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Chromatin immunoprecipitation (ChIP) assay

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Introduction

Chromatin immunoprecipitation (ChIP) assay is a powerful tool to study protein-DNA interaction and chromatin changes associated with gene expression. In the related article, we used ChIP assay to evaluate CCA1 binding to the CCA1, LHY, TOC1 and GI upstream region, as well as histone modifications at these loci in *A. thaliana*, *A. arenosa* and the two independently re-synthesized allotetraploids (Allo733 and Allo738). The ChIP protocol is modified from those of Gendrel *et al*. (2002)[1] and Lawrence *et al*. (2004)[2].

Subject terms: Plant biology

Keywords: chlorophyll starch EMSA ChIP circadian clock gene expression epigenetics hybrid vigor allopolyploid

Reagents

Plant Materials and Growth Conditions

Harvest young fresh leaves (~2 grams) prior to bolting (6-8 rosette leaves in *A. thaliana*, 10-12 leaves in *A. arenosa*, and 12-15 leaves in allotetraploids). Grow plants under 16/8 hours (light/dark) cycles and harvest samples at Zeitgeber time 6 (noon), unless noted otherwise.

ChIP Antibodies

• Anti-CCA1 antibody (Elaine Tobin, University of California, Los Angels, also made by the Chen Laboratory at The University of Texas at Austin)
• Anti-dimethyl-H3-Lys4 antibody (Upstate, 07-030)
• Anti-trimethyl-H3-Lys4 antibody (Abcam, ab8580)
• Anti-dimethyl-H3-Lys9 antibody (Upstate, 07-521; Abcam, ab1220)
• Anti-dimethyl-H3-Lys27 antibody (Abcam, ab24684)
• Anti-trimethyl-H3-Lys27 antibody (Abcam, ab6002)
• Anti-acetyl-H3-Lys9 antibody (Upstate, 07-352)

Stock Solutions and Buffers

100mM PMSF: Dissolve 0.0871 g PMSF in 5 ml ethanol (note: PMSF is unstable in aqueous solution, always add it fresh to extraction buffer prior use).

Extraction buffer 1: 0.4 M sucrose, 10 mM Tris-HCl pH8, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, protease inhibitor cocktail tablet (1 tablet/10 ml solution) (note: freshly add β-mercaptoethanol, PMSF and protease inhibitor cocktail tablet before use).

Extraction buffer 2: 0.25 M sucrose, 10 mM Tris-HCl pH8, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, protease inhibitor cocktail tablet (1 tablet/ 10 ml solution).

Extraction buffer 3: 1.7 M sucrose, 10 mM Tris-HCl pH8, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, protease inhibitor cocktail tablet (1 tablet/10 ml solution).

Lysis buffer: 50 mM Tris-HCl pH8, 10 mM EDTA, 1% SDS, 1 mM PMSF, protease inhibitor cocktail tablet (1 tablet/10 ml solution) (note: include 10mM sodium butyrate if evaluating histone acetylation; sodium butyrate inhibits histone deacetylation).

ChIP dilution buffer: 16.7 mM Tris-HCl pH8, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, protease inhibitor cocktail tablet (1 tablet/10 ml solution) (note: include 10 mM sodium butyrate if evaluating histone acetylation).

Low salt buffer: 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.

High salt buffer: 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.

LiCl wash buffer: 0.25 M LiCl, 1% Nonidet-P40 (NP40), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH8.

Elution buffer: 0.1 M $NaHCO_3$, 1% SDS (Note: prepare the buffer fresh and heat up to 65°C prior to use).

TE buffer: 10 mM Tris-HCl pH8, 1 mM EDTA

Procedure

Day 1: *In vivo* formaldehyde cross-linking of samples

1. Place 2 grams of leaves in a 50 ml Falcon tube.
2. Rinse the leaves twice with 40 ml of nanopure water.
3. Add 37 ml of 1% formaldehyde solution (note: make sure leaves are submerged).
4. Put tube inside a desiccator and apply vacuum for 10 minutes (note: air bubbles should start forming on the leaf surface; gently tap the desiccator to occasionally remove air bubbles).
5. Slowly release vacuum after 10 minutes (note: leaves should appear translucent).
6. Add 2.5 ml of 2 M glycine to a final concentration of 0.125 M glycine to stop cross-linking.
7. Apply vacuum for another 5 minutes and slowly release vacuum.
8. Rinse the leaves twice with 40 ml nanopure water.
9. Dry the leaves with paper towel to remove excess water.
10. Freeze cross-linked leaves in liquid nitrogen.

Nuclei and chromatin isolation

(Note: keep samples cold all the time on ice)

11. Grind leaves (~2 grams) into fine powder with liquid nitrogen in mortar and pestle.
12. Transfer ground powder into a 50 ml falcon tube and add 30 ml of extraction buffer 1 (note: add at least 10-fold volume of extraction buffer relative to the amount of ground samples) and vortex.
13. Filter through a layer of Miracloth into a new 50 ml falcon tube.
14. Centrifuge extract at 4,000 rpm for 20 minutes at 4°C.
15. Gently remove supernatant and resuspend the pellet in 1 ml of extraction buffer 2 by pipetting up and down (note: the pellet should be completely resuspended).
16. Transfer the solution into a new 1.5 ml microcentrifuge tube.
17. Centrifuge at 13,000 rpm for 10 minutes at 4°C.
18. Remove supernatant and resuspend the pellet in 400 µl of extraction buffer 3 by pipetting up and down.
19. Add 400 µl of extraction buffer in a new 1.5 ml microcentrifuge tube and carefully layer the extract (400 µl) on the top of the buffer.
20. Centrifuge at 13,000 rpm for 1 hour at 4°C.
21. Remove supernatant and resuspend pellet in 500 µl of cold nuclei lysis buffer (note: include 10 mM sodium butyrate in the lysis buffer when analyzing histone acetylation).
22. Carefully resuspend the pellet by pipetting up and down (note: keep the chromatin solution cold on ice).
23. Keep an aliquot (10 µl) of the chromatin solution for agarose gel electrophoresis in step 27 (note: examine the quality of the isolated chromatin before sonication).

Sonication

24. Sonicate chromatin solution 4 times for 10 seconds (continuous) with power output setting at 10 using Fisher sonic 60 dismembrator (notes: to prevent from overheating the chromatin solution during sonication, return the solution on ice for at least 1 minute between each sonication; avoid foaming during sonication; chromatin solution can be stored at -20°C after this step).
25. Centrifuge chromatin solution at 13,000 rpm for 5 minutes at 4°C to remove debris.
26. Transfer supernatant into a new 1.5 ml microcentrifuge tube.
27. Take an aliquot (10 µl) of the sonicated chromatin solution for agarose gel electrophoresis with the aliquot from step 23 (notes: to determine sonication efficiency; DNA sizes should appear smear at a range of 200-bp to 1-kb range).
28. Keep another aliquot (30 µl) of sonicated chromatin for input DNA control (note: add 470 µl of TE buffer to this aliquot and continue with reverse crosslink in step 55).

Immunoprecipitation

29. Split the remaining chromatin solution (450 µl) into 3 tubes with 150 µl each. Add 1,350 µl ChIP dilution buffer to each tube (note: to dilute SDS from 1% to 0.1% in the buffer).

30. For each tube from step 29, prepare a 40 µl of 50% salmon sperm DNA/ protein A agarose beads by rinsing three times with 1 ml of ChIP dilution buffer (without protease inhibitor or sodium butyrate) and resuspend the beads in 40 µl of ChIP dilution buffer (note: after each wash, spin down beads at 6,000 rpm for 2 minutes at 4°C).
31. Incubate the chromatin solution (1.5 ml) with 40 µl of protein A agarose beads for 1 hour at 4°C in a rotisserie shaker.
32. Centrifuge at 6,000 rpm for 2 minutes at 4°C to pellet beads from the chromatin solution.
33. Combine 3 aliquots of chromatin solution into a 14 ml falcon tube (note: avoid pipetting pellet protein A agarose beads).
34. For each chromatin IP, mix 600 µl pre-cleared chromatin solution with antibody in a 1.5 ml microcentrifuge tube (notes: the amount of antibodies used varies among different sources and should be determined empirically; we used 10 µl anti-H3K9Ac, 5 µl anti-H3K9Me2, 5 µg anti-H3K4Me2 or 10 µl anti-CCA1 per IP).
35. Set up another chromatin solution (600 µl) without antibodies as a control for ChIP.
36. Incubate chromatin and antibodies overnight at 4°C in a rotisserie shaker.

Day 2: IP complexes pull down and washing

37. Prepare 40 µl of 50% salmon sperm DNA/protein A agarose beads as in step 30.
38. Add 40 µl of protein A agarose beads to the ChIP tube and incubate for 1.5 hours at 4°C in a rotisserie shaker (note: pull down the ChIP-complexes formed).
39. Centrifuge at 6,000 rpm for 1 minute at 4°C to collect ChIP-beads complexes, discard supernatant.
40. Add 1 ml of low salt buffer to ChIP-beads complexes.
41. Wash the complexes for 10 minutes at 4°C with rotation.
42. Centrifuge at 6,000 rpm for 1 minute at 4°C to collect ChIP-beads complexes, discard supernatant
43. Add 1 ml of high salt buffer to ChIP-beads complexes
44. Repeat steps 41 and 42
45. Add 1 ml of LiCl washing buffer to ChIP-beads complexes
46. Repeat steps 41 and 42
47. Add 1 ml of TE buffer to IP-beads complexes
48. Repeat steps 41 and 42
49. Repeat washing with TE buffer (steps 47 and 48), collect ChIP-beads complexes and discard supernatant.
50. Add 250 µl of elution buffer to elute ChIP complexes (note: prepare elution buffer prior washing steps and store it at 65°C).
51. Vortex briefly and incubate the samples at 65°C for 15 minutes.
52. Centrifuge the samples at 13,000 rpm for 2 minutes at room temperature.

53. Carefully transfer the supernatant (eluate) to a new 1.5 ml screw cap tube.
54. Repeat elution (steps 50 to 53) and combine the two eluates in a single tube.

Reverse crosslink and DNA purification

55. Add 20 µl of 5 M NaCl to the eluate and reverse cross-link at 65°C for at least 6 hours to overnight (note: also perform reverse-crosslink for input DNA control from step 28).
56. Add 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl (pH6.5) and 1 µl of 20 mg/ml proteinase K.
57. Incubate at 45°C for 1-3 hours.
58. Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).
59. Mix and centrifuge the samples at 13,000 rpm for 5 minutes at room temperature.
60. Transfer the supernatant to a new 1.5 ml microcentrifuge tube and add an equal volume of chloroform/isoamyl alcohol (24:1).
61. Mix and centrifuge the samples at 13,000 rpm for 5 minutes at room temperature.
62. Carefully transfer supernatant to a new 2 ml microcentrifuge tube (note: avoid residue chloroform as it may affect PCR).
63. Add 0.1 of volume 3 M NaoAC (pH5.2) and 2 µl of glycoblue (15 mg/ml; Ambion) (note: the use of glycoblue increases the visibility and recovery of DNA precipitates).
64. Add 2.5 volumes of absolute ethanol and precipitate DNA at -80°C for 2 hours (note: alternatively, precipitate DNA at -20°C overnight).
65. Centrifuge DNA at 13,000 rpm for 15 minutes at 4°C.
66. Remove the supernatant and wash the pellet with 1 ml of 75% ethanol (note: DNA pellet should appear blue with the use of glycoblue).
67. Centrifuge DNA at 13,000 rpm for 5 minutes at 4°C.
68. Air-dry DNA pellet for 10 minutes (note: alternatively, dry DNA under vacuum for 3 minutes).
69. Resuspend DNA in 50 µl of TE buffer with 10 µg/ml RNaseA.
70. Use 0.5-1 µl of DNA in a 25 µl PCR reaction (note: the amount of DNA needed for PCR depends on ChIP efficiency).

References

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Associated Publications

This protocol is related to the following articles:

- **Altered circadian rhythms regulate growth vigor in hybrids and allopolyploids**

See other protocols related to this article

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Competing financial interests

The authors declare no competing financial interests.

Readers' Comments

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