Video Article

Chromatin Immunoprecipitation Assay for the Identification of Arabidopsis Protein-DNA Interactions *In Vivo*

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Abstract

Intricate gene regulatory networks orchestrate biological processes and developmental transitions in plants. Selective transcriptional activation and silencing of genes mediate the response of plants to environmental signals and developmental cues. Therefore, insights into the mechanisms that control plant gene expression are essential to gain a deep understanding of how biological processes are regulated in plants. The chromatin immunoprecipitation (ChIP) technique described here is a procedure to identify the DNA-binding sites of proteins in genes or genomic regions of the model species *Arabidopsis thaliana*. The interactions with DNA of proteins of interest such as transcription factors, chromatin proteins or posttranslationally modified versions of histones can be efficiently analyzed with the ChIP protocol. This method is based on the fixation of protein-DNA interactions *in vivo*, random fragmentation of chromatin, immunoprecipitation of protein-DNA complexes with specific antibodies, and quantification of the DNA associated with the protein of interest by PCR techniques. The use of this methodology in Arabidopsis has contributed significantly to unveil transcriptional regulatory mechanisms that control a variety of plant biological processes. This approach allowed the identification of the binding sites of the Arabidopsis chromatin protein EBS to regulatory regions of the master gene of flowering *FT*. The impact of this protein in the accumulation of particular histone marks in the genomic region of *FT* was also revealed through ChIP analysis.

Video Link

The video component of this article can be found at http://www.jove.com/video/53422/

Introduction

During recent years a wide range of genetic, molecular and genomic tools have been developed in the model species *Arabidopsis thaliana*. This technology has facilitated enormously the progress in understanding how plant development is regulated. Among the developmental processes studied using Arabidopsis as a model, the genetic control of flowering time has been extensively analyzed. These studies have shown that plants modulate very precisely the time of flowering in response to endogenous cues such as hormones and the age of the plant, and also to environmental signals such as photoperiod and temperature that synchronize flowering time with the natural cycle of seasons[1,2]. The isolation and characterization of Arabidopsis mutants with alterations in the time of flowering has been determinant in unveiling a complex network of genes that regulate the flowering time in response to endogenous and environmental factors. These genetic circuits are integrated at the level of a few master genes that act as switches of flowering, and the exact timing of the floral initiation depends on the balance of flowering promoting and repressing activities that work upstream of the floral integrator genes[1,3].

The functional characterization of genes identified for their role in the control of flowering initiation, aided by the recent use of genomic approaches, have revealed the central role of transcriptional regulation in the modulation of flowering time. In fact, many of the master genes of flowering encode transcription factors[4]. In addition, a number of chromatin remodeling protein complexes influence the expression of master genes of flowering. A number of the Arabidopsis mutants isolated for their altered flowering time turned out to carry mutations in genes encoding a variety of chromatin modifiers. Different chromatin remodelers that introduce posttranslational modifications in histone tails, reposition nucleosomes relative to the DNA or exchange canonical histones by histone variants are necessary for the proper regulation of flowering in Arabidopsis[5,6]. Some of these chromatin remodeling activities catalyze the deposition or removal of covalent modifications such as acetylation or methylation in specific histone residues. These histone marks are specifically recognized by specialized effectors that recruit other chromatin remodeling complexes, transcription factors or components of the transcriptional machinery to regulate the transcriptional activity of flowering genes.

Chromatin ImmunoPrecipitation (ChIP) allows the identification of *in vivo* DNA-binding sites for proteins of interest (**Figure 1**). This procedure takes advantage of the ability of certain chemicals to cross-link the proteins to the DNA. The resulting DNA-protein complexes can be then immunoprecipitated by using specific antibodies against transcription factors, chromatin-binding proteins, or particular modifications and heterologous epitopes (commonly referred to as "tags") attached to the protein of choice. The DNA purified from these immunoprecipitates

can be used as a template in quantitative PCR (qPCR) reactions to assess for enrichment of particular sequences of interest. In this way, the binding sites of transcription factors or the distribution of histone marks in particular genes can be established[7,8]. In addition, combined with Next Generation Sequencing (NGS) that enables massive parallel sequencing, ChIP technology has made possible the genome-wide identification of the binding sites of transcription factors as well as unveiling epigenomic landscapes of histone modifications. Furthermore, the simultaneous analysis of gene expression allows monitoring how the binding of transcriptional regulators or the deposition of particular histone marks correlate with the transcriptional activity of genes[9].

The use of ChIP protocols in Arabidopsis has allowed assessing the impact that a variety of transcriptional regulators have on the chromatin organization of master genes of flowering and how these structural changes influence gene expression[5,6]. Previous results showed that the Arabidopsis locus *EARLY BOLTING IN SHORT DAYS* (*EBS*) acts as a repressor of flowering and mutants in this gene show an acceleration of flowering and upregulation of the master gene of flowering *FT*. In addition, loss-of-function mutations in *FT* fully suppress the early flowering phenotype of the *ebs* mutant plants, indicating that *FT* is required for the premature flowering of *ebs* mutants and that EBS is necessary for the repression of this master gene of flowering[10,11]. *EBS* encodes a PHD-containing protein that can specifically bind histone H3 di- and trimethylated in the lysine 4 residue (H3K4me2/3), suggesting a role for EBS in the chromatin-mediated repression of *FT*[12]. The use of the ChIP approach demonstrated that the Arabidopsis PHD-containing protein EBS[10,11] binds regulatory regions of the floral integrator gene *FT* to repress its expression[12]. Additional data obtained through the use of ChIP technology showed that this protein is required to maintain low levels of histone acetylation, a hallmark of active transcription, in the chromatin of this master gene of flowering during initial stages of Arabidopsis development. These observations, together with genetic and gene expression data, demonstrate that this Arabidopsis PHD-containing protein has a central role in the fine tuning of flowering time by modulating the expression of the floral integrator gene *FT*[12]. The work presented here provides an optimized method useful not only for the analysis of histones but also for other chromatin associated proteins, and with increased efficiency and reduced experimental time. Furthermore, this report illustrates how the use of ChIP protocols has provided new insights into the relationship between changes in chromatin modifications and transcriptional states of plant genes, and how these chromatin-mediated mechanisms of gene expression control impact on the onset of flowering in Arabidopsis.

Protocol

1. Crosslinking of the Plant Material (1 hr)

1. Grow the Arabidopsis lines used in the experiment (wild type — WT — versus mutants, and/or lines expressing the tagged version of your protein of interest versus lines expressing the tag not fused to any protein) for 12-18 days on large Petri dishes (150 mm) with MS-agar medium (1 L: 1x Murashige & Skoog salts, 10 g sucrose, 0.5 g MES, pH 5.7 (KOH), 1% agar). Alternatively, grow plants on pots containing 3:1 mix of soil and vermiculite.
   CAUTION! Formaldehyde is toxic by inhalation, in contact with skin and if swallowed, and should be handled in a chemical hood wearing suitable personal protective equipment. Steps 1.2 – 1.6 should be performed under the fume hood.
2. Make little holes in the lid of the 50 ml tubes with a laboratory burner-heated needle. Add 40 ml of 1x PBS buffer and 1.08 ml of formaldehyde to a final concentration of 1% (stock 37% solution). Collect 1.5 g of plant material in those 50 ml tubes. Keep the tubes on ice during crosslinking.
3. Close the 50 ml tubes with the lid. Place the tubes in a desiccator and apply vacuum. Vacuum infiltrate the tissue for 10 min, then release the vacuum carefully, to prevent churning up the solution. Mix the sample. Repeat twice. After infiltration, observe the plant material and confirm that it becomes slightly translucent and tend to sink to the bottom of the tube (**Figure 2**).
4. Add 2.5 ml of 2 M Glycine to achieve a final concentration of 0.125 M. Apply vacuum for 5 min. Glycine acts as a competitive inhibitor of formaldehyde crosslinking.
5. Discard the PBS formaldehyde-glycine solution by inverting the closed 50 ml tube with holes in the lid.
6. Rinse the plantlets twice with 1x PBS and once with water discarding the washing solution as described in step 1.5. Dry them on a paper towel and freeze in liquid nitrogen.
   NOTE: Frozen tissue can be kept at –80 °C for weeks.

2. Preparation of the Antibodies (1 day)

NOTE: For immunoprecipation, the use of magnetic beads conjugated with antibodies via a protein G or protein A linker with high affinity for the constant domain of the antibody heavy chain is recommended. The DNA-protein complexes can attach unspecifically to the surface of the beads-antibody conjugates. For that reason, it is necessary to perform controls without specific antibodies to quantify the non-specific background binding.

1. For each immunoprecipitation, prepare 15 µl of magnetic beads in a 1.5 ml microcentrifuge tube. The amount of beads depends on the quantity of the chromatin used for immunoprecipitation and also on the antibodies.
2. To wash, add 1 ml of Chromatin Immunoprecipitation (ChIP) dilution buffer to the beads, mix by rotation and place the 1.5 ml microcentrifuge tube in a magnetic rack. Wait around 1 min to let the beads attach to the magnet. With the 1.5 ml microcentrifuge tubes still in the rack, remove the supernatant by pipetting. Repeat twice.
   NOTE: For each plant line two immunoprecipitation sets are needed: one with the beads and antibody (Ab) and a second no antibody (No-Ab) control with magnetic beads only.
3. Resuspend the beads in 200 µl of ChIP dilution buffer. Add the required amount of the specific Ab to one of the two tubes prepared for each plant line (the exact dilution differs for each Ab and has to be optimized experimentally or, in the case of commercial antibodies, follow the instructions of the manufacturer). Use this sample for immunoprecipitation. Add the same amount of unspecific IgG to the other tube, which will be used as negative control.
4. Incubate O/N on a rotating wheel at 4 °C to let the antibody attach to the beads.

3. Chromatin Extraction (4 hr)

1. Grind thoroughly the frozen plant material in liquid nitrogen using mortar and pestle until the powder becomes homogenous and light green. Transfer the powder to a new 50 ml tube.
2. For steps 3.2 – 3.6, work under the fume hood. Add 30 ml of Extraction buffer 1 (ExB 1), and mix well to soak the tissue powder. From this step on, keep the samples at 4 °C at all times. Frozen tissue and liquid nitrogen leftovers will cause the buffer to freeze. Make sure to thaw the frozen tissue completely by inverting the tubes 4-5 times every 2 min.
3. Clear the slurry by passing it through a filtration tissue with pore size of 22-25 µm into a new 50 ml tube and centrifuge it at 1,000 x g for 20 min at 4 °C.
   NOTE: In this step, the nuclei and all the organelles will pellet. ExB 1 contains enough sucrose to provide high density and prevent the disruption of organelle structure.
4. Gently remove the supernatant by decantation. At this stage, observe a green pellet of around 2 ml.
5. Resuspend the pellet in 5 ml of ExB 2. Resuspension of the pellet will be difficult at this point. Centrifuge at 1,000 x g for 10 min at 4 °C.
   NOTE: ExB 2 contains 1% Triton X-100 to help burst the chloroplasts open and remove chlorophyll.
6. Resuspend the pellet in 300 µl of Extraction Buffer 3 (ExB 3).
7. In a clean microfuge tube, add 600 µl of ExB 3. Take the resuspended pellet from step 3.6 and carefully layer it on top of the clean ExB 3.
8. Spin for 1 hr at 16,000 x g at 4 °C.
   NOTE: This step helps removing the detergent from the sample.
9. Remove the supernatant by aspirating with a pipette. Resuspend the nuclear pellet in 300 µl of sonication buffer by slowly pipetting up and down to avoid bubble formation, which may affect sonication efficiency. Carefully pipette all the suspension out and transfer it to a clean 1.5 ml microcentrifuge tube.
   NOTE: This buffer contains SDS, to lyse the nuclei and release the chromatin.
10. Sonicate the suspension to lyse the nuclei and randomly shear the chromatin into small fragments. Use sonication conditions that render chromatin enriched in fragments between 200-800 bp (20-30 cycles with the settings 30 sec ON / 30 sec OFF at 4 °C for the sonication device available described in the Table of materials/reagents). Check the efficiency of sonication by electrophoretic separation of the obtained DNA fragments on an agarose gel[13], loading 2 µl of sonicated chromatin (**Figure 3**).
    CAUTION! Make sure to wear ear protection equipment during the sonication step in case the ultrasound device is not contained in a soundproof cabinet.
    Crucial step! The chromatin fragmentation largely depends on the sonication equipment. The sonication conditions should be adjusted to achieve enrichment in the appropriate DNA sizes. See **Figure 3** for an example of how to optimize chromatin fragmentation.
11. Spin the solution once at 12,000 x g for 10 min at 4 °C. Transfer the supernatant by pipetting to a clean 1.5 ml microcentrifuge tube. Discard the pellet. Take 1/10 of the supernatant to a new 1.5 ml microcentrifuge tube, mark it as Input and freeze at -20 °C.
12. Dilute the chromatin 10x with ChIP dilution buffer to dilute SDS to final concentration of 0.1%.
    Note: Samples can be frozen and stored at -20 °C for several weeks.

4. Immunoprecipitation of the Protein of Interest and Rescue of DNA (1 day, 3 hr)

1. Wash the beads coated with the Ab from step 2.4 three times with 1 ml of ChIP dilution buffer to remove excess of antibody as described in step 2.2. Resuspend in 200 µl of ChIP dilution buffer. Add 50 µl of isolated chromatin. Incubate O/N on a rotating wheel at 4 °C.
   NOTE: Check the concentration of the chromatin in microvolume UV-Vis spectrophotometer. 50 µl of isolated chromatin should contain more than 10 µg of DNA.
2. For steps 4.2-4.5 work in 4 °C. Wash the beads twice in 1 ml of Low Salt Washing buffer as described in step 2.2.
3. Wash the beads once in 1 ml of High Salt Washing buffer.
4. Wash the beads once in 1 ml of LiCl Washing buffer.
5. Wash the beads twice in 1 ml of TE buffer. After the last wash, make sure to fully remove the TE buffer left in the tube by aspiration with a pipette.
6. Take out the INPUT samples (step 3.9). Transfer 5 µl of the INPUT to a new 1.5 ml microcentrifuge tube and continue as with the ChIP samples. Reverse crosslinking by adding 200 µl of 5% chelating ion exchange resin, and incubate for 10 min at 95 °C shaking every 2-3 min. Spin down at 16,000 x g for 30 sec and carefully transfer the supernatant into a new microcentrifuge tube by pipetting.
   NOTE: While the traditional method for crosslinking reversal involves an O/N incubation with NaCl, the incubation with a chelating ion resin at 95 °C allows for efficient decrosslinking and elution of DNA in 10 min, therefore making the protocol one day shorter.
7. Add 2 µl of proteinase K (10 mg/ml) and incubate at 37 °C for 30 min to digest proteins and release DNA.
8. Stop the reaction by incubating at 95 °C for 10 min.
9. Quickly spin at 16,000 x g for 30 sec and transfer supernatant to a new tube by pipetting.
10. Clean the DNA isolated using a standard DNA fragments purification kit.
    NOTE: Proceed according to the manufacturer's instructions.
11. Transfer the DNA binding column from the kit to a new 1.5 ml microcentrifuge tube. Elute the purified DNA by adding 20 µl of DNase-free water to the center of the column and spinning at 16,000 x g for 60 sec. Stop point: Samples can be stored at -80 °C for several weeks.

5. Measuring Abundance of Binding Sites in the Immunoprecipitated DNA by qPCR (4 hr)

NOTE: DNA isolated from the precipitated chromatin has to be analyzed to determine which DNA fragments have been ChIP-ed from the total chromatin, due to its binding to the protein of interest.

1. Design primers for the genomic regions of interest with a melting temperature (Tm) of 60 °C and a GC content of 30%-80%. As chromatin is fragmented by sonication, the length of amplified sequence should not be longer than 150-200 nucleotides (**Table 1**).
   NOTE: A useful tool for primer design is Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0/). Additional guidelines for primer design are available in previous reports[8,14,15].

2. Use a BLAST program to make sure that the primers are specific (especially promoters can share similar TF binding sequences) and test the primer efficiency using 1:10 dilutions in water of the input DNA (step 4.9). Certain regions of the genome will be purified better than others. It is therefore important to design PCR primers and check them on the diluted input DNA.
3. Dilute the purified DNA 1:10 with water and pipette 5 µl of the diluted DNA in a PCR tube for each reaction.
   NOTE: Dilute only the amount needed, as DNA can attach to the walls of the 1.5 ml microcentrifuge tubes. Multiple thawing-freezing cycles increase the number of attached DNA molecules. For each primer pair, amplification in the input, Ab-ChIP and No-Ab ChIP has to be analyzed.
4. To complete the PCR mix, add Sybr Green PCR master mix to a final concentration of 1x, and 0.5 µM of each primer. Fill up to 20 µl with DNase-free water.
5. Perform the qPCR reaction following the instructions of the Sybr Green PCR master mix manufacturer.

6. Data Analysis

NOTE: Among the existing ways of analyzing ChIP-data, two are most commonly used. The first of them is the fold enrichment method, also named as 'relative enrichment', 'signal over background' or 'relative to the no-antibody control'. The second method is named as "% of input".

1. Data analysis by fold enrichment method.
   1. Create a spreadsheet with samples names and raw Ct values that have been obtained after qPCR reaction.
   2. For each sample, subtract from its raw Ct value the raw Ct value obtained for the corresponding No-Ab control. NOTE: After this mathematical operation, Ct value for No-Ab sample will equal 0.
   3. Calculate fold enrichment by exponentiation operation with base 2 and exponent (power) equal to the negative remainder obtained in step 6.1.2 (**Table 2**).
      $$fold\ enrichment = 2^{-\ (Ct(sample)\ -\ Ct(No\text{-}Ab))}$$
      NOTE: In this method the abundance of a specific DNA fragment in the ChIP-ed sample is compared with the abundance of this fragment in No-Ab control. The assumption of this method is that the level of background signal is reproducible between different primer sets, samples, and replicate experiments. However, this ´noise´ signal levels do vary between primer sets, samples, and experiments.

2. Data analysis by % of input method.
   1. Create a spreadsheet with samples names and raw Ct values that have been obtained for them after qPCR reaction.
   2. Adjust raw Ct values for input samples by subtracting a value of logarithm base 2 from the fraction of total extracted chromatin that was taken as an input.
      NOTE: In this protocol the subtracted value equals 3.32, as a 10% of total chromatin was taken as an input in this protocol. $Log_2(10)$ equals 3.32.
   3. Calculate % of input by multiplying by 100 the value of exponentiation operation with base 2 and exponent (power) equal to remainder of adjusted input values subtracted from raw Ct values of the sample (**Table 3**).
      $$\%\ of\ input = 100*2^{(adjusted\ input\ –\ Ct(sample))}$$
      NOTE: With this procedure, the relation between specific DNA abundance in the ChIP-ed sample to the total isolated chromatin (input sample) is calculated. Further details on ChIP data analysis can be found in Haring et al.[8].

Representative Results

Eight main steps can be singled out in this ChIP protocol for the identification of in vivo protein-DNA interactions, including growing and harvesting of plant material, cross-linking of chromatin, chromatin isolation, chromatin fragmentation, selective isolation of the complexes between DNA and the protein of interest by immunoprecipitation, protein digestion, DNA purification, and qPCR analysis (**Figure 1**). A crucial step in the ChIP protocol is the fixation of DNA-protein interactions in a cross-linking reaction. Typically, formaldehyde cross-linking is used in plant ChIP. Formaldehyde penetrates the tissue and creates methylene bridges between NH_2 groups of proteins or proteins and nucleotides in the DNA[16]. An efficient fixation of nucleoprotein complexes in vivo requires that formaldehyde reaches the chromatin within plant nuclei, and it is essential that plant tissue is fully submerged in the formaldehyde buffer to ensure that during immunoprecipitation the protein-DNA complexes are effectively pulled down. Arabidopsis leaves are covered in wax layers that difficult the penetrance of the crosslinking agent into the cells. In addition, trichomes favor the formation of air bubbles on the surface of the leaves, preventing the formaldehyde solution from getting inside of the plant tissue. To overcome this limitation, in this step vacuum is applied to the samples, preventing the formation of bubbles in the fixation solution and improving the penetration of formaldehyde into the nuclei of all cells. Seedlings after efficient cross-linking treatment are shown on **Figure 2**.

Another key step in the ChIP protocol is chromatin shearing. Large chromatin fragments can result in low resolution mapping of the binding sites of the protein of interest in the DNA sequence. In contrast, very small fragments can prevent efficient amplification of the bound DNA by qPCR. According to empirical experience, the optimal chromatin size range for ChIP analysis should be between 200 and 600 bp, and the conditions to achieve the appropriate fragmentation of the chromatin have to be set depending on the sonication device available in the laboratory. **Figure 3** illustrates the procedure to optimize chromatin shearing to achieve the maximum enrichment in the desired range of DNA sizes. Before sonication, chromatin consists mainly of very large fragments. An increased number of sonication cycles progressively reduces the average size of the DNA in ChIP samples, reaching the optimum enrichment in the range of 200-600 bp after 20-30 cycles (section 3.8 of the protocol and **Figure 3**).

Here, the identification of the EBS protein binding sites to regulatory regions of the floral integrator *FT* is shown as an example of the usefulness of the ChIP technique. The binding of EBS to the *FT* locus was unraveled through ChIP assays with Arabidopsis lines expressing a cMyc-EBS fusion protein under the control of the native *EBS* promoter (pEBS::cMyc-EBS). This tagged version of EBS is fully functional as shown by the wild type phenotype of *ebs* mutant plants expressing this construct. Four regions within the genomic *FT* locus were tested for EBS binding; one in the promoter region of the gene (FTII), two around the transcriptional start site (FTIV, FTVI), and another one in the first intron of *FT* (FTVII)

(**Figure 4A**). As shown in **Figure 4B**, ChIP results demonstrate that the EBS protein can bind a regulatory sequence close to the transcriptional start site of the master gene of flowering *FT*[12]. Furthermore, the EBS protein can interact both *in vitro* and *in vivo* with histone deacetylases such as HDA6, another chromatin-related protein involved in the regulation of flowering time in Arabidopsis[17]. In order to assess the possible influence of EBS on the levels of histone acetylation in the genomic region of *FT*, ChIP assays were performed using an antibody directed against histone H3 acetylated in lysines 9 and 14, a histone mark correlated with transcriptionally active chromatin[12]. Moreover, these data illustrate how ChIP approaches can contribute to shed light on the molecular mechanisms that modulate the transcriptional regulation of plant developmental processes.
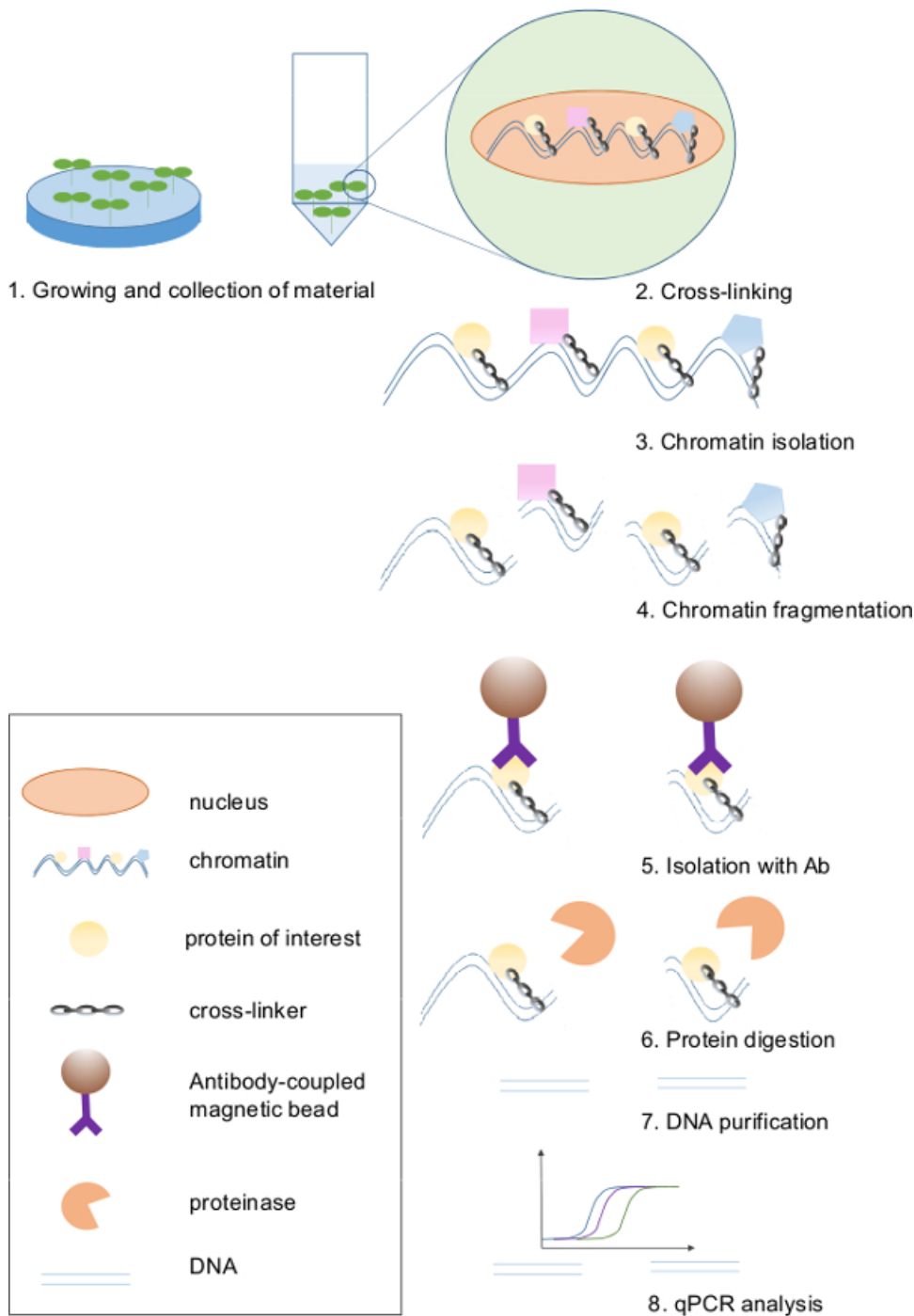

Figure 1. Scheme of the Chromatin Immunoprecipitation protocol. ChIP is a technique commonly used to investigate interactions between proteins and DNA, fixed by a chemical compound. Chromatin with preserved interactions is isolated from the nuclei and fragmented. By using antibodies against the protein of interest, we can select fragments of DNA attached to it. The antibody is conjugated to magnetic beads that enable fast and simple isolation using a magnetic rack. In the next step, proteins are removed by proteinase treatment and DNA is released. Purified DNA fragments are then used as templates in qPCR reactions to measure their relative abundance. Please click here to view a larger version of this figure.
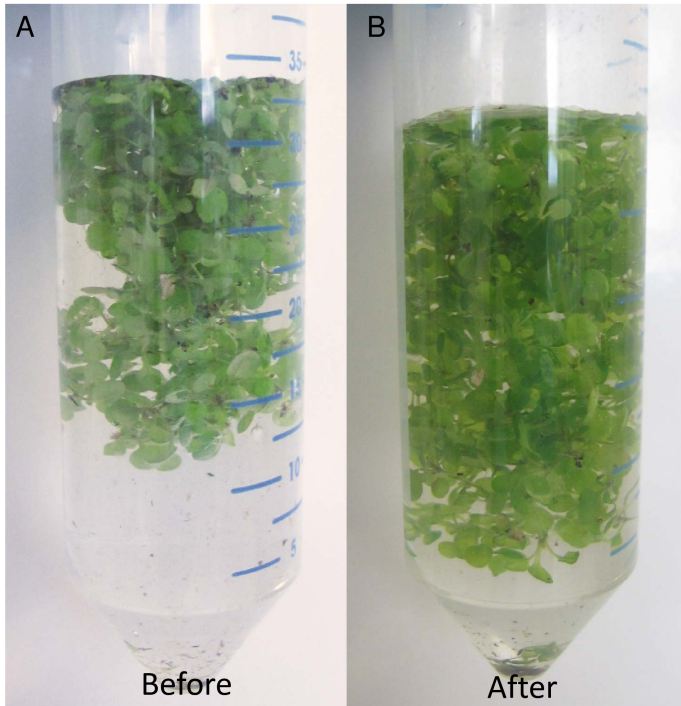
Figure 2. Example of a tissue after cross-linking with formaldehyde. In order to investigate interactions between DNA and protein, *in vivo* complexes have to be fixed to make them stable and durable through the multiple steps of the protocol. In this version of the protocol, fixation occurs by formaldehyde treatment. Efficient penetration of formaldehyde through the tissue is crucial for the success of the ChIP method. (**A**) Before the cross-linking step the plant material floats on the surface of the solution and is covered with small air bubbles; the abaxial and adaxial surfaces of the leaves differ in color. (**B**) After fixation, plantlets should be visibly soaked in the solution, slightly transparent, and evenly immersed in the crosslinking solution. Please click here to view a larger version of this figure.
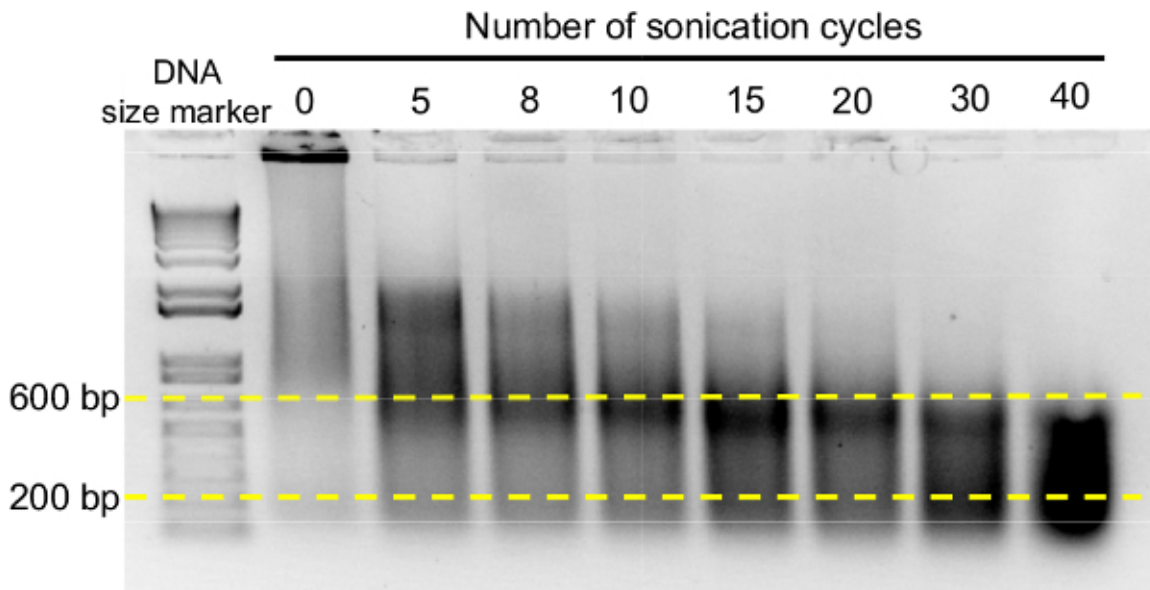

Figure 3. Optimization of the sonication conditions for ChIP analysis. Chromatin isolated from fixed material consists of broad range of fragments lengths with a strong band at the top of the gel representing extremely long chromatin sizes (second line on the left). With increasing number of sonication cycles, the peak of chromatin fragments sizes is shifted towards smaller fragments. In the experiment shown, optimal cycle numbers vary between 20 and 30, after which the optimal chromatin fragments ranging from 200 to 600 bp are more abundant in the ChIP sample. Please click here to view a larger version of this figure.
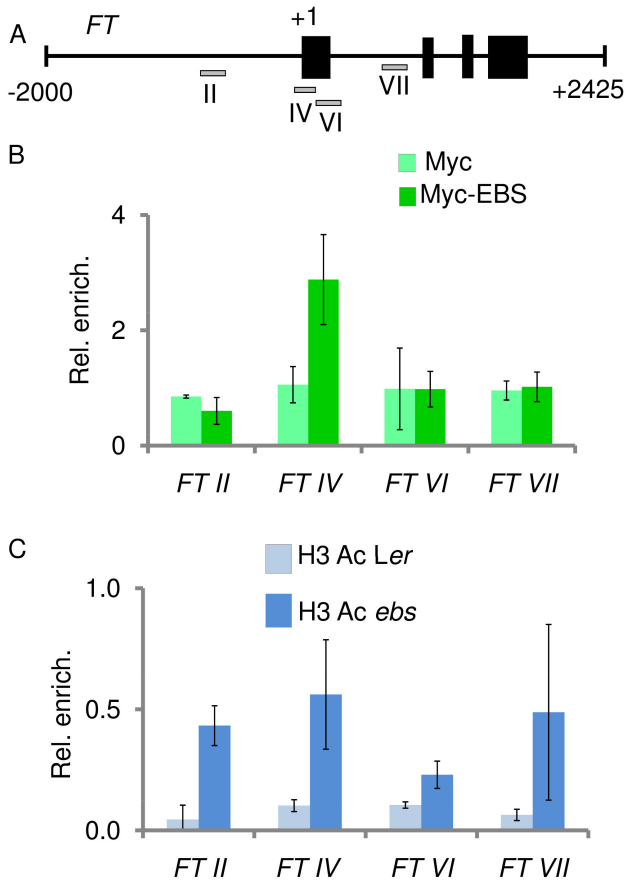
Figure 4. ChIP analyses show that EBS binds *FT* and *ebs* mutations alter the level of histone H3 acetylation in this floral integrator gene. (**A**) Genomic region of *FT* with black boxes representing exons. Four fragments spanning regulatory regions of this floral integrator gene were tested (grey boxes) by designing specific qPCR primers. (**B**) EBS binds the *FT* IV region, located close to the transcriptional start site. Myc-EBS corresponds to *ebs* mutant plants expressing the cMyc-tagged version of EBS; Myc denotes transgenic plants expressing the cMyc epitope not fused to any Arabidopsis gene. (**C**) *ebs* mutant plants display higher abundance of H3K9,14Ac than wild type Landsberg *erecta* (L*er*) in all four *FT* regions assessed. Error bars show standard deviation (**B and C**). Modified from previously published data in *The Plant Cell*[12]. Please click here to view a larger version of this figure.

Forward	Reverse	Region tested
TCGTGCAAATGGATGGTTAG	TTTTTTATAAACAAGCGGCC	FT II
TGATTTCACCGACCCGAGTTAATGCAAATC	AACTCTGCTTACTATAAGAGGGTCTC	FT IV
AAACCACCTGTTTGTTCAAGATC	TCCTGAGGTCTTCTCCACCA	FT VI
GGGATTTTTCTTTGTTCCTCC	ATTCCACAACAGAGATTCATCA	FT VII

Table 1. Primers used in this study.

Sample	Raw Ct Values	Ct-Ct(No-Ab)	Fold enrichment $2^{-(Ct(sample) - Ct(No-Ab))}$
No-Ab	34.5	0	1
Ab	29.2	-5.3	39.4

Table 2. Example of ChIP data analysis by "fold enrichment" method. Ct Values for No-Ab samples represent the background signal caused by unspecific binding of DNA to the Ab/magnetic beads. These Ct values should exceed 30. Difference between Ct values of No-Ab and Ab sample should be higher than 1.

Step 1 – Adjust the input			
Adjusted input = Raw $Ct_{(input)}$ - Log_2(fraction of total chromatin)			
	Raw Ct	input from total chromatin	$Log_2 10$
Input	29.5	10%	3.32
Adjusted input = 29.5 – 3.32 = 26.18			
Step 2 - % of input calculations			
Sample	Raw Ct	$Ct_{(adjusted\ input)} - Ct_{(sample)}$	$2^{Ct(adjusted\ input) - Ct(sample)} * 100\%$
Input	26.18		
ChIP-Ab	31.45	-5.27	2.59
No-Ab	34.5	-8.32	0.31

Table 3. Example of ChIP data analysis by "% of input" method. Inputs represent the total cross-linked chromatin isolated from nuclei, so the Ct values should be equal for all the primer pairs. Conventionally, 5% or 10% of the total chromatin is taken as an input. No-Ab represents a negative control sample — the beads not coated by specific antibodies against the protein or tag of interest. ChIP-Ab stands for the sample after the immunoprecipitation with the antibodies against the protein or tag of interest.

PBS 10x
1.3 M NaCl
30 mM Na_2HPO_4
30 mM NaH_2PO_4
pH 7

Extraction buffer 1 (ExB 1)
0.4 M sucrose
10 mM Tris-HCl pH 8
10 mM MgCl
5 mM β-Mercaptoethanol
Protease inhibitors

Extraction buffer 2 (ExB 2)
0.25 M sucrose
10 mM Tris-HCl pH 8
10 mM $MgCl_2$
1% Triton X-100
5 mM β#Mercaptoethanol
Protease inhibitors

Extraction buffer 3 (ExB 3)
1.7 M sucrose
10 mM Tris-HCl pH 8
0.15% Triton X-100
2 mM $MgCl_2$
5 mM β#Mercaptoethanol
Protease inhibitors

Sonication buffer
50 mM Tris-HCl pH 8
10 mM EDTA
1% SDS
Protease inhibitors

ChIP dilution buffer
1.1% Triton X-100
1.2 mM EDTA
16.7 mM Tris-HCl pH 8
167 mM NaCl
Protease inhibitors

Low Salt buffer
150 mM NaCl
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8

High Salt Washing buffer
500 mM NaCl
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8

LiCl Wash buffer
0.25 M LiCl
1% NP-40
1% sodium deoxycholate
1 mM EDTA
10 mM Tris-HCl pH 8

TE buffer
10 mM Tris-HCl pH 8
1 mM EDTA

Table 4. Buffer composition.

Discussion

The ChIP protocol described here is a reproducible and powerful technique to analyze interactions between proteins and specific DNA sequences *in vivo* in Arabidopsis plants. A successful identification of binding sites for the proteins of interest requires an adequate selection of plant organs or developmental stages where the relevant interactions are actually taking place. In addition, it is critical to obtain an appropriate fixation of the plant material and an optimal shearing of the chromatin by sonication. Highly specific antibodies for the efficient immunoprecipitation of the selected protein/tag are also essential.

The use of ChIP approaches in plants faces some difficulties related to the presence of secondary metabolites and cell walls that can potentially interfere with different steps in the protocol. Moreover, the complexity of plant organs, often containing multiple cell types where the DNA-binding protein of interest can be differentially present or active, have hindered the use of this methodology. For that reason, whenever possible it is most advantageous to use in ChIP approaches homogeneous tissues where the presence and/or activity of the protein of interest have been already demonstrated. Complex plant materials containing different tissues tend to cause a dilution of the cell types where the protein actually functions. Tissue sectioning[20] have been used in plants for studies in individual tissues or cell types. However, during recent years, different procedures have been developed for the isolation of cell-type specific chromatin in Arabidopsis[8] to a range of optimal fragments (see Representative Results section)[7]. As discussed above, the conditions to achieve this size distribution greatly depend on the specific ultrasound equipment used. In any case, chromatin should be kept at low temperature to prevent reversal of the crosslinking favored by heat generated during sonication. The sonication device of choice should provide a good level of reproducibility, which together with optimal fragmentation conditions, is essential for ChIP analysis (**Figure 3**).

Another key factor in successful ChIP experiments is the chosen antibody for immunoprecipitation of the protein of interest. Antibodies raised against plant transcription factors can be useful for ChIP analysis, but these sera should be thoroughly tested because antibodies checked in Western blot experiments are not necessarily valid for ChIP. In addition, antibodies raised against different tags are frequently used for this

protocol. ChIP-grade antibodies specifically recognizing a variety of tags are commercially available, and have the advantage that they have been tested in several laboratories. The drawback for the use of these commercial antibodies is that the protein of interest has to be fused to the corresponding epitope and expressed in transgenic lines (**Figure 4B**). In this case, it is advisable to ensure that the chimeric protein is functional by generating Arabidopsis transgenic lines bearing the fusion protein in a genetic background deficient in the gene encoding our protein of interest. The complementation of the phenotypic abnormalities of the mutant line in the transgenic plants will confirm that the fusion protein retains the normal activity of the endogenous protein. When transgenic lines are produced to identify the binding sites of transcriptional regulators, it is preferable to use the endogenous promoter to drive the expression of the tagged protein, preventing the problems associated with the utilization of constitutive strong promoters (see above). Specific antibodies for histone posttranslational modifications such as methylation or acetylation of specific residues are also frequently used in ChIP analyses to determine the epigenomic landscapes of genes involved in the regulation of a number of developmental processes, including the control of flowering time (**Figure 4C**). As for the antibodies against tags, ChIP-grade commercial antibodies are the preferred option in the case of histone marks.

ChIP experimental approaches coupled to gene expression analyses have been instrumental in increasing our knowledge on how transcription factors, chromatin remodeling proteins, and histone covalent modifications modulate the expression of genes involved in the regulation of plant biological processes and responses. Initially developed as a method to analyze the interaction of proteins present in the chromatin with particular loci or gene regions, the burst of genomic resources and NGS technology has allowed ChIP to become a powerful tool for the genome-wide profiling of DNA-binding proteins such as transcription factors or modified histones and histone variants. ChIP-chip and ChIP-seq have provided a new global dimension to the analyses of interactions between chromatin proteins and DNA in Arabidopsis[25]. ChIP-seq approaches are considered the alternative of choice to ChIP-chip, which is based on hybridization arrays and introduces some bias, given that these arrays contain a restricted number of probes.

Disclosures

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