Chapter 15

Three-Dimensional, Live-Cell Imaging of Chromatin Dynamics in Plant Nuclei Using Chromatin Tagging Systems

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Abstract

In plants, chromatin dynamics spatiotemporally change in response to various environmental stimuli. However, little is known about chromatin dynamics in the nuclei of plants. Here, we introduce a three-dimensional, live-cell imaging method that can monitor chromatin dynamics in nuclei via a chromatin tagging system that can visualize specific genomic loci in living plant cells. The chromatin tagging system is based on a bacterial operator/repressor system in which the repressor is fused to fluorescent proteins. A recent refinement of promoters for the system solved the problem of gene silencing and abnormal pairing frequencies between operators. Using this system, we can detect the spatiotemporal dynamics of two homologous loci as two fluorescent signals within a nucleus and monitor the distance between homologous loci. These live-cell imaging methods will provide new insights into genome organization, development processes, and subnuclear responses to environmental stimuli in plants.

Key words Chromatin dynamics, Chromatin tagging system, *lacO*/LacI-EGFP, Live-cell imaging, Time-lapse imaging

1 Introduction

The spatiotemporal dynamics and arrangement of chromatin directly contribute to the regulation of biological processes in the nuclei of eukaryotes, including plants [1, 2]. In plants, respective chromatin domains are randomly organized in nuclei, whereas they are organized non-randomly in animal nuclei [3, 4]. Recently, a genome-wide chromosome confirmation capture (Hi-C) method revealed the three-dimensional genome structure in interphase nuclei of *Arabidopsis thaliana* [5, 6]. Hi-C revealed that any local interaction domains, called topological associated domains, were not found in the nuclei of *A. thaliana*, unlike in humans and flies. Therefore, the three-dimensional chromatin structure in plants might be more complicated than in animals. Fluorescence in situ hybridization (FISH) has contributed to the investigation of the

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subnuclear distribution of DNA sequences, such as centromeric or telomeric repetitive sequences and ribosomal genes. However, FISH requires the fixation of cells and high temperatures for DNA denaturation. Unfortunately, FISH cannot be used to analyze chromatin dynamics in living cells.

The chromatin tagging system was developed as a technique to monitor chromatin dynamics and arrangement in living cells [7]. This technique is based on a bacterial operator/repressor system. The repressor, which is fused to a fluorescent protein, binds a tandem array of operators that are inserted into a specific genomic locus. Visualization of the specific genomic loci by the chromatin tagging system enables investigation into the dynamics of RNA transcription, DNA replication, and DNA repair at different genomic loci in mammalian cells and yeasts [8–10]. In a plant chromatin study, this technique was used to observe the spatial arrangement of chromatin domains in *A. thaliana* plants that contained a *lacO* operator/LacI repressor-enhanced green fluorescent protein (*lacO*/LacI-EGFP) system or a *tetO* operator/TetR repressor-enhanced yellow fluorescent protein (*tetO*/TetR-EYFP) system under the control of the cauliflower mosaic virus (CaMV) 35S promoter [11–13]. However, in these lines, overexpression of the repressor protein-fluorescent protein (RP-FP) often induced silencing, and the pairing frequency of the tandem operator array was higher than that of other genomic loci [14]. A recent refinement of promoters for the chromatin tagging system solves these problems. RP-FP expression under the RPS5A and GC1 promoters, which are specifically activated in meristematic tissues or guard cells, respectively, does not induce silencing or effect the chromatin arrangement [15]. Here, we introduce a method to analyze chromatin dynamics and arrangement in the nuclei of roots or leaves via three-dimensional, live-cell imaging using a *lacO*/LacI-EGFP system. Importantly, we used this system to quantitatively analyze the distance between homologous loci, as well as the movements of genomic loci in living plant cells. Recently, our experiments using this system revealed that chromatin arrangement was changed by DNA damages in *A. thaliana* [16]. Therefore, our method could reveal the function of various chromatin regulators and improve our knowledge of chromatin dynamics in response to plant development, growth, and environmental stimuli.

2 Materials

2.1 The lacO/LacI-EGFP System in A. thaliana

1. The line expressing *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP [15]. This transgenic line was used to image chromatin dynamics in the nuclei of roots.
2. The line expressing *lacO*/*pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP [15]. This transgenic line was used to image chromatin dynamics in the nuclei of a guard cell in a cotyledon.

2.2 Seeding for Live-Cell Imaging in Roots

1. 70% Ethanol.
2. A sterile solution: 0.1% [v/v] Triton X-100, 5% sodium hypochlorite.
3. 1/2 Murashige and Skoog (MS) gellan gum medium in a 35 mm glass dish (*see* **Note 1**).
4. Surgical tape.
5. A black stand tilted to 45° (Fig. 1).

2.3 Seedling for Live-Cell Imaging in Cotyledons

1. 70% Ethanol.
2. A sterile solution: 0.1% Triton X-100, 5% sodium hypochlorite.
3. A 1/2 MS agar medium: 1/2 MS salts, 1% [w/v] sucrose, 1.0% [w/v] agar.
4. 1/2 MS liquid solution: 1/2 MS Salts, 1% [w/v] sucrose.
5. A 1-well glass chamber slide.
6. Paper wipes.
7. Surgical tape.

2.4 Live-Cell Imaging of Chromatin Dynamics

1. An inverted fluorescence microscope equipped with 40× and 60× objectives, a confocal scanning unit, and a scientific complementary metal oxide semiconductor CMOS (sCMOS) camera.

3 Methods

3.1 Seeding for Imaging of Chromatin Dynamics in the Nuclei of Roots

1. Add 1 mL of 70% ethanol to a tube containing seeds of a plant that expresses *lacO/pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. Invert the tube a few times to mix.
2. Remove the ethanol from the tube. Add 1 mL of the sterile 0.1% Triton X-100 and 5% sodium hypochlorite solution to the tube and invert it approximately five times to mix.
3. After 5 min, remove the sterile solution from the tube. Wash the seeds three times with sterile distilled water.
4. Prepare the medium in the dish (*see* Subheading 2.2), and hollow out one-third of the medium using a spatula. Place approximately five seeds on the side of medium and place the lid on the dish (Fig. 1). Wind surgical tape around it.
5. Place the dish at 4 °C for 1 day and then move it to a 22 °C incubator. Put the dish on the stand tilled at 45° (Fig. 1).
6. Grow the seeds at 22 °C on a 16-h light/8-h dark cycle for 5 days.

3.2 Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Roots

1. Put the dish on the stage of an inverted fluorescence microscope with a 40× objective.
2. Move the region of interest (e.g., the meristematic or elongation zone) to the center of the visual field and determine the *z* range.

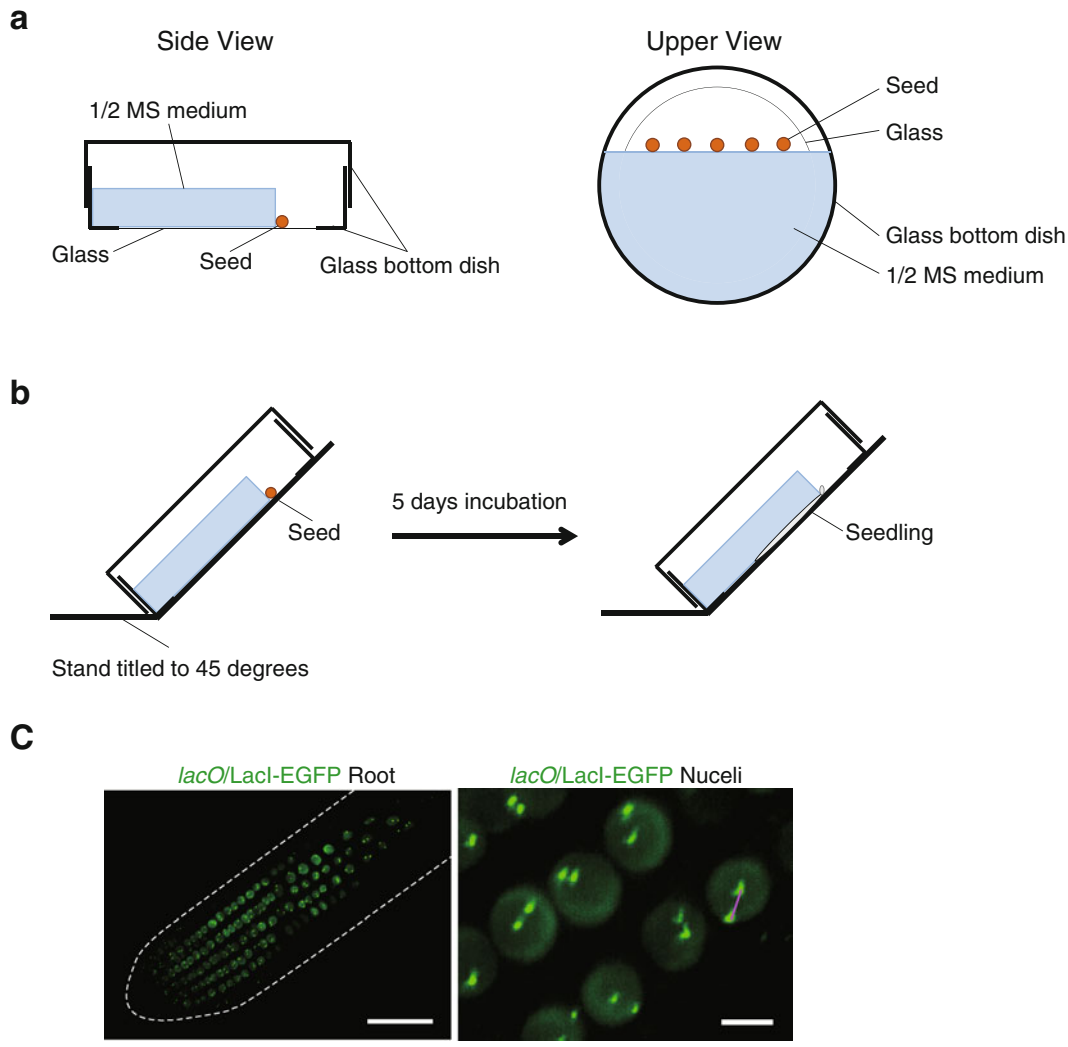

Fig. 1 Preparation for the imaging of chromatin dynamics in root nuclei. (**a**) A schematic figure of *A. thaliana* seeding for the imaging of chromatin dynamics in root nuclei. Sterilized seeds were placed on the edge of the 1/2 MS medium at even intervals in a glass-bottom dish. (**b**) A schematic figure of the incubation of *A. thaliana* seeds for the imaging of chromatin dynamics in root nuclei. The glass-bottom dish is placed onto a stand tilted to 45°. After 5 days, the root extends into the space between the 1/2 MS medium and the glass. (**c**) A root (*left*, scale bar = 50 μm) and nuclei (*right*, scale bar = 5 μm) of *A. thaliana* expressing *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. The bright foci of LacI-EGFP can be detected in the green nuclei. A *magenta line* indicates the distance of homologous loci (*right*)

For example, in the case of the root epidermis, the z range is approximately 15 μm (*see* **Note 2**).

3. Set the conditions for the time-lapse imaging, time interval, and duration. For example, to observe cell division in the meristematic zone, the time interval is 10 min and the duration is 3 h (*see* **Note 3**).

4. Perform the time-lapse imaging. As roots continue to grow during observation, the object being measured might need to be moved to the center of the visual field (*see* **Note 4**).

3.3 Seeding for Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Add 1 mL of 70% ethanol to the tube containing the seeds of plants expressing *lacO/pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP.
2. Remove the ethanol from the tube. Add 1 mL of the sterile 0.1% Triton X-100 and 5% sodium hypochlorite solution to the tube and invert it approximately five times to mix.
3. After 5 min, remove the sterile solution from the tube. Wash the seeds three times with distilled water.
4. Prepare the 1/2 MS agar medium. Sow the seeds on the medium.
5. Store the plate at 4 °C for 1 day and then move it to a 22 °C incubator. Grow the seeds at 22 °C on a 16-h light/8-h dark cycle for 5 days.

3.4 Preparation of Samples for Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Five days after moving the medium to the incubator, excise the cotyledons from the seedlings using tweezers.
2. Put ~5 cotyledons, adaxial side up, on a 1-well slide chamber and add the 1/2 MS liquid solution.
3. Soak paper wipes in the 1/2 MS liquid solution and fold them in accordance with the size of the slide.
4. Cover the cotyledons with the wet paper wipes and place the lid on the slide chamber (Fig. 2). Wind surgical tape around it (*see* **Note 5**).

3.5 Time-Lapse Imaging of Chromatin Dynamics in the Nuclei of Guard Cells of a Cotyledon

1. Place the chamber on the stage of an inverted fluorescence microscope with a 60× objective.
2. Cotyledons cannot be visualized by light from above because they are covered with paper wipes. Therefore, visualize the cotyledons using their intrinsic fluorescence under an excitation light.
3. Determine the *z* range. For example, in the case of guard cells, the *z* range is about 5–10 μm (*see* **Note 2**).
4. Perform the time-lapse imaging. Because leaves do not move during observation, the position of the object being measured does not need adjustment (*see* **Note 5**).

4 Notes

1. Mix 1/2 MS salts, 1% sucrose, and 0.6% gellan gum in sterile distilled water and adjust the pH to 5.9 with 0.1 N KOH. Autoclave the medium for 20 min at 121 °C. After autoclaving, pour 6 mL of the medium into a 35 mm glass dish. Store the medium in the dish at 4 °C. The transparency of gellan gum in the medium is

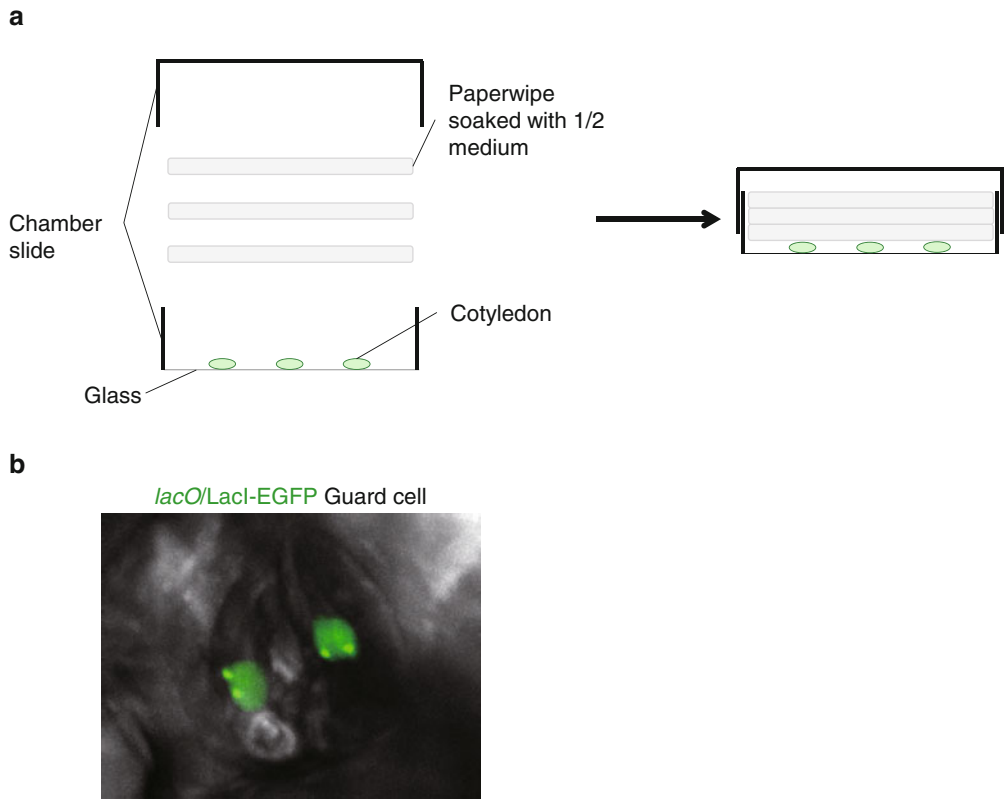

Fig. 2 Preparation for the imaging of chromatin dynamics in the nuclei of guard cells of a cotyledon. (**a**) A schematic figure of the seeding for the imaging of chromatin dynamics in guard cell nuclei. Excised cotyledons of *A. thaliana* are placed onto the *bottom* of the glass chamber. Accumulated Kimwipes soaked with 1/2 MS liquid solution are placed gently on the cotyledons. Finally, the Kimwipes are pressed down by the chamber cover. (**b**) Guard cell nuclei in *A. thaliana* expressing *lacO*/*pro35S*::LacI-EGFP and *proGC1*::LacI-EGFP. The fluorescent image merged with the bright-field image shows guard cells on the underside of a cotyledon. The bright foci of LacI-EGFP can be detected in the green nuclei

higher than that of agar. Therefore, it is desirable to use gellan gum to make the medium in the experiment.

2. The exposure time should be short to prevent *lacO*/LacI-EGFP signals from shifting in the z-stacks.
3. Mitotic nuclei can be observed in meristematic zone of plant lines transfected with *lacO*/*pro35S*::LacI-EGFP and *proRPS5A*::LacI-EGFP. However, it is difficult to discern their phase in detail. We recommend producing double-labeled cell lines, for example, the line transfected with the *lacO*/LacI-EGFP and the histone H2B-red fluorescent protein (H2B-RFP) systems.
4. For image analysis, we usually use ImageJ software (http://imagej.nih.gov/ij/). LP -StackLine (LPixel, http://lpixel.net/) and manual tracking (http://rsb.info.nih.gov/ij/plugins/track/track.html) can be used to measure chromatin dynamics.

5. The materials and methods used to observe the *lacO*/LacI-EGFP signal in the nuclei of cotyledons are mainly based on those in a previously report [17, 18]. This method is also effective for long-time observations of cellular dynamics, such as stomatal aperture, cell division, and organellar movement in plants.

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