

Protein electroporation of Cre recombinase into cultured Arabidopsis cells with an intact cell wall

Yoshio Kato (✉ y-kato@aist.go.jp)

Yoshio Kato Lab

Yuichi Furuhashi

Yoshio Kato Lab

Ayako Sakai

Yoshio Kato Lab

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Abstract

Cre recombinase is widely used in a variety of organisms to manipulate DNA both in vitro and in vivo. Combined with its consensus DNA sequences, named loxP, it allows the DNA modification including deletions, insertions, translocations and inversions at specific DNA locus of cells. To promote its use in plant cultured cells, we report here a detailed protocol for direct protein delivery of Cre recombinase into cultured *Arabidopsis thaliana* cells with an intact cell wall. Electroporation with the optimized buffer and program enables the efficient delivery of Cre protein and following recombination in cultured *Arabidopsis thaliana* cells. Specifically, we describe the purification of Cre protein, the preparation of electroporation samples, and the assessment of electroporated cells. This simple, economical, and effective technology will contribute to the biological analysis of genes and cellular functions in cultured *Arabidopsis thaliana* cells. This protocol accompanies Furuhashi et al (DOI: 10.1038/s41598-018-38119-9), *Scientific Reports*, published online on February 15th 2019.

Introduction

The Cre/loxP system is a powerful genome engineering tool that has been extensively used in numerous areas of the life sciences. Cre recombinase catalyzes recombination between two of its consensus DNA sequences, named loxP¹⁻³, and can be used for various applications including lineage/cell type-specific gene knockout, selectable marker removal, and chromosomal rearrangement¹⁻⁸. Although the Cre/loxP system has been widely used in a variety of organisms including plant cells⁹⁻¹⁷, its application in cultured plant cells has been limited compared to that in animal cells. One major obstacle is cell wall, which protects plant cells from mechanical stress and the entry of exogenous substances. Methods have been developed to deliver biomaterials into cultured plant cells through the cell wall, such as the delivery of DNA using a particle gun or *Agrobacterium* (*Rhizobium*)-mediated techniques are widely used in plant research^{18,19}. However, exogenous DNA fragments are often or invariably incorporated into the genomic DNA and may induce unexpected effects that interfere with subsequent analyses. To overcome these obstacles, we focus on direct protein delivery to cultured plant cells and show an efficient protein-electroporation technique for Cre recombinase into cultured *Arabidopsis thaliana* cells with an intact cell wall.

Reagents

Escherichia coli (*E. coli*) strain BL21(DE3) (312-06534, Nippon Gene, Japan) pET-HNCre(A207T)²⁰ (Constructed in our lab and available upon request) LB Broth (Lennox) (L7275, Sigma, United States) SOC outgrowth medium (B9020, New England Biolabs, United States) Kanamycin Sulfate Solution (50mg/ml) (117-00961, FUJIFILM Wako, Japan) Isopropyl β-D-1-thiogalactopyranoside (IPTG) (094-05144, FUJIFILM Wako, Japan) Triton X-100 (168-11805, FUJIFILM Wako, Japan) Ni-NTA agarose (30230, QIAGEN, Germany) Coomassie staining solution: EzStain Aqua (AE-1340, Atto, Japan) *Arabidopsis thaliana* T87 cell line (RIKEN Bio Resource Center, Japan) *Arabidopsis thaliana* T87-

xGxGUS cell line²¹ (Established with a binary vector pCAMBIA-N-xGxGUS²¹, and available upon request) Murashige and Skoog Plant Salt Mixture (392-00591, Nihon Pharmaceutical, Japan) Murashige and Skoog Modified Vitamin Solution 1000X (2625149, MP Biomedicals, United States) Hygromycin B (084-07681, FUJIFILM Wako, Japan) Opti-MEM I (51985034, Thermo Fisher Scientific, United States) X-Glucuronide (X-Gluc) (EB04274, Carbosynth, United Kingdom) 6-chloro-4-methylumbelliferyl β -D-glucuronide (CMUG) (29064, Glycosynth, United Kingdom) Black 96-well plate (137101, Thermo Fisher Scientific, United States) MightyAmp DNA Polymerase Ver.3 (R076A, Takara Bio, Japan) DNA oligonucleotides (FASMAC, Japan) Agarose S Tablet (316-06071, Nippon Gene, Japan) 50 \times TAE (313-90035, Nippon Gene, Japan) GelGreen (41004, Biotium, United States) ****Reagent setup**** 0.1 M IPTG: Dissolve IPTG powder in H₂O and stored in -20°C until use. 10% (v/v) Triton X-100: Dilute viscous solution in H₂O for easier handling. 2 M imidazole (pH 8.0): Adjust pH with HCl. Lysis10 buffer: 50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM benzylsulfonyl fluoride, 1 mM dithiothreitol, pH 8.0 Wash20 buffer: 50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.0 Elute500 buffer: 50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 500 mM imidazole, pH 8.0 Buffer A: 20 mM HEPES, 500 mM NaCl, 10% glycerol, 1 mM dithiothreitol, pH 7.4 HBS: 20 mM HEPES, 150 mM NaCl, 5 mM KCl, 25 mM glucose Liquid NT1 medium (pH 5.8): 30 g/L sucrose, 0.1 mM KH₂PO₄, 1 \times Murashige and Skoog Plant Salt Mixture, 1 \times Murashige and Skoog Modified Vitamin Solution, 2 μ M 2,4-dichlorophenoxyacetic acid, Adjust pH with KOH, autoclave after pH adjustment. Store at 4°C. Liquid NT1 medium (pH 7.0): Adjust pH with KOH Staining buffer: 20% (w/v) methanol, 50 mM NaH₂PO₄, pH 7.0 70% (w/v) ethanol 250 mM NaOH and 0.1% Tween20 1 M Tris-HCl (pH6.5)

Equipment

Heat block Shaker incubator with cooling function (BR-43FL-MR TAITEC, Japan) Centrifuges Sonicator (model 250DA equipped with temperature probe, Branson, United States) Empty column: Polyprep (732-1010, Bio-Rad, United States) Chromatography system (ÄKTA start, GE Healthcare, United States) HiPrep 16/60 Sephacryl S-200 HR Gel filtration column (17116601, GE healthcare, United States) Tissue culture incubator with light source (MIR-154 equipped with illuminator, Panasonic, Japan) Electroporator NEPA21 Type (Nepa Gene, Japan) 4-mm-wide cuvette (EC-004, Nepa Gene, Japan) Phase contrast microscope Cell density meter (CO8000 biowave, Biochrom, United Kingdom) Microplate reader (Wallac 1420 ARVOsx, PerkinElmer, United States) Thermal cycler (T100, Bio-Rad, United States) Electrophoresis equipment (Mupid-one, Mupid, Japan) Transilluminator (Chemi-Doc XRS+, Bio-Rad, United States)

Procedure

****Purification of Cre protein**** Day 1 1) Mix 1 ng of a plasmid pET-HNCre(A207T)²⁰ and 20 μ L of *Escherichia coli* (*E. coli*) strain BL21(DE3) competent cells in a 1.5 mL-tube. Incubate the competent cell/DNA mixture on ice for 5 min. 2) Heat-shock the mixture by incubation at 42°C for 30 sec. Put the tube back on ice for 2 min. 3) Add 100 μ L of SOC media to the mixture and incubate at 37°C for 30-60 min. 4) For the pre-culture, put the 100 μ L of the mixture into 15 mL of LB with kanamycin. Incubate the

LB with shaking at 37°C overnight. Rest of the transformation mixture can be plated on LB agar plate containing kanamycin in case of backup. Cre protein is less toxic to *E. Coli*, so that the supplementation of glucose, a catabolic repressor for leaky expression, to the preculture is not necessary. Day 2 1) Pour the 15 mL of pre-culture to 1 L of LB with kanamycin in a baffled flask. 2) Incubate at 37°C with shaking for 2-3 h, until absorbance A_{600} becomes 0.6. 3) Shift the temperature to 18°C and incubate with shaking for 30 min. 4) Add 1 mL of 0.1 M IPTG (0.1 mM of final concentration) to induce protein expression and incubate at 18°C with shaking overnight. Day 3 1) Collect cells by centrifugation at 5,000 g for 20 min at 4°C. Remove supernatant. 2) Resuspend cells in 40 mL of ice-cold Lysis10 buffer. 3) Add 800 μ L of 10% Triton X-100 towards final concentration of 0.2% Triton X-100. 4) Sonicate the suspension to shear the DNA for 10 min on ice. We do not add lysozyme or nuclease to the lysate, since these components are difficult to be removed completely from samples. 5) Centrifuge the suspension at 9,000 g for 10 min at 4°C to pellet the cellular debris. 6) Transfer the supernatant to a new tube on ice. 7) Repeat Steps 5 and 6. 8) Transfer 10 ml of a 50% slurry of Ni-NTA Agarose into a clean empty column. 9) Wash and equilibrate the column by running 20 ml of lysis buffer through the column. 10) Add the supernatant of Step 7 lysate to the column. 11) Wash the column with 15 mL of Wash20 buffer. 12) Repeat Step 11. 13) Elute the protein with 3 mL of Elute500 buffer. 14) Repeat Step 13. 15) Analyze each fraction to determine the purity of Cre protein by SDS-PAGE followed by CBB stain. Day 4 1) Purify the eluted HNCre protein with a gel filtration column and Buffer A using chromatography system and HiPrep 16/60 Sephacryl S-200 HR Gel filtration column. 2) Freeze the purified HNCre protein using liquid N_2 and store it at -80°C 3) Thaw the frozen protein and dialyze it with HBS before use. Note: It is important to use buffers containing 500 mM NaCl because Cre protein, especially at lower salt concentration, tends to bind and thus copurified with nucleic acids, and also tends to aggregate during purification process. ****Electroporation**** 1) T87 cells are maintained with rotary shaker (120 rpm) under constant light at 22°C. Passage T87 cells into fresh NT1 medium (pH 5.8) 1–5 days before electroporation. Time for pre-culture does not largely affect the electroporation efficiency. 2) Collect cells in a fresh 1.5 mL-tube. Cells can be left for 5 min to settle naturally. 3) Remove the supernatant and visually estimate the packed cell volume (pcv). Wash cells once with Opti-MEM I (at least 1 volume of pcv). 4) Discard the supernatant. Add 200 μ L of Opti-MEM I with 1–5 μ M HNCre protein to the 20 μ L pcv of cells on ice. Note: The delivery efficiency is increased with increasing concentrations of Cre protein. 5) Transfer cells into a 4-mm-wide cuvette on ice. Note: A 4-mm-wide cuvette is recommended because T87 cells often form large aggregates. 6) Place the cuvette into NEPA21 Type. 7) Use the electroporation program; 375 V/cm (150 V setting/0.4 cm width), 10 ms, 5 times, and a 50 ms interval for poring pulse, and 20 V, 50 ms, 20 times, and a 50 ms interval for transfer pulse. Note: The delivery efficiency is increased with increasing the field strength or/and duration of poring pulse. 8) Immediately after electroporation, add 800 μ L of NT1 medium (pH 5.8) to the cuvette and transfer all cells to a new 1.5 mL-tube. 9) Discard the supernatant and resuspend cells with 1 mL of NT1 medium (pH 5.8). Discard the supernatant and resuspend cells with 2 mL of NT1 medium (pH 5.8). Transfer cells to a 12-well plate. 10) Culture cells at 22°C with shaking until the assay. Note: When T87-xGxGUS cells is used, the delivery efficiency of HNCre protein can be measured by β -glucuronidase (GUS) activity using X-Glucuronide or 6-chloro-4-methylumbelliferyl β -D-glucuronide, or genomic PCR primers flanking the two *loxP* sites as follows. ****GUS staining**** 1) Two days after electroporation,

discard the supernatant and resuspend cells with 0.5 mg/mL of X-Glucuronide dissolved in 300 μ L of staining buffer. Note: At least 2 days of incubation after electroporation are necessary for the detection of GUS activity. 2) Incubate cells for 30 min at 37°C. 3) Transfer cells into 70% (w/v) ethanol. 4) Image cells using a phase contrast microscope. ****Fluorescence quantification of GUS activity**** 1) Two days after electroporation, wash cells once with NT1 medium (pH 7.0). 2) Suspend cells with 5 μ L of PCV in 100 μ L of NT1 medium (pH 7.0). 3) Transfer cells to a black 96-well plate. 4) Add 5 μ L of 200 μ M CMUG (final concentration of 10 μ M CMUG) to each well. 4) Measure fluorescence using a microplate reader. The excitation wavelength and the emission wavelength are set at 355 nm and at 460 nm, respectively. ****Genomic PCR**** 1) Two days after electroporation, discard the supernatant and resuspend 10 μ L pcv of cells with 10 μ L of 250 mM NaOH and 0.1% Tween20. 2) Incubate cells for 10 min at 98°C to extract genomic DNA. 3) Add 5 μ L of 1M Tris-HCl (pH 6.5). 4) Centrifuge cells at 10,000 g for 5 min at RT. 5) Collect the supernatant. 6) Perform genomic PCR using MightyAmp DNA Polymerase Ver.3 and the supernatant as a template DNA with primers TCCTTCGCAAGACCCTTCCTC and GGATGGCAAGAGCCAAATGCTTAG. 7) Analyze PCR products by agarose gel electrophoresis. 8) Stain gels with GelGreen. 9) Image gels using a transilluminator.

Timing

Purification of HNCre protein: 4 d Preculture of T87 cells: 1-5 d Electroporation: 1 h Post-electroporation culture: 2 d GUS staining: 1 h Fluorescence quantification of GUS activity: 2 h Genomic PCR: 3 h

Troubleshooting

Low protein expression – Inoculate from fresh bacterial cultures, since higher protein yields are generally obtained from a fresh bacterial colony. Low delivery efficiency – Prolonged storage of Cre protein at 4°C will result in protein denature. Do not keep the Cre protein over one month at 4°C after dialysis with HBS.

Anticipated Results

Cre protein will be efficiently delivered into *Arabidopsis thaliana* cells with about 80% efficiency.

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Figures

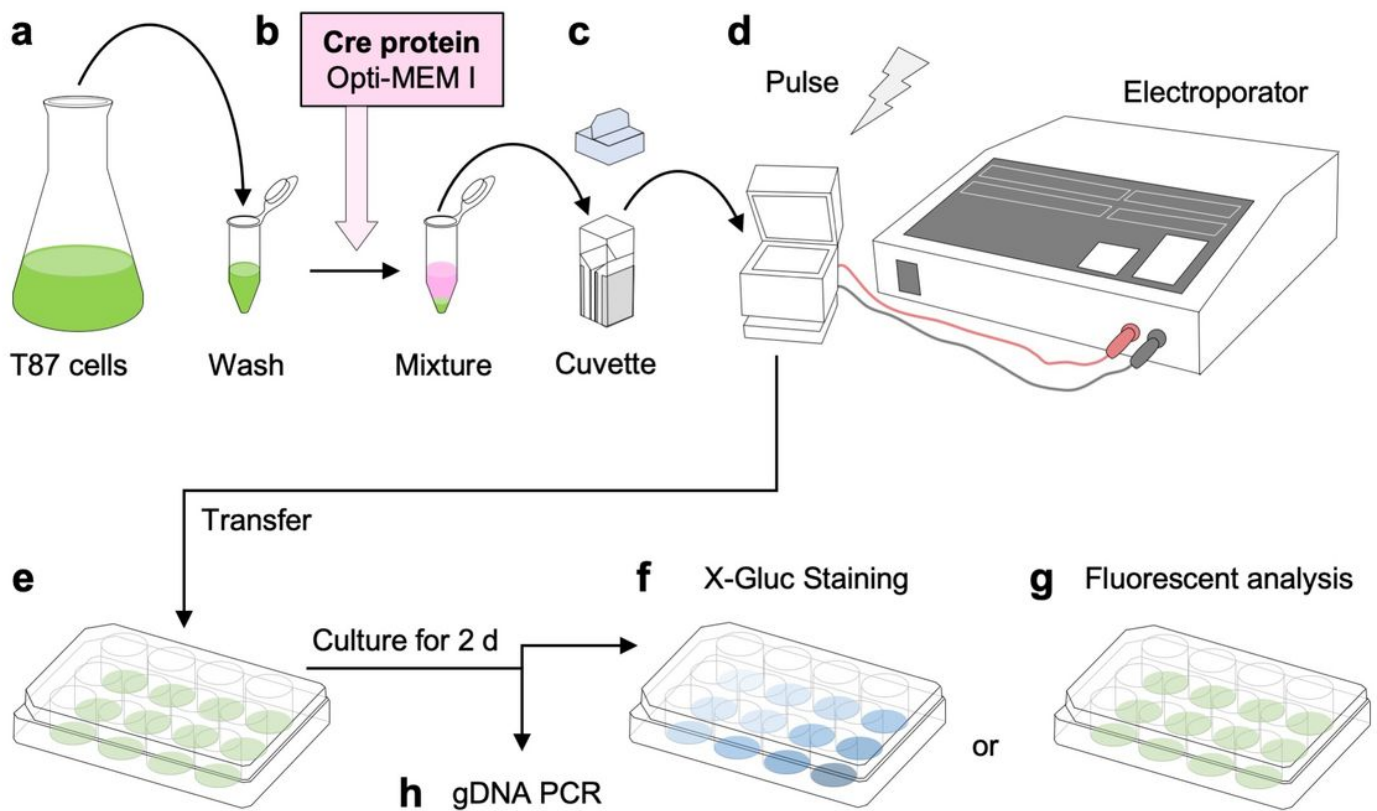
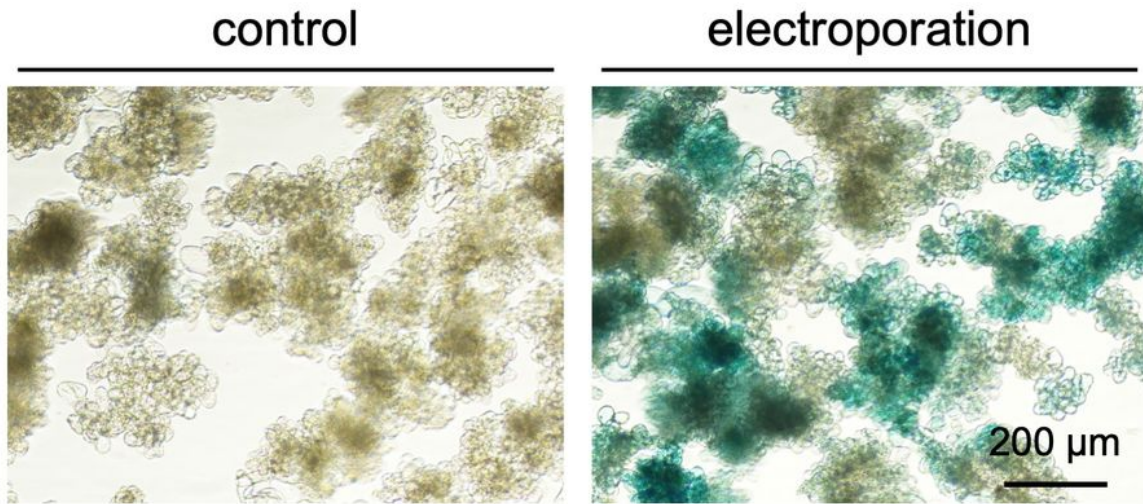
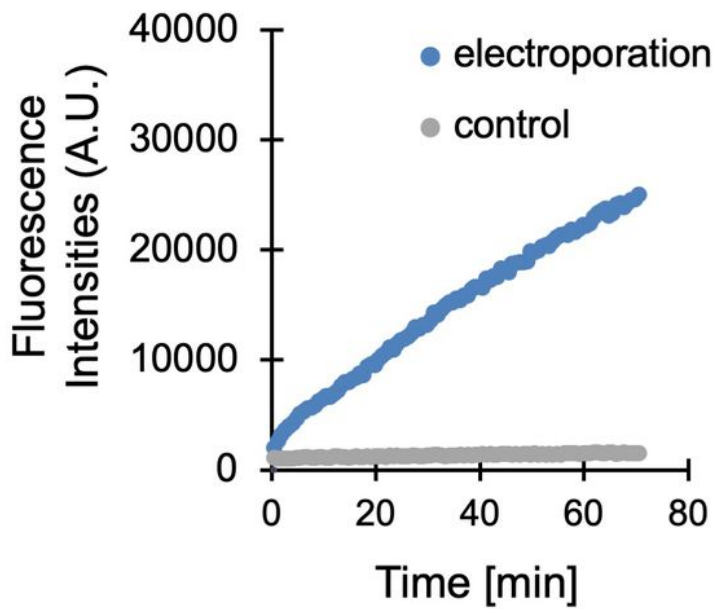
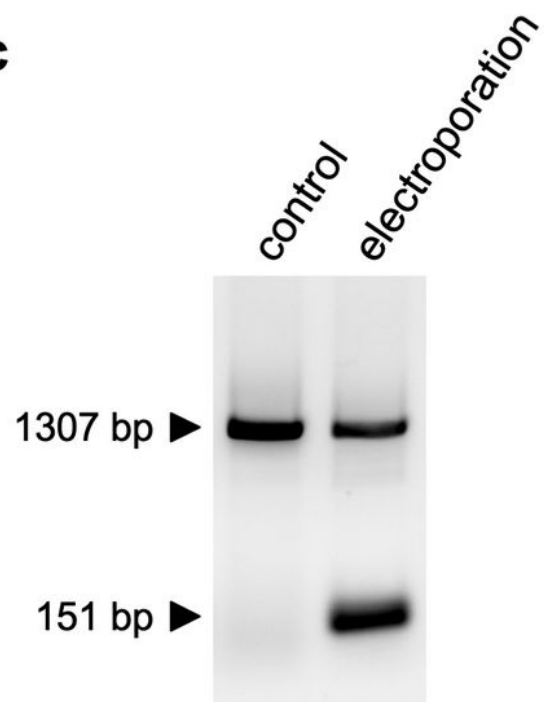


Figure 1

Electroporation procedure (a) Cultured T87-xGxGUS cells used for electroporation. Step 1-2. (b) 20 μ L PCV of T87-xGxGUS cells suspended in 200 μ L Opti-MEM I with HNCre protein. Step 3-4. (c) T87-xGxGUS cells transferred into a 4-mm-wide cuvette on ice. Step 5. (d) The cuvette placed in NEPA21 Type[®] followed by electroporation. Step 6-7. (e) Transfer cells after washing with NT1 medium (pH5.8). Step 8-10. (f) X-Gluc staining or (g) Fluorescent analysis. (h) genomic DNA (gDNA) analysis.

a**b****c****Figure 2**

Analysis of electroporation efficiency (a) GUS staining of T87-xGxGUS cells. Scale bar represents 200 μm . (b) Time-resolved fluorescence data for GUS activity of electroporated cells. (c) Agarose gel electrophoresis of genomic DNA PCR products from T87-xGxGUS cells. The 1307 bp and 151 bp fragments represent the reporter gene cassette before and after Cre-mediated recombination, respectively. In all assays, cells were electroporated in Opti-MEM I containing 5 μM Cre protein using 5 poring pulses of

150 V and 10 ms. Assays were performed at 2 days after electroporation. Control indicates untreated T87-xGxGUS cells.