube to serve as the non-digested control when assessing MNase efficiency by gel electrophoresis and keep on ice. Incubate chromatin in a ThermoMixer (Eppendorf) for 2-5 minutes at 37˚C with shaking at 1,200 rpm, for optimal MNase activity. Add MNase (Fisher cat# 88216) to the chromatin to a final concentration of 0.6 U/ml and incubate 10 minutes in the ThermoMixer at 37˚C, 1,200 rpm. Stop the digestion by adding 5 µl of 0.5 M EDTA pH 8 (5 mM final concentration), invert the tube several times to mix and immediately place on ice for 5 minutes. The optimal MNase enzyme concentration and incubation time to obtain predominantly mono-nucleosomes should be tested and optimised for different tissues and different concentrations of chromatin. For the optimisation of MNase digestions, we recommend using 1 ml of chromatin and carrying out digestions in 100 µl aliquots with varying concentrations of MNase (0.2 U/ml to 1 U/ml) and incubation times (5 to 20 minutes). Transfer 50 µl of digested chromatin to a PCR tube, which will be used to check the MNase efficiency. The rest of the digested chromatin should be stored at -80°C and can be kept at –80 °C for 3 months without significant loss of chromatin quality.

iii. MNase digestion analysis

Complete the digested sample (50 µl) and non-digested (20 µl) aliquots to 55.5 µl with TE buffer, add 4.5 µl of 5M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Add 2 µl of 10 mg/ml RNase A and incubate at 37°C for 30 minutes. Add 2 µl of 20 mg/ml proteinase K and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Proceed to the DNA extraction, using SPRI beads as explained before in the sonication analysis section (ii). Add 2.8 µl of 6× Loading dye to 14 µl of DNA. Separate the DNA by electrophoresis on a 1.5% agarose gel for at least 1 h at 70 V. The most abundant band should be 150-200 bp in size (Figure 6 A), which corresponds to chromatin in mono-nucleosome form, with a less abundant 300-350 bp band (di-nucleosomes) and a faintly visible ~500 bp band (tri-nucleosomes). For optimum sequencing results, approximately 80% of chromatin should be in mono-nucleosome form. If this is judged not to be the case from the gel, it is not possible to carry out further MNase digestions on the chromatin, as EDTA sequesters calcium ions that are required for MNase activity.

Immunoprecipitation (IP): TIMING 7-8 hours (+ overnight incubation)

Transfer 50 µl of protein A- and/or protein G-coupled magnetic beads (Invitrogen cat# 10-002D and cat# 10-004D, respectively) per IP to a 2 ml tube. Wash the beads with 1 ml of Binding buffer [0.5% (wt/vol) BSA, 0.5% (vol/vol) Tween-20 in PBS (without Ca2+, Mg2+)] during 5 minutes at 4°C on a rotating wheel (low speed, around one rotation every 5-6 seconds). Place the tubes on a magnetic rack (Thermo Fisher cat# 12321D) until the liquid is clear and remove the supernatant. Repeat three times. After the washes, resuspend the beads in 250 µl of Binding buffer. Add 5 µl of antibody per IP to the beads. In our study, we used anti-trimethyl-histone 3 Lys 27 antibody (Millipore cat# 07-449) and anti-trimethyl-histone 3 Lys 4 antibody (Millipore cat#17-614,). Incubate 4 hours on a rotating wheel at 4°C (low speed). During this incubation time, centrifuge the sonicated or digested chromatin at 15,800 × g for 5 minutes at 4°C to pellet debris, and carefully recover supernatant into a new tube. Transfer 100 µl of sonicated chromatin to a new 2 ml tube for one IP, add 900 µl of Binding buffer and keep on ice. Or transfer 200 µl of MNAse-digested chromatin to a new 2 ml tube for one IP, add 800 µl of Binding buffer and keep on ice. Transfer 20 µl of sonicated or MNAse-digested chromatin as an input fraction (no immunoprecipitation) in a PCR tube and store at -80°C. The rest of the chromatin can be used for another IP or stored at -80°C. After completion of the incubation of protein A/G beads with the antibody, place the tubes containing the antibody-bead complexes on a magnetic rack, remove the supernatant and wash the beads with 1 ml of Binding buffer during 5 minutes at 4°C on a rotating wheel (low speed). Repeat three times. Resuspend the beads in 50 µl of Binding buffer per IP, then add them to the 1 ml of diluted chromatin. Incubate overnight on a rotating wheel at 4°C (low speed). Briefly centrifuge the tube (<3 seconds) to pull down the liquid in the lid of the tube. Place on a magnetic rack and remove the supernatant. Wash the beads to reduce unspecific interactions by incubating 5 minutes at 4°C on a rotating wheel (low speed) with 1ml of the following buffers and total number of washes: a. 5 washes with Low Salt Wash buffer [150 mM NaCl, 0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8]; b. 2 washes with High Salt Wash buffer [500mM NaCl, 0.1% SDS, 1% triton X-100, 2 mM EDTA, 20mM Tris-HCl pH 8]; c. 2 washes with LiCl Wash buffer [0.25 M LiCL 1% NP-40 (IGEPAL), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8]; d. 2 washes with TE buffer. After the second wash in TE buffer, resuspend the beads in 100 µl of TE buffer and transfer the beads to a PCR tube. Place the tube on a magnetic rack, remove the TE buffer and resuspend the beads in 60 µl of Elution buffer [10 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 300 mM NaCl, 0.5% SDS].

Reverse cross-linking and Elution by proteinase K treatment: TIMING 2 hours (+ 8 hours of incubation)

Defrost the input fraction on ice (20 µl of sonicated or MNAse-digested chromatin). Complete the input fraction to 60 µl with the Elution buffer. Incubate the input fraction and the IP sample (60 µl beads-Elution buffer) at 65°C for 8 hours in a

PCR machine or thermocycler to reverse crosslink. Add 2 µl of RNase A (10 mg/ml) and incubate at 37°C for 30 minutes. Add 2 µl of Proteinase K (20 mg/ml) and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Place the tubes on a magnetic rack to collect the beads, transfer 60 µl of supernatant from each well to a new PCR tubes (or a new 96 wells-plate).

DNA extraction using SPRI beads and qPCR: TIMING 1 hour

Proceed to the DNA extraction, using SPRI beads as explained before in the sonication analysis section (ii) until just before the elution. Remove the tubes from the magnetic rack and for the elution, resuspend the beads in 50 µl of 10mM Tris-HCl (pH 8.0) by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature and place on the magnetic rack for 4 minutes to capture the beads. Carefully transfer 49 µl of supernatant containing DNA to a new tube. For qPCR analysis, use 1 µl of DNA per 10 µl reaction, from the IP and input. The percentage of enrichment of DNA in the ChIP fraction relative to the input fraction is calculated according to the formula: $(2^{-\text{Cp ChIP}} / 2^{-\text{Cp input}}) \times 100$. Keep the rest of the DNA for sequencing (continue to "ChIP library preparation and size selection section").

ChIP library preparation and size selection: TIMING 2-3 days

i. ChIP library

For the preparation of sequencing libraries, use the entire volume from the IP and 5–10 ng from the input fraction. We recommend carrying out ChIP-seq library preparation using the TruSeq ChIP Sample Prep Kit (Illumina 48 samples, 12 indexes, Illumina, cat# IP-202-1012) with minor modifications.

1- The "Purify Ligation Products" section using the gel electrophoresis is eliminated to minimise DNA loss.
2- DNA size selection is carried out after the "Enrich DNA fragments" section. This is a required step to increase the visualisation of nucleosome positioning. Smaller and larger reads might disturb the MNase input profile after analysis.

The Illumina TruSeq ChIP Sample Preparation protocol is available at the following URL: http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqchip/truseq-chip-sample-prep-guide-15023092-b.pdf

ii. Size selection

Size selection is performed using SPRI beads to remove fragments larger than 500 bp and fragments smaller than 100 bp, the latter being most likely adapters contaminations. This size selection improves the resolution of the ChIP-seq peaks

obtained from MNase but is not required when using sonication, except in the case of adapter contaminations. Complete the 15 µl of libraries to 100 µl with Resuspension buffer from the TruSeq ChIP Sample Prep Kit. During this step, allow the SPRI beads to equilibrate at room temperature (at least 30 minutes before use) and vortex the them until they are well dispersed. Add 65 µl of SPRI beads (0.65× ratio; to eliminate > 500 bp fragments). Incubate at room temperature for 5 minutes. Place on a magnetic rack for 5 minutes. Transfer 155 µl of the supernatant (containing < 500 bp fragments) to a new tube. Add 50 µl of SPRI beads (1.15× ratio; to eliminate fragments < 100 bp). Incubate at room temperature for 5 minutes. Place on a magnetic rack for 5minutes. Remove and discard the supernatant. Without removing the tube from the magnetic rack, add 200 µl of freshly prepared 80 % ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove and discard the supernatant. Repeat the ethanol wash once more and then remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. The beads must be completely free from ethanol as the latter can interfere with downstream processes. Remove the tube from the magnetic rack and resuspend the beads with 17.5 µl of Resuspension buffer. Gently pipette the entire volume up and down at least 10 times to mix. Incubate the tube at room temperature for 2 minutes. Place the tube on a magnetic rack at room temperature for 5 minutes. Transfer 15 µl of the clear supernatant to a new tube. Check the quality of libraries using a 4200 TapeStation or Bioanalyzer instruments (Agilent) following manufacturer's. See Suppl. Fig. 2 A) and B) as a comparison profiles with and without adapter contaminations. If the libraries are contaminated with adapters, repeat again the "size selection" step to remove them, otherwise proceed directly with the "quantification and pool of libraries" section. DNA for sequencing can be frozen at -20°C for 3 months without significant loss of quality.

RNA EXTRACTION AND LIBRARY PREPARATION SECTION

RNA extraction: TIMING 2-5 hours

We recommend for the RNA extraction the use of RNeasy® Plant Mini kit from Qiagen (cat# 74904) for less than 50 samples with the following minor modifications:

1- Start from 50-70 mg of buds powder with scales or 30-50 mg of buds without scales. Only remove the tubes from the liquid nitrogen when the RNA Extraction buffer is prepared.
2- Add 1.5 % of PVP-40 (polyvinylpyrrolidone) in the RLT buffer to chelate phenolic compounds and thus prevent any interaction. Then add the appropriate volume of ß-mercaptoethanol mentioned in the Qiagen protocol.
3- Add 750 µl of RNA Extraction buffer (RLT buffer + PVP-40 + ß-mercaptoethanol) instead of 450 µl if the starting material contains scales to increase the RNA yield.

Alternatively, RNA can be extracted using the MagMAX™-96 Total RNA Isolation Kit from Thermo Fisher (cat# AM1830) for more than 50 samples following manufacturer's instructions. RNA can be frozen at -80°C for 2-3 months without significant loss of quality.

RNA library preparation: TIMING 3-4 days

i. RNA library

We recommend carrying out RNA-seq library preparation using the Truseq Stranded mRNA Library Prep Kit from Illumina (96 samples, 96 indexes, Illumina cat# RS-122-2103). Check the quality of libraries using 4200 TapeStation or Bioanalyzer instruments (Agilent) following manufacturer's instructions. See Suppl. Fig. 3 A) and B) for profiles of libraries with and without adapter contaminations. If the libraries are contaminated with adapters, continue with the following "size selection" step to remove them, otherwise proceed directly with the "quantification and pool of libraries" section.

ii. Size selection

Add 20 µl of Resuspension buffer from the Illumina kit to 30 µl of libraries. Allow the SPRI beads to equilibrate at room temperature (at least 30 minutes before use) and vortex them until they are well dispersed. Add 47,5 µl of SPRI beads (0.95 × ratio; to eliminate < 150 bp fragments). Incubate at room temperature for 5 minutes. Place on a magnetic rack for 5 minutes. Remove and discard the supernatant. Without removing the tube from the magnetic rack, add 200 µl of freshly prepared 80 % ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove and discard the supernatant. Repeat the ethanol wash once more and then remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. The beads must be completely free from ethanol, as the latter can interfere with downstream processes. Remove the tube from the magnetic rack and resuspend the beads with 32.5 µl of Resuspension buffer. Gently pipette the entire volume up and down at least 10 times to mix. Incubate the tube at room temperature for 2 minutes. Place the tube on a magnetic rack at room temperature for 5 minutes. Transfer 30 µl of the clear supernatant to a new tube. Check the quality of libraries using a D1000 ScreenTape kit on 4200 TapeStation instrument following manufacturer's instructions or Bioanalyzer instruments. DNA for sequencing can be frozen at -20°C for 3 months without significant loss of quality.

QUANTIFICATION AND POOL OF LIBRARIES SECTION

Quantification of RNA and ChIP libraries:

From this step, the quantification and the pool for RNA and ChIP libraries are the same. However, ChIP-seq libraries on one-hand and RNA-seq libraries on the other should be quantified, pooled and sequenced separately. Libraries are quantified Qubit Fluorometer from Thermo Fisher (DNA high sensitivity). Dilute the DNA high sensivity dye to 1/200 in the DNA high sensitivity buffer (e.g. for 10 samples: mix 1.990 ml of DNA high sensivity buffer and 10 µl of DNA high sensitivity dye). Add 198 µl of mix in Qubit tubes (Thermo Fisher cat# Q32856) and 2 µl of DNA (for standard: 190 µl of mix + 10 µl of standard). Vortex and spin down. Quantification is performed using Qubit fluorometer following manufacturer's instructions.

Pool of libraries: TIMING 1 hour

According to the quantification results, dilute libraries at 10 nM using this calcul to convert from ng/µl to nM: (concentration*10^6)/(size*617.96+36.04), where concentration is in ng/µl and size in bp. And pool the libraries using 5 µl of each library. Quantify the pool by Qubit as explained before and dilute the pool to the concentration required by the sequencing facility/company or the sequencer system used.

Data analysis

(i) RNA-seq

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. Firstly, we determined the quality of reads using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, possible adaptor contaminations and low quality trailing sequences were removed using Trimmomatic (24), before alignment to the *Prunus persica* v.1 or *Malus domestica* v.3 reference genome using Tophat (25). Possible optical duplicates resulting from library preparation were removed using the Picard tools (https://github.com/broadinstitute/picard). For each gene, raw reads and TPM (Transcripts Per Million) were computed (26). To finish, data are represented using the Integrative Genome Viewer (27) as a tool for visualising sequencing read profiles.

(ii) -ChIP-seq

Sequenced ChIP-seq data were analysed in house, following the same quality control and pre-processing as in RNA-seq. The adaptor-trimmed reads were mapped to the *Prunus persica* or *Malus domestica* reference genome using Bowtie2 (28). Possible optical duplicates were removed using Picard, as described earlier. Data are represented using the Integrative Genome Viewer (27).

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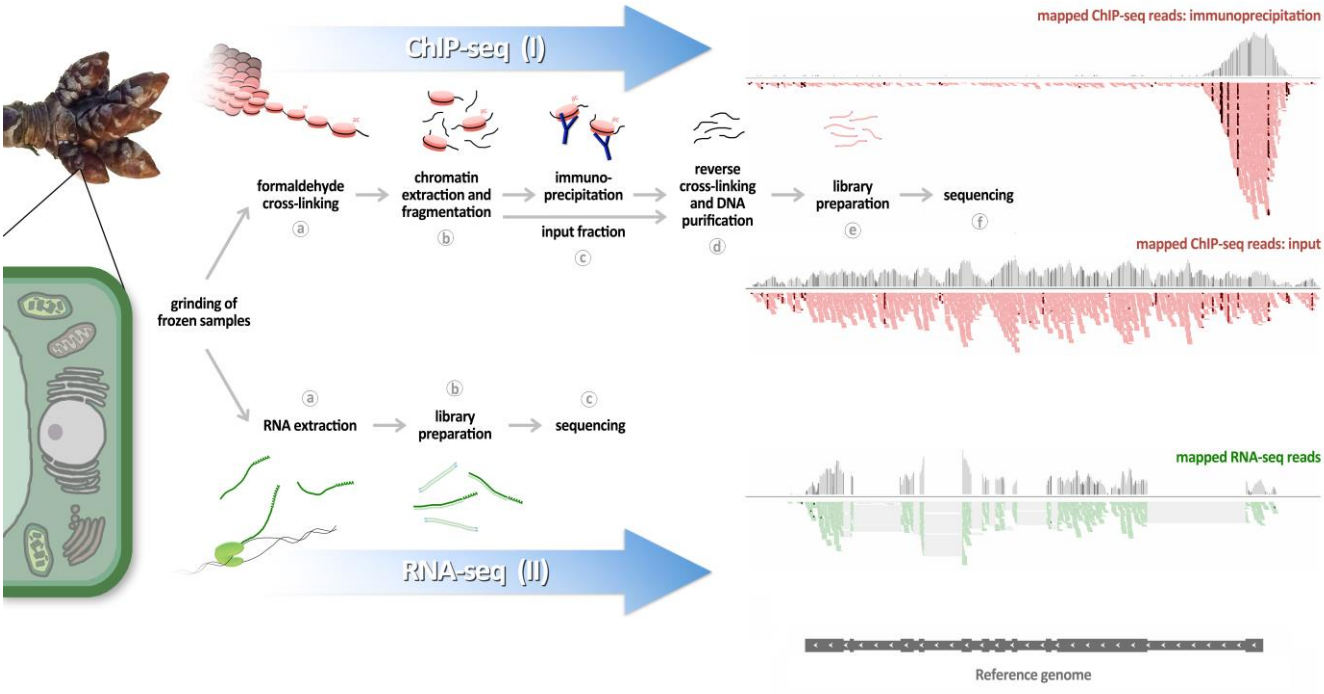


Figure 1

Figure 1| WORFLOW

Outline of the two main modules of the protocol: (I) ChIP-seq (top) and (II) RNA-seq (bottom).

Each module starts with the same biological material (ground frozen material).

(I) (a) The ChIP-seq module starts with a cross-linking step on frozen powder to stabilise interactions between DNA and proteins. (b) The chromatin is extracted using different buffers and then fragmented by sonication or MNase digestion. (c) Proteins of interest, among the protein/DNA complexes, are immunoprecipitated using specific antibodies coupled to magnetic beads. An aliquot of chromatin is set aside as an input fraction, later used for normalisation. (d) After different wash steps, a reverse cross-linking step is performed, and the DNA is isolated using SPRI beads. (e) The purified DNA is used in library preparation, and (f) is then sequenced.

(II) (a) The RNA-seq module starts with RNA extraction from the frozen powder. (b) This DNAse treated RNA is then used in library preparation, and (c) sequenced.

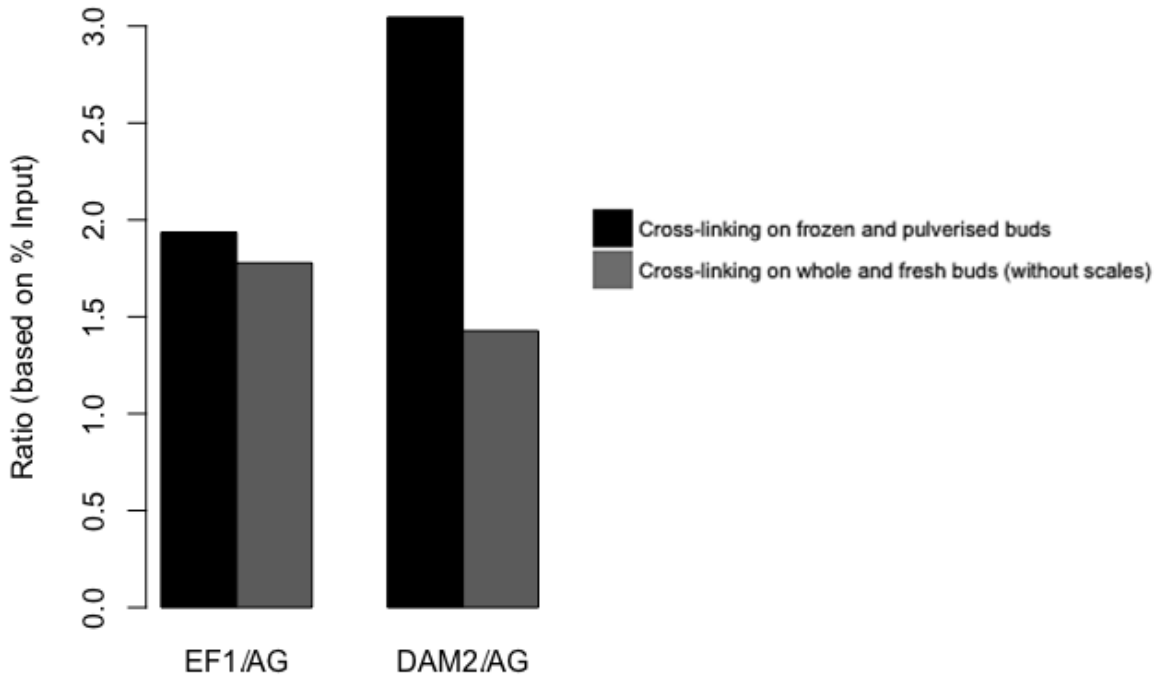
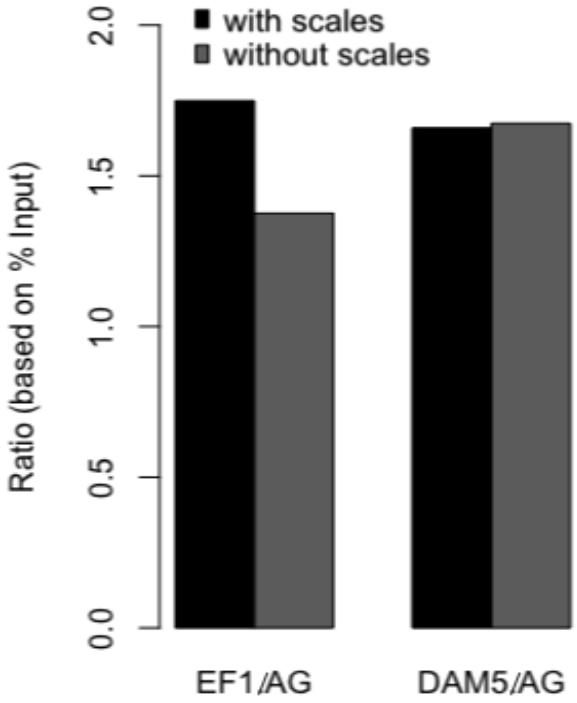


Figure 2

Figure 2| Impact of the two different cross-linking procedures on ChIP-qPCR efficiency

ChIP-qPCR for samples with the cross-linking performed on frozen and pulverised buds (black), or on whole and fresh buds without scales (grey). Quantitative PCR has been performed on *ELONGATION FACTOR 1* as a positive control for H3K4me3, *AGAMOUS* as a negative control for H3K4me3 and *DAM2* (*Dormancy Associated MADs-box 2*). Results are shown for one replicate.

A)



B)

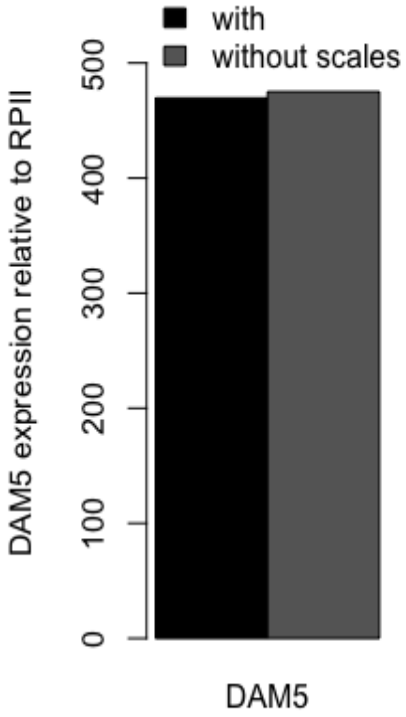


Figure 3

Figure 3| Impact of removing or not bud scales on ChIP-qPCR and RNA extraction efficiency

(A) ChIP-qPCR for H3K4me3. Black histograms represent results for chromatin extracted from buds with scales and grey histograms represent results for chromatin extracted from buds for which the scales were removed. Quantitative PCR has been performed on *ELONGATION FACTOR 1* as a positive control of H3K4me3, *AGAMOUS* as a negative control for H3K4me3 and *DAM5* (*Dormancy Associated MADs-box 5*). Results are shown for one replicate.

(B) RT-qPCR for DAM5. Black histograms represent results for chromatin extracted from buds with scales and grey histograms represent results for chromatin extracted from buds for which the scales were removed. Quantitative PCR has been performed on *DAM5* (*Dormancy Associated MADs-box 5*). The expression is relative to a constitutive gene, *RPII* (*RNA polymerase II*). Results are shown for one replicate.

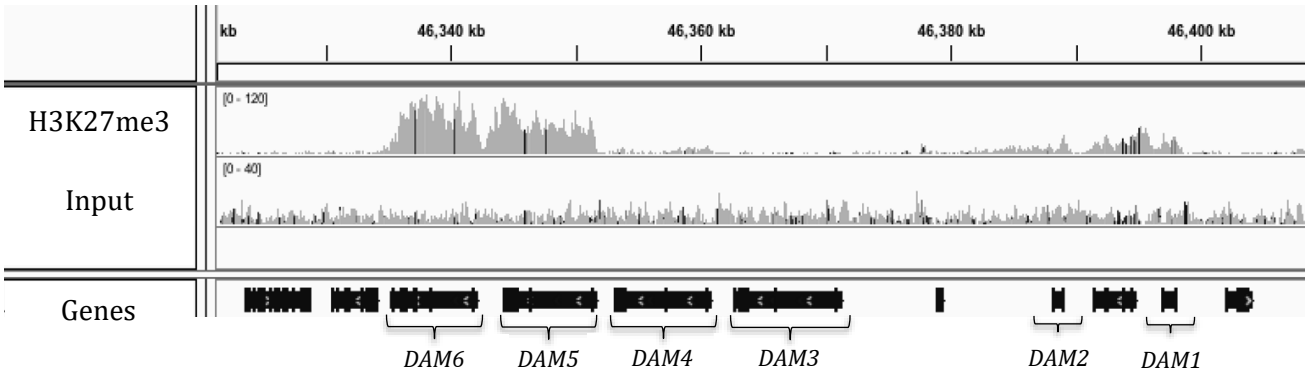
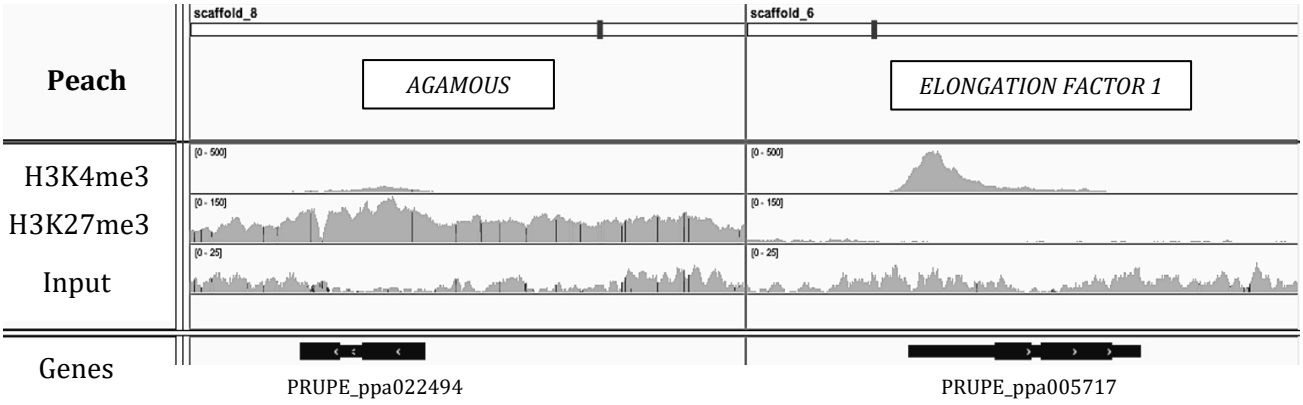


Figure 4

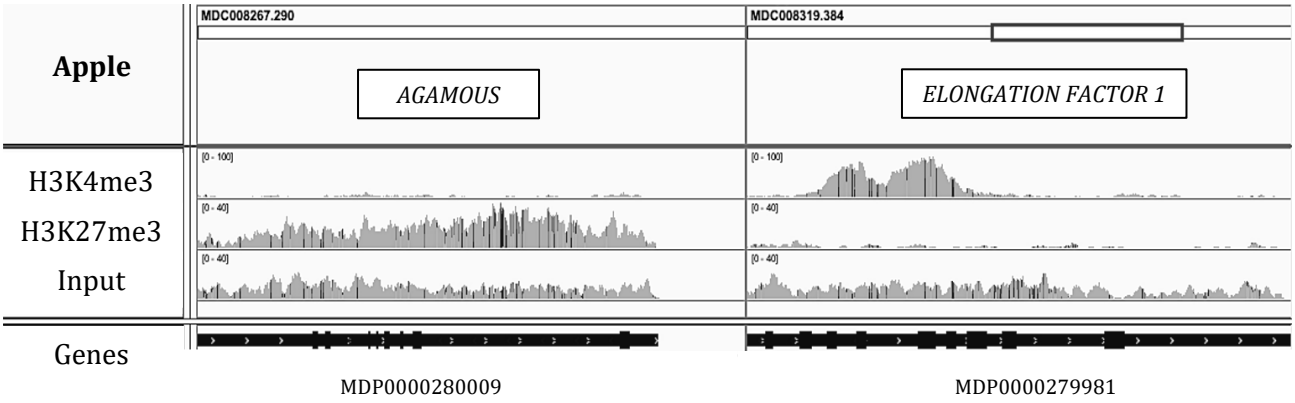
Figure 4| H3K27me3 ChIP-seq profile at the *DAM* gene cluster in peach

IGV screenshot of ChIP-seq data for H3K27me3 and its corresponding input performed on non-dormant peach buds (*Prunus persica*) at a cluster of *DAM* genes. Genes are represented by black rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

A)



B)



C)

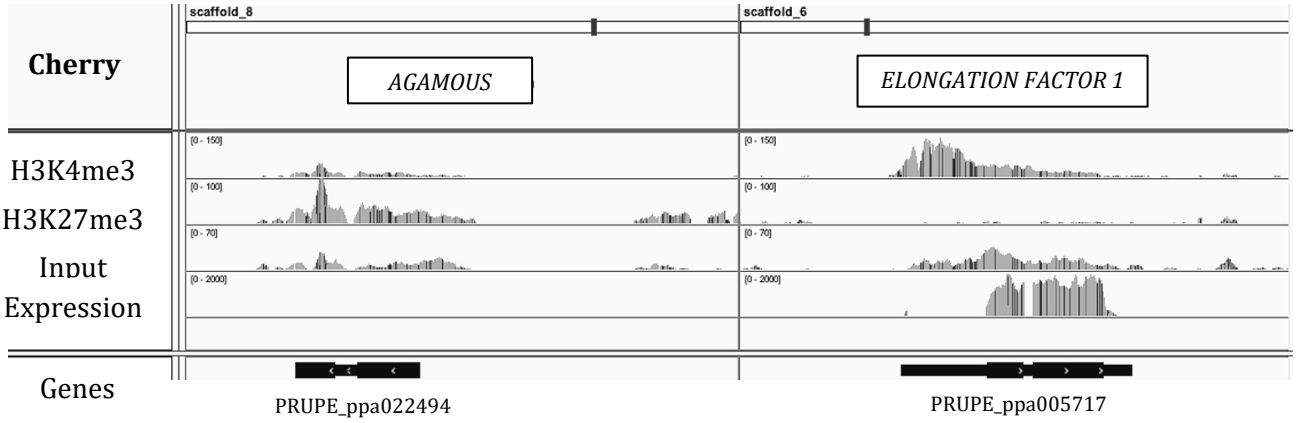


Figure 5

Figure 5| H3K27me3 and H3K4me3 ChIP-seq and RNA-seq profiles three fruit tree species

IGV screenshot of ChIP-seq data for H3K27me3 and H3K4me3 and their corresponding inputs performed on peach (A), apple (B) and sweet cherry buds (C) for two control genes: *ELONGATION FACTOR 1* as a positive control of H3K4me3 and *AGAMOUS* as a positive control of H3K27me3. RNA-seq was carried out on sweet cherry buds (C). Genes are represented by black rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

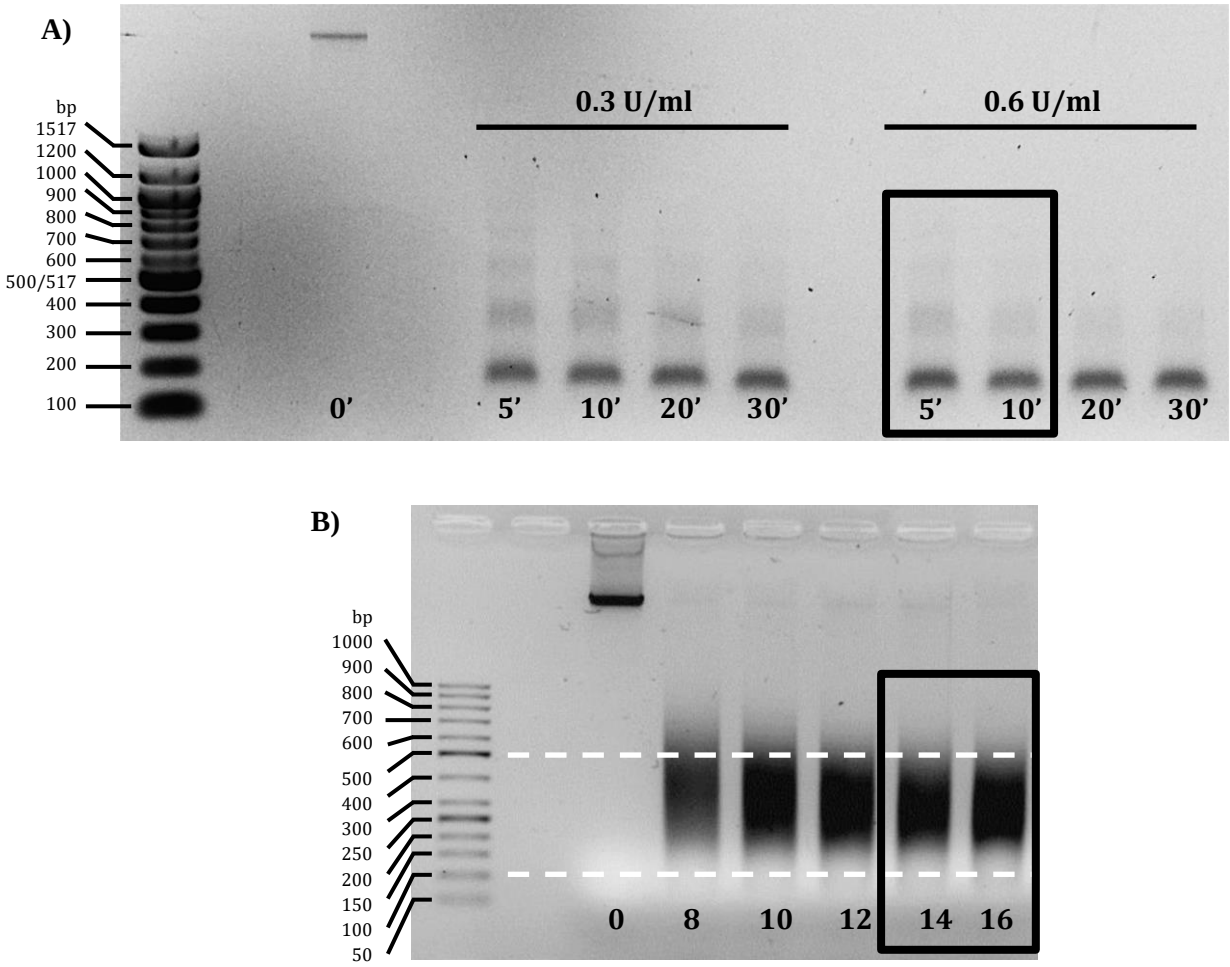


Figure 6

Figure 6| DNA profiles for MNase-digested and sonicated chromatin

A) DNA profiles of chromatin digested with different concentrations of MNase (0.3 and 0.6 U/ml) for various durations (5, 10, 20 and 30 minutes). Optimal MNase digestion profiles, with ~80% of chromatin in mono-nucleosome form, are indicated with the black rectangle.

B) DNA profiles of chromatin fragmented with different numbers of sonication cycles (0-16). The dotted white lines represent the optimal range of DNA fragments size (100-500 bp). Optimal sonication profiles are indicated with the black rectangle.