

Chapter 19

Trimolecular Fluorescence Complementation (TriFC) Assay for Visualization of RNA-Protein Interaction in Plants

Jun Sung Seo and Nam-Hai Chua

Abstract

RNA-protein interactions play important roles in various eukaryotic biological processes. Molecular imaging of subcellular localization of RNA-protein complexes in plants is critical for understanding these interactions. However, methods to image RNA-protein interactions in living plants have not yet been developed until now. Recently, we have developed a trimolecular fluorescence complementation (TriFC) system for in vivo visualization of RNA-protein interaction by transient expression in tobacco leaves. In this method, we combined conventional bimolecular fluorescence complementation (BiFC) system with the MS2 system (phage MS2 coat protein [MCP] and its binding RNA sequence [MS2 sequence]) to tag lncRNA. Target RNA is tagged with 6xMS2, and MCP and RNA-binding protein are fused with YFP fragments. DNA constructs encoding such fusion RNA and proteins are infiltrated into tobacco leaves with *Agrobacterium* suspensions. RNA-protein interaction in vivo is observed by confocal microscopy.

Key words Long noncoding RNA, RNA-protein interaction, TriFC, Tobacco transient expression, In vivo visualization

1 Introduction

Recently, a variety of types of long noncoding RNAs (lncRNAs) has been identified and shown to play important roles in transcriptional regulation and chromatin modification [1]. So far, most of the molecular mechanisms for lncRNA-mediated functions are closely related to RNA-protein interactions [1]. Therefore, characterizing RNA-protein interaction is a key step in the functional study of lncRNAs. In plants, molecular functions of lncRNAs are only beginning to be characterized, and the molecular basis of lncRNA-mediated gene regulation is still poorly understood. Although techniques for RNA visualization in plants have been well developed, a visual assay for RNA-protein interaction in the plant is still poorly developed [2]. To develop a visual assay for RNA-protein interaction in plants, we modified and combined the MS2 system for RNA imaging technique with a conventional BiFC

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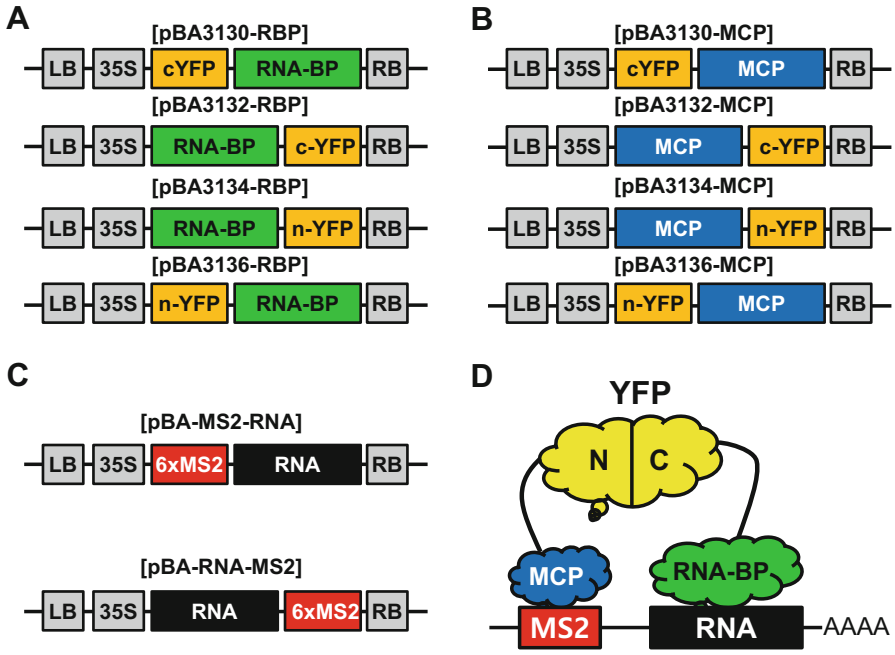

Fig. 1 Schematic representation of the TriFC system. (**a**) A schematic drawing of an expression vector encoding RNA-binding protein (RNA-BP) fused with YFP fragments. (**b**) A schematic drawing of an expression vector encoding MCP (MS2 coat protein) fused with YFP fragments. (**c**) A schematic drawing of MS2-tagged RNA expression vectors. (**d**) Illustration of the TriFC system

system for protein-protein interaction [3] (Fig. 1d). The MS2 coat protein (MCP) binds to a 19-nucleotide RNA hairpin that is recognized with high specificity [4] and with an affinity sufficient for in vivo detection [5]. Additional advantages of this RNA-binding protein are that no potential endogenous target sequences are known from eukaryotes and a fusion of MCP to GFP does not produce a specific subcellular localization by itself. For RNA imaging, the MCP-FP (fluorescence protein) fusion is co-introduced with a construct of the target RNA "tagged" by the introduction of MS2 binding sites [6, 7]. To increase the sensitivity of detection, usually, 6–24 repeats of the MS2 binding site are introduced into target RNAs, although as many as 96 repeats have been used to enable single-molecule sensitivity [8]. We generated binary Gateway vectors (pBA3130, pBA3132, pBA3134, and pBA3136) for transient BiFC assay [9] and obtained binary Gateway vectors (pBA-GW-6xMS2 and pBA-6xMS2-GW) for RNA tagging from Dr. Ulrich Z. Hammes (Fig. 1) [3]. We tested and confirmed this TriFC assay was working well in plants with lncRNA, ELENA1, and MED19a protein [9]. TriFC assay in plants will provide new insights on the interaction between lncRNAs and proteins.

2 Materials

2.1 TriFC Vector Construction Components

1. Destination vectors for TriFC: pBA3130 (35S:cYFP-DC; spectinomycin), pBA3132 (35S:DC-cYFP; spectinomycin), pBA3134 (35S:DC-nYFP; spectinomycin), pBA3136 (35S:nYFP-DC; spectinomycin) [9], pBA-6xMS2-DC (kanamycin), and pBA-DC-6xMS2 (kanamycin) [3].
2. Entry clones: pENTR-ELENA1 (kanamycin), pENTR-Med19a (kanamycin), and pENTR-MCP (kanamycin).
3. LR Clonase II (Thermo Fisher Scientific).
4. *E. coli* competent cells (strain DH5α).
5. LB agar media.
6. LB broth media.
7. Antibiotics: Spectinomycin (Sigma), Kanamycin (Sigma).
8. QIAprep Spin Miniprep Kit (Qiagen).
9. Incubator (37 °C) and shaking incubator (37 °C).

2.2 Agrobacterium Preparation Components

1. *Agrobacterium* competent cells (strain GV3101).
2. Incubator (28 °C) and shaking incubator (28 °C).
3. 10 mL culture tubes and 50 mL Falcon tubes.
4. Spectrometer.
5. Centrifuge for 50 mL tubes.
6. LB-MES (pH 5.6): LB broth with 10 mM MES (pH 5.6).
7. 100 mM acetosyringone stock: 20 mg acetosyringone (Sigma) in 1 mL ethanol or DMSO, stored at −20 °C (*see* **Note 1**).
8. Resuspension solution: 10 mM $MgCl_2$, 10 mM MES-KOH (pH 5.6), and 100 μM acetosyringone (*see* **Note 2**).

2.3 Tobacco Infiltration and Confocal Microscope Components

1. 1 mL syringe (BD).
2. *Nicotiana benthamiana* (*N. benthamiana*) plants; 2–4 weeks old (6–10 leaves stage).
3. Slide glasses and cover glasses for microscope.
4. Scissor and forceps.
5. Confocal laser scanning microscope system (ZEISS, model: LSM 780).
6. ZEN image analyzer (ZEISS).

3 Methods

3.1 TriFC Vector Construction

1. Prepare the entry and destination vectors for construction of TriFC vectors.
2. LR reaction MCP and RNA-BP entry clones with Gateway BiFC vectors (pBA3130, pBA3132, pBA3134, and pBA3136; Fig. 1a, b) following the manufacturer's instruction (*see* **Note 3**). In this study, we used pENTR-MED19a for RNA-BP entry clone and pENTR-MCP for MCP entry clone.
3. LR reaction RNA entry clones with Gateway MS2 tagging vectors (pBA-6xMS2-DC or pBA-DC-6xMS2; Fig. 1c) following the manufacturer's instruction (*see* **Note 3**). In this study, we used pENTR-ELENA1 for RNA entry clone. After LR reaction, transform competent *E. coli* cells (strain DH5α) by heat shock method.
4. Spread the cells on the LB/spectinomycin (or kanamycin) agar media, and grow overnight in an incubator at 37 °C.
5. Screen colony by PCR with specific primers.
6. Inoculate one single colony of *E. coli* in 5 mL LB with appropriate antibiotics. Grow overnight in a shaking incubator with 200 rpm at 37 °C.
7. Extract plasmid with plasmid extraction kit (Qiagen).

3.2 Agrobacterium Preparation for Tobacco Infiltration

1. Transform 50 μL *Agrobacterium* competent cells (strain GV3101) with each DNA construct (50 ng) (three different vectors; Fig. 1a–c) by heat shock method [10].
2. Spread the cells on the LB/spectinomycin (or kanamycin) agar media, and grow for 2 days in an incubator at 28–30 °C.
3. Inoculate one single colony of *Agrobacterium* in 5 mL LB with appropriate antibiotics. Grow cells overnight in a shaking incubator with 200 rpm at 28–30 °C.
4. Use 250 μL of the overnight culture to inoculate 10 mL LB-MES (pH 5.6) (with same antibiotics) plus 4 μL of 100 mM acetosyringone, and grow the culture overnight in a shaking incubator with 200 rpm at 28–30 °C.
5. Measure the A600 of overnight cultures (OD = 0.8–1.0).
6. Collect bacterial cells (4000 *g*, 10 min) at room temperature, and resuspend the pellet in resuspension solution. Adjust the final A600 to 0.4.
7. Leave on a bench (room temperature) for over 4 h (or overnight) before infiltration.

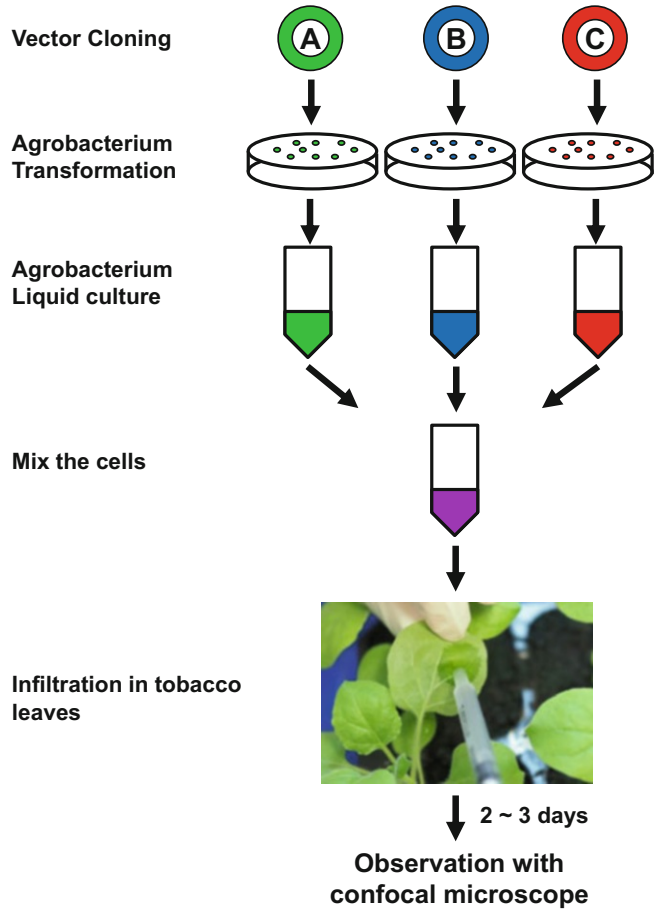

Fig. 2 Work flow of TriFC assay in *N. benthamiana*. Cloning the vectors for TriFC assay [*A* is RNA-BP construct (Fig. 1a), *B* is MCP construct (Fig. 1b), and *C* is MS2-tagged RNA construct (Fig. 1c)] and transformation of *Agrobacterium* with each vector. Selecting the colony and growing the cells. Mix three different *Agrobacterium* cells. Infiltration in tobacco leaves with syringe, and keep the plant in the growth room for 2–3 days. Observe the infiltrated region with confocal microscope

3.3 Tobacco Infiltration and Confocal Microscopy Observation

1. Transfer 1 mL of each *Agrobacterium* cell suspension into a 10 mL tube, and mix well (*see* **Note 4**).
2. Perform the infiltration with a 1 mL syringe. Simply press the syringe (no needle) on the underside of a leaf (Note: Avoid cotyledons), and exert a counter-pressure with a finger on the other side. Successful infiltration is often observed as a spreading of "wetting" area in the leaf and labels the infiltrated region (Fig. 2) (*see* **Note 5**).
3. Keep infiltrated plants in the greenhouse or growth chamber for 2–3 days.

4. Observe the YFP signals under a confocal laser scanning microscope 2–3 days after infiltration (*see* **Note 6**). The excitation wavelength is 514 nm, and detection range of emission wavelength is 520–550 nm (*see* **Note 7**).

4 Notes

1. We recommend making acetosyringone stock solution freshly before using.
2. Add acetosyringone solution after autoclaving and immediately before using.
3. We performed LR reaction through the following procedures: Add 100 ng entry vector and 100 ng destination vector in 1.5 mL tube and make total volume to 10 μL with distilled water. Then add 2 μL LR Clonase II enzyme mix, and keep the reaction mix for 1–2 h. (Optional) Add 1 μL of the proteinase K solution to each sample, and incubate samples at 37 °C for 10 min.
4. An optimal combination of 6xMS2 tag, nYFP, and cYFP orientation is sometimes critical for the TriFC assay. Therefore, other combinations should be tried if the signal is not detected.
5. Do not press the leaf strongly with a syringe. It will damage the leaf and cause cell death.
6. Posttranscriptional gene silencing (PTGS) is a major cause for the lack of efficiency in transient expression experiments [11]. Therefore, we usually co-infiltrate with *pUBQ:p19*, which suppresses PTGS, together with other constructs as it greatly improves the efficiency.
7. Adjust the detection range of emission wavelength depending on the signal intensity.

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