Visualization of Chromatin Loci with Transiently Expressed CRISPR/Cas9 in Plants

Satoru Fujimoto and Sachihiro Matsunaga*

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278–8510, Japan

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Summary Labeling of specific DNA sequences in living organisms is important for understanding the mechanism of gene regulation during development, differentiation, and environmental adaptation. Here we describe a CRISPR/Cas9-based chromatin visualization system in plants. We adapted the mammalian nuclease-dead Cas9-fluorescent protein (dCas9-FP) system to a plant expression vector. Transient expression of dCas9-FP and single guide RNA (sgRNA) for telomeric sequences efficiently labeled telomere repeats in tobacco.

Key words CRISPR-dCas9, Chromatin dynamics, Live cell imaging, Nicotiana tabacum.

The spatial organization and temporal dynamics of chromatin play important roles in cell functions in plants (Santos et al. 2011, Rosa et al. 2013, Pontvianne et al. 2013, Matsunaga et al. 2013, Feng et al. 2014, Bourboussie et al. 2015). Labeling specific genomic regions in living cells has been a major challenge in studying the spatiotemporal regulation of chromatin in the nucleus. Fluorescent in situ hybridization (FISH) has been widely used to detect specific genomic loci. However, FISH cannot be adapted to in vivo monitoring of chromatin dynamics because it requires the fixation of cells and a high-temperature treatment for hybridization. Several live imaging methods for chromatin loci have been developed based on programmable DNA-binding proteins derived from genome editing techniques (Fujimoto and Matsunaga 2016). Engineered transcription activator-like effectors (TALEs) were fused with fluorescent proteins (FPs) to successfully visualize repetitive sequences (Miyanari et al. 2013, Ma et al. 2013, Thanisch et al. 2014, Yuan et al. 2014, Fujimoto et al. 2016). The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has also been used for genome visualization (Chen et al. 2013, Anton et al. 2014). CRISPR/Cas9 is a two-component system consisting of Cas9 and a single guide RNA (sgRNA), which directly interacts with the Cas9 nuclease at a specific locus matching the sgRNA in the genome. The sgRNA contains a targeting sequence and a Cas9 nuclease-recruiting hairpin loop sequence. Additionally, instead of Cas9, nuclease-dead Cas9 (dCas9) has been fused with FPs and co-introduced with sgRNAs to label a target site.

Here we present a CRISPR/Cas9-based approach for visualizing telomere repeats in plants. Furthermore, different structures for the sgRNAs targeting telomeres were examined to optimize the dCas9-FP system in plants.

Materials and methods

Vector construction

pDe-Cas9 and pEnChimera were used as the backbone system (Fauser et al. 2014). dCas9-3°GFP was amplified from the pHAGE-TO-dCas9-3°GFP vector (Addgene #64107) (Ma et al. 2015) with the primers pDe-dCasGFP_F, TGT GCA GCG AAT TCG GCG CGA TCG CGC CAT GGC CCC TAA AAA G and dCasGFP-pDe_R, AGG CCT GGA GCT CGG CGC GCT ACT CGA GTT TGT ACAG. The amplicon and Ascl-digested pDe-Cas9 were combined with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to produce pDe-dCas9-3°GFP (Fig. 1A).

Then, the each pEnChimera for telomere was introduced into pDe-dCas9-3°GFP (Fig. 1A).

pEnChimera was modified using a QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) with the primers pEn-TtoG, TGT TTG AGA GCT AGA AAT AGA AAG TTT GCT GTT TCC AGC AGT GTC TTC TCG AAG AC and Sp5iP, TTA AAT AAG GCT AGT CCG TTA TCA ACT TGA AAA AGT GCC ACC GAG CGG GTGC, and self-ligated to produce pEnChimera-hairpin. Telomere-target protospacers (Fig. 1B) were synthesized as oligonucleotides with 4 bp 5′overhangs and ligated into the BbsI restriction site of pEnChimera (Fauser et al. 2014). Then, each pEnChimera for telomere was introduced into pDe-dCas9-3°GFP with Gateway LR clonase (Thermo Fisher Scientific) to produce a dCas9-FP vec-

* Corresponding author, e-mail: sachi@rs.tus.ac.jp
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Transient expression of dCas9-FP

Transient expression in tobacco (*Nicotiana tabacum* L. cv. Xanthi) was performed according to the FAST Agrobacterium-mediated seedling transformation method (Li et al. 2009) with some modifications. dCas9-FP for the telomere expression vector was transformed into *Agrobacterium tumefaciens* strain GV3101. Four days after germination, tobacco seedlings were inoculated with the *Agrobacterium* in 0.25º MS salt, 1% sucrose, 150 µM acetosyringone and 0.0025% Silwet L-77. After two days co-cultivation, claforan (100 mg L⁻¹) was added to kill the bacterial cells.

Stable transformation of dCas9-FP

Transformation of *A. thaliana* accession Col-0 was performed by the floral dip method (Clough and Bent 1998). The transformants were selected on 1/2 MS agar containing claforan (100 mg L⁻¹) and phosphinothricin (10 mg L⁻¹).

Microscopy

Transformed tobacco seedlings or roots of *A. thaliana* expressing dCas9-3×GFP were observed using a confocal microscope (FV1200 Olympus, Tokyo, Japan) equipped with a 60× objective (UPLSAPO 60XW). Images were processed with Fiji (Schindelin et al. 2012) to generate maximum intensity projection images and to add color.

Results and discussion

Transient expression of dCas9-FP and sgRNA for telomeres in tobacco

To visualize chromosome loci with the CRISPR/Cas9 system, the mammalian dCas9-FP system was adapted to a plant CRISPR/Cas9 binary vector (Fauser et al. 2014). The *Streptococcus pyogenes* Cas9 gene of the pDe-Cas9 vector was replaced with the *S. pyogenes* dCas9-3×GFP gene from pHAGE-TO-dCas9-3×GFP (Ma et al. 2015). After co-cultivation of tobacco seedlings with *Agrobacterium* harboring the dCas9-FP binary vector, some strong signals were observed in addition to strong nucleolar signals (Fig. 1C). In plants, the distribution patterns of telomeres in interphase are dependent on the species (Dong and Jiang 1998). For example, wheat, rye, barley, and oats show the Rabl orientation, in which all the centromeres are grouped at one side of the nucleus while the telomeres are gathered in the opposite hemisphere. In contrast, sorghum, rice, and maize show non-Rabl patterns. In the case of tobacco, the telomeres are localized at random in the nucleus in a typical non-Rabl pattern (Fujimoto et al. 2005, Lee and Kim 2013, Schrumpfová et al. 2014). The signal pattern observed with dCas9-FP for telomeres was similar to previous telomere localization patterns in tobacco. During the preparation of this manuscript, the transient expression of dCas9-FP for telomeres in plants was reported by Dreissig et al. (2017), in which telomeres were labeled in *N. benthamiana*, and similar distribution patterns in the nucleus were observed.

Comparison of sgRNA structures for dCas9-FP localization

In mammalian cells, stabilization of the sgRNA structure was reported to improve dCas9-FP localization (Chen et al. 2013, Ma et al. 2016). The original sgRNA hairpin stem loop contains a four base U stretch. RNA Polymerase III terminates after synthesis of a poly-U stretch, so the four base U stretch may potentially terminate the transcription of the sgRNA halfway. Replacement of the fourth U with G was proposed to stabilize the sgRNA structure (Ma et al. 2016). Another target for
modification is the hairpin length. Increasing the loop length of the hairpin can also stabilize the sgRNA (Chen et al. 2013).

In addition to the original sgRNA, sgRNA-T to G (nucleotide modification) and sgRNA-hairpin (hairpin extension) for telomeres were co-expressed with dCas9-3-GFP transiently in N. tabacum. Signals were observed in the nucleus with all three sgRNAs. In comparison with the original sgRNA, the modified versions of these sgRNAs increased the signal-to-noise ratio in dCas9-FP telomere localization (Fig. 2). Similar to mammalian cells, modifications of the sgRNA were effective for improving dCas9-FP localization in plants.

Stable expression of dCas9-FP and sgRNA for telomeres in Arabidopsis

We also obtained stable transformants with the same construct (dCas9-3-GFP/sgRNA for telomeres) in A. thaliana. However, in all transformants the GFP signals were localized over the entire nuclei (Fig. 3). This indicated that successful expression of dCas9-FP/sgRNA may affect the normal cell cycle in plants.

Here we have shown a method of chromatin visualization for telomere repeats with dCas9-FP. We also successfully used different sgRNA structures for dCas9-FP to improve the fluorescence signals. TALE-FP is a powerful tool for visualization of chromatin in live plants (Fujimoto et al. 2016). However, TALE-FP is limited to highly repetitive sequences because of the laboriousness of TALE construction. By comparison, dCas9-FP is a desirable technique for the visualization of chromosome loci at the length of individual genes with multiple sgRNAs (Chen et al. 2013). Furthermore, to enable simultaneous visualization with higher sensitivity, three-component systems have been developed using dCas9, sgRNAs with stem loop motifs, and stem loop RNA-binding coat proteins with fluorescent proteins (Ma et al. 2016, Shao et al. 2016, Wang et al. 2016, Fu...
et al. 2016). Although it is difficult to obtain transgenic lines expressing dCas9-FP that reflect an exact genomic position, future improvements will promote dCas9-FP as a visualization technique for analyzing chromatin dynamics in plants.

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