Video Article

Detection of Histone Modifications in Plant Leaves

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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[1-2]. H3 and H4 histone modifications include methylation of lysine and arginine, acetylation of lysine, and phosphorylation of serine residues[1-2]. 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.)[3-7].

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[8,9], extraction and sonication of chromatin, chromatin immunoprecipitation (ChIP) with modification-specific antibodies[9,10], 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 C_4 photosynthesis in maize[5,11] and systemic immunity in Arabidopsis[3].

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 with a paint brush in 1mL extraction buffer #2. First add a small volume of buffer #2, then suspend pellet with a paint brush, and then add the rest of 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

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	
Sodium butyrate	10mM
Sucrose	250mM
Tris-HCl, pH 8.0	10mM
β-Mercaptoethanol	5mM
$MgCl_2$	10mM
Triton X-100	1% (w/v)
Complete Protease Inhibitor	1x

Extraction buffer #3	
Sodium butyrate	10mM
Sucrose	1.7M
Tris-HCl, pH 8.0	10mM
β-Mercaptoethanol	5mM
$MgCl_2$	2mM
Triton X-100	0.15% (w/v)
Complete Protease Inhibitor	1x

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

Antibody binding buffer	
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	
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	
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[12], was induced by benzothiadiazole (BTH), a synthetic analog of the plant hormone salicylic acid[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[13], histone acetylation values were normalized for the signal obtained with an antibody to an invariant region of histone 3.

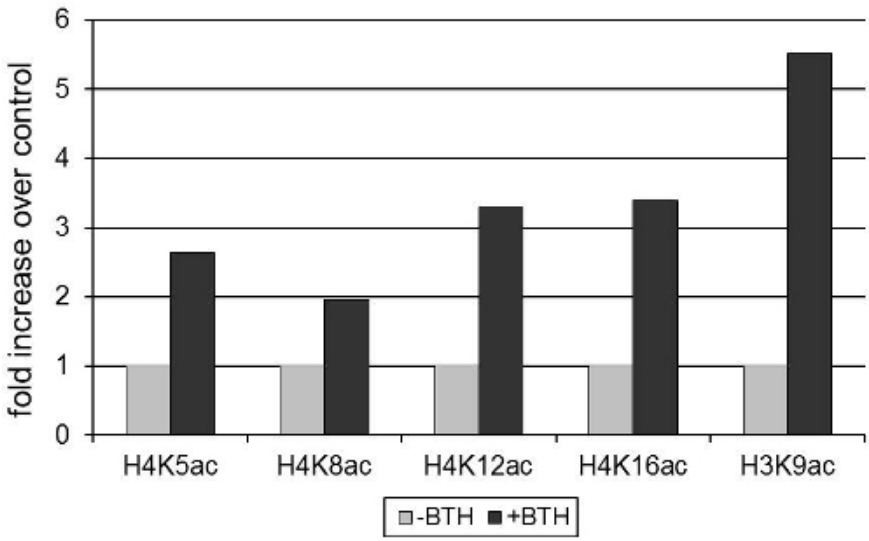

Figure 1 BTH induces histone acetylation on the *PR2* promoter in Arabidopsis. Plants were treated with 100μM BTH or a wettable powder control. After 72h, leaves were collected and chromatin was isolated. The signal obtained after precipitation with acetylation-specific antibodies (as indicated in the figure) was normalized to the signal obtained by precipitation with an antibody to an invariant domain of histone H3. Precipitated chromatin was quantified by RT-qPCR. Data are standardized for histone modification levels in the absence of BTH treatment.

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.

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