Detection of Histone Modifications in Plant Leaves

Michal Jaskiewicz¹², Christoph Peterhansel³, Uwe Conrath²

¹Department of Botany, RWTH Aachen University
²Department of Plant Physiology, RWTH Aachen University
³Department of Botany, Leibniz University

Correspondence to: Christoph Peterhansel at cp@botanik.uni-hannover.de, Uwe Conrath at uwe.conrath@bio3.rwth-aachen.de

URL: http://www.jove.com/video/3096
DOI: doi:10.3791/3096

Keywords: Molecular Biology, Issue 55, chromatin, chromatin immunoprecipitation, ChiP, histone modifications, PCR, plant molecular biology, plant promoter control, gene regulation

Date Published: 9/23/2011

Citation: Jaskiewicz, M., Peterhansel, C., Conrath, U. Detection of Histone Modifications in Plant Leaves. J. Vis. Exp. (55), e3096, doi:10.3791/3096 (2011).

Abstract

Chromatin structure is important for the regulation of gene expression in eukaryotes. In this process, chromatin remodeling, DNA methylation, and covalent modifications on the amino-terminal tails of histones H3 and H4 play essential roles. H3 and H4 histone modifications include methylation of lysine and arginine, acetylation of lysine, and phosphorylation of serine residues. These modifications are associated either with gene activation, repression, or a primed state of gene that supports more rapid and robust activation of expression after perception of appropriate signals (microbe-associated molecular patterns, light, hormones, etc.). Here, we present a method for the reliable and sensitive detection of specific chromatin modifications on selected plant genes. The technique is based on the crosslinking of (modified) histones and DNA with formaldehyde, extraction and sonication of chromatin, chromatin immunoprecipitation (ChiP) with modification-specific antibodies, de-crosslinking of histone-DNA complexes, and gene-specific real-time quantitative PCR. The approach has proven useful for detecting specific histone modifications associated with C₄ photosynthesis in maize and systemic immunity in Arabidopsis.

Video Link

The video component of this article can be found at http://www.jove.com/video/3096/

Protocol

1. Crosslinking of plant material

1. Harvest 1-2g of Arabidopsis leaves (6 to 10cm rosette diameter) in a 50-ml plastic tube and fill up to 40mL with crosslinking buffer.
2. Make sure leaves stay immersed, for example by stuffing a tailored filter sponge right above buffer surface. Then place tubes in a desiccator.
3. Vacuum infiltrate for 10min. To each tube add 2.5mL of 2M glycine to stop crosslinking, and invert tubes to warrant equal dispersion of glycine.
4. Vacuum infiltrate for another 5min and discard fluid while harvesting leaves on a sieve.
5. Wash leaves twice with 1L of water in a glass beaker, then dry leaves thoroughly with paper towels.
6. Collect leaves in a fresh plastic tube and freeze in liquid nitrogen. Store leaves at -80° C until chromatin isolation.

2. Isolation of chromatin

1. Leaves were ground with mortar and pistil that were kept in liquid nitrogen until use.
2. Transfer powder to a 50-ml plastic tube and suspend in 30mL extraction buffer #1.
3. Incubate for 15min at 4°C on an overhead shaker.
4. Filtrate suspension through four layers of miracloth into a fresh 50-ml plastic tube.
5. Spin for 20min at 2,800 x g at 4°C.
6. Remove supernatant and suspend pellet in 300μL of extraction buffer #3. First add a small volume of buffer #3, then suspend pellet with a paint brush, and then add the remaining buffer.
7. Transfer suspension to a 1.5mL microfuge tube and spin for 10min at 12,000 x g at 4°C.
8. Meanwhile, prepare 2mL-microfuge tubes containing 1.5mL extraction buffer #3.
9. Remove supernatant from the spun tube (step 2.7) and suspend pellet in 300μL of extraction buffer #3. First add a small volume of buffer #3, suspend pellet with a paint brush, and then add the remaining buffer.
10. Carefully pipet the suspended pellet (step 2.9) on top of the 1.5 mL extraction buffer #3 prepared in step 2.8. Make sure the two phases won't mix. Spin for 1h at 16,000 x g at 4°C.
11. Remove supernatant and suspend chromatin pellet with a paint brush in 300μL of sonication buffer.
12. Sonicate chromatin with a sonotrode, or in an ultrasonic bath, to a DNA size of approximately 400bp. When sonicating, make sure chromatin will not be heated above approximately 30 °C to protect heat-sensitive crosslinks. The duration of sonication needs to be determined experimentally, because it varies significantly between different sonication devices. For guidance, settings for two different devices are provided:

For sonication with a Bandelin brand sonotrode expose chromatin in 0.6mL microfuge tube five times to 20 bursts with settings 25% power, cycle = 50. While doing so, cool sample after every 20th bursts in an ice bath.

For sonication with a Diagenode Bioruptor ultrasonic bath, fill bath with water and ice and sonicate ten times for 30 seconds in 1.5mL microfuge tubes with setting 'high'. After every 30 seconds, cool samples for another 30 seconds.

13. Spin sonicated sample for 5min at 16,000g at 4°C to precipitate undissolved material. The supernatant can directly be used for immunoprecipitation. Alternatively, samples can be shock frozen in liquid nitrogen and stored at -80°C until further use.

3. Immunoprecipitation

1. Prepare 2-mL microfuge tubes containing 40μL protein-A agarose and 1.8mL of antibody-binding buffer. Add 200μL of the chromatin preparation (step 2.13).
2. Incubate tubes for 1h on an overhead shaker.
3. Discard protein-A agarose beads and use supernatant for immunoprecipitation.
4. Remove a 40μL-aliquot to determine chromatin concentration ('input').
5. Add 30μL protein-A agarose and the amount of modification-specific antibody that is indicated in the below table of specific reagents and equipment to 400μL sonicated chromatin suspension.
6. Incubate for 2.5h or overnight on an overhead shaker at 4°C.
7. Spin down beads for 2min at 440 x g.
8. Wash bead pellet with 900μL of each wash buffer (see the below list). For each buffer, incubate beads in the buffer for 10min on an overhead shaker at 4°C, then spin for 2min at 440 x g, discard supernatant, and add the next buffer.
   1. Low salt wash buffer
   2. High salt wash buffer
   3. LiCl wash buffer
   4. TE wash buffer
9. After the final wash, completely remove any remaining buffer.

4. De-crosslinking of histones and DNA, and purification of precipitated DNA

1. Add 100μL of de-crosslinking buffer to the agarose pellet and the 40μl 'input' sample from step 3.4, mix for 5min on a vortex mixer, spin samples, and incubate at 65°C overnight.
2. Purify DNA with a commercial DNA or PCR purification kit.
3. Typically 5μl of a 1:5 dilution of the final column eluate are sufficient to perform conventional locus-specific real-time quantitative RT-PCR (RT-qPCR).

5. Table of buffers

| Buffer Type          | Component          | Concentration |
|----------------------|--------------------|---------------|
| **Crosslinking buffer** | Sodium butyrate    | 10mM          |
|                      | Sucrose            | 400mM         |
|                      | Tris-HCl, pH 8.0   | 10mM          |
|                      | β-Mercaptoethanol  | 5mM           |
|                      | Formaldehyde       | 3% (v/v)      |
| **Extraction buffer #1** | Sodium butyrate    | 10mM          |
|                      | Sucrose            | 400mM         |
|                      | Tris-HCl, pH 8.0   | 10mM          |
|                      | β-Mercaptoethanol  | 5mM           |
|                      | Complete Protease Inhibitor | 1x         |
### Extraction buffer #2

| Component                  | Concentration |
|----------------------------|---------------|
| Sodium butyrate            | 10mM          |
| Sucrose                    | 250mM         |
| Tris-HCl, pH 8.0           | 10mM          |
| β-Mercaptoethanol          | 5mM           |
| MgCl₂                      | 10mM          |
| Triton X-100               | 1% (w/v)      |
| Complete Protease Inhibitor| 1x            |

### Extraction buffer #3

| Component                  | Concentration |
|----------------------------|---------------|
| Sodium butyrate            | 10mM          |
| Sucrose                    | 1.7M          |
| Tris-HCl, pH 8.0           | 10mM          |
| β-Mercaptoethanol          | 5mM           |
| MgCl₂                      | 2mM           |
| Triton X-100               | 0.15% (w/v)   |
| Complete Protease Inhibitor| 1x            |

### Sonication buffer

| Component                  | Concentration |
|----------------------------|---------------|
| Tris-HCl, pH 8.0           | 25mM          |
| EDTA-NaOH, pH 8.0          | 5mM           |
| SDS                        | 0.5% (w/v)    |
| Complete Protease Inhibitor| 1x            |

### Antibody binding buffer

| Component                  | Concentration |
|----------------------------|---------------|
| Tris-HCl, pH 8.0           | 50mM          |
| EDTA-NaOH, pH 8.0          | 1mM           |
| NaCl                       | 150mM         |
| Triton X-100               | 0.1% (w/v)    |

### Low salt wash buffer

| Component                  | Concentration |
|----------------------------|---------------|
| NaCl                       | 150mM         |
| SDS                        | 0.1% (w/v)    |
| Triton X-100               | 1% (w/v)      |
| EDTA-NaOH, pH 8.0          | 2mM           |
| Tris-HCl, pH 8.0           | 20mM          |

### High salt wash buffer

| Component                  | Concentration |
|----------------------------|---------------|
| NaCl                       | 500mM         |
| SDS                        | 0.1% (w/v)    |
| Triton X-100               | 1% (w/v)      |
| EDTA-NaOH, pH 8.0          | 2mM           |
| Tris-HCl, pH 8.0           | 20mM          |
**LiCl wash buffer**

- Lithium chloride 250mM
- Nonidet-P40 1% (v/v)
- Sodium desoxycholate 1% (w/v)
- EDTA-NaOH, pH 8.0 1mM
- Tris-HCl, pH 8.0 20mM

**TE wash buffer**

- EDTA-NaOH, pH 8.0 10mM
- Tris-HCl, pH 8.0 1mM

**Decrosslinking buffer**

- Tris-HCl, pH 6.8 62.5mM
- NaCl 200mM
- SDS 2% (w/v)
- DTT 10mM

---

**6. Representative results:**

Expression of the Arabidopsis thaliana PR2 defense gene encoding a β-1,3-glucanase with antimicrobial activity\(^\text{12}\), was induced by benzothiadiazole (BTH), a synthetic analog of the plant hormone salicylic acid\(^\text{3}\). Figure 1 demonstrates that activation of PR2 expression is associated with an increase in the acetylation of lysine residues on histone H3 and H4 on the PR2 promoter. Since nucleosome density decreases during gene activation\(^\text{13}\), histone acetylation values were normalized for the signal obtained with an antibody to an invariant region of histone 3.

---

**Discussion**

Most critical steps in the protocol are the crosslinking of DNA and histones, the type and amount of leaf material, grinding of the material, and the sonication of chromatin. For a more detailed discussion of these issues, see refs. 9 and 10.

**Sonication and crosslinking:**

It is important to disrupt chromatin during sonication to fragments of about 400bp. Exhaustive sonication will destroy chromatin by disrupting DNA and histones, whereas insufficient sonication will leave long chromatin fragments. These affect the specificity of the method, because histone modifications distal from the tested locus cannot be discriminated from local modifications. Sonication efficiency is best tested by collecting 20μL chromatin from samples before and after sonication, de-crosslinking DNA and histones, isolating the DNA and determining...
the length of the sheared DNA by agarose gel electrophoresis. RNAse should be added to the samples before electrophoresis, because the chromatin preparation still contains large amounts of RNA at this stage of purification that might interfere with the visualization of fragmented DNA. The samples are useful for chromatin immunoprecipitation if DNA from non-sheared chromatin is longer than 10kbp, whereas DNA from sheared chromatin forms a smear with maximum signal intensity around 400bp of DNA.

We routinely use 3% (v/v) formaldehyde for chromatin crosslinking in intact leaves, but 1% (v/v) formaldehyde might be sufficient in most cases. In our hands, low formaldehyde concentrations allow for shorter sonication times and background signals in consequent PCR reactions. However, insufficient amounts of formaldehyde often result in low reproducibility of the PCR signal between independent experiments. This might be due to insufficient penetration of leaves with formaldehyde at low concentrations. Make sure to exchange your formaldehyde stock frequently as the compound tends to polymerize with time. Formaldehyde solutions are stable only for approximately one month, and this period is hardly prolonged by methanol stabilization. It is advised to test different formaldehyde concentrations when setting up experimental conditions.

**Amount of plant material and grinding:**

It is important to infiltrate low amounts of leaf material in a large volume of buffer to avoid leaves sticking to each other. Leaf sticking often results in insufficient infiltration of formaldehyde. Furthermore, too much plant material will block pores of the miracloth tissue. The grinding efficiency considerably depends on using a mortar and pestle with the perfect fit. We routinely grind with a liquid nitrogen-cooled pestle and mortar in the absence of additional liquid nitrogen three times for 50 seconds until the material is ground to a fine powder.

**Disclosures**

No conflicts of interest declared.

**Acknowledgements**

This work was funded by the German Science Foundation (DFG) and the Excellence Initiative of the German federal and state governments.

**References**

1. Eberharter, A. & Becker P.B. Histone acetylation: a switch between repressive and permissive chromatin. *EMBO rep.* 3, 224-229 (2002).
2. Ruthenburg, A.J., et al. Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol. Cell.* 25, 15-30 (2007).
3. Jaskiewicz, M., et al. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO rep.* 12, 50-55 (2011).
4. Ng, D.W., et al. Ordered histone modifications are associated with transcriptional poising and activation of the phaseolin promoter. *Plant Cell.* 18, 119-132 (2006).
5. Offermann, S., et al. Illumination is necessary and sufficient to induce histone acetylation independent of transcriptional activity at the C4-specific phosphoenolpyruvate carboxylase promoter in maize. *Plant Physiol.* 143, 1660-1668 (2007).
6. Deng, W., et al. Involvement of the histone acetyltransferase AtHAC1 in the regulation of flowering time via repression of FLOWERING LOCUS C in Arabidopsis. *Plant Physiol.* 143, 1660-1668 (2007).
7. Benhamed, M., et al. Arabidopsis GCN5, HD1, and TAF1/HAF2 interact to regulate histone acetylation required for light-responsive gene expression. *Plant Cell.* 18, 2893-2903 (2006).
8. Bowler, C., et al. Chromatin techniques for plant cells. *Plant Journal.* 39, 776-789 (2004).
9. Orlando, V. Mapping chromosomal proteins in vivo by formaldehyde decrosslinked-chromatin immunoprecipitation. *Trends Biochem. Sci.* 25, 99-104 (2000).
10. Haring, M., et al. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Meth.* 3, 11 (2007).
11. Danker, T., et al. Developmental information but not promoter activity controls the methylation state of histone H3 lysine 4 on two photosynthetic genes in maize. *Plant J.* 53, 465-474 (2008).
12. Uknes, S., et al. Acquired resistance in Arabidopsis. *Plant Cell.* 4, 645-656 (1992).
13. Workman, J.L. Nucleosome displacement in transcription. *Genes Develop.* 20, 2009-2017 (2006).