Video Article

Detection of Protein Interactions in Plant using a Gateway Compatible Bimolecular Fluorescence Complementation (BiFC) System

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Abstract

We have developed a BiFC technique to test the interaction between two proteins *in vivo*. This is accomplished by splitting a yellow fluorescent protein (YFP) into two non-overlapping fragments. Each fragment is cloned in-frame to a gene of interest. These constructs can then be co-transformed into *Nicotiana benthamiana* via *Agrobacterium* mediated transformation, allowing the transit expression of fusion proteins. The reconstitution of YFP signal only occurs when the inquest proteins interact [1-7]. To test and validate the protein-protein interactions, BiFC can be used together with yeast two hybrid (Y2H) assay. This may detect indirect interactions which can be overlooked in the Y2H. Gateway technology is a universal platform that enables researchers to shuttle the gene of interest (GOI) into as many expression and functional analysis systems as possible[8,9]. Both the orientation and reading frame can be maintained without using restriction enzymes or ligation to make expression-ready clones. As a result, one can eliminate all the re-sequencing steps to ensure consistent results throughout the experiments. We have created a series of Gateway compatible BiFC and Y2H vectors which provide researchers with easy-to-use tools to perform both BiFC and Y2H assays[10]. Here, we demonstrate the ease of using our BiFC system to test protein-protein interactions in *N. benthamiana* plants.

Video Link

The video component of this article can be found at https://www.jove.com/video/3473/

Protocol

1. Preparation of *Agrobacterium* culture

1. *Agrobacterium* strain GV3101 previously transformed with Gateway compatible BiFC vector pEarleyGate201-YC and pEarleyGate202-YN, each containing a fusion construct of GOI.
2. Inoculate a 5-ml YEB (5g L^{-1} Beef extract, 1g L^{-1} Yeast extract, 5g L^{-1} Peptone,5g L^{-1} Sucrose, 2mM $MgSO_4$, pH 7.2) culture of BiFC fusion protein constructs transformed *agrobacterium* with the appropriate antibiotic selection. Grow culture overnight at 28°C shaking at 200 rpm.
3. Remove 1 ml of culture into a sterile 1.5 ml centrifuge tube.
4. Pellet cells at 1,000g for 10 min at room temperature, discard supernatant.
5. Wash the pellet by adding 1 ml of infiltration media (5 g L^{-1} D-glucose, 50 mM MES (Sigma), 2 mM Na_3PO_4, 0.1 mM acetosyringone) [11]. Resuspend pellet by pipetting, pellet cells again at 1,000g for 10 min.
6. Repeat the wash step two more times.
7. Resuspend the pellet in 0.5 ml infiltration media.
8. Measure the absorbance at 600 nm and adjust final OD_{600} to 1.0 to 1.2.
9. Aliquot an equal amount of *agrobacterium* suspension of each construct into a new 1.5 ml tube, vortex for 10 sec. For a single infiltration, 100 µL of each construct is more than enough.
10. The *agrobacterium* mixture is now ready for infiltration.

2. Infiltration

11. *N. benthamiana* plants are grown at 21°C, with 16-h light & 8-h dark cycles. Five to six-week-old plants should be used for infiltration.
12. Remove plants from the growth room and place under white light for 1 h before infiltratring allowing the stomata to fully open.
13. Draw up resuspended *agrobacterium* mixture in a 1-ml slip-tip tuberculin syringe without the needle.
14. Place the tip of the syringe against the underside of the leaf and gently depress the plunger while directly supporting the upper side of the leaf with one finger. The liquid will diffuse through the leaf as it fills the mesophyllar air spaces.
15. Label the infiltrated area with a marker pen for future identification.

16. When infiltrating with different constructs, make sure to either change your gloves or clean them with 70% ethanol between infiltrations. If possible, leave one midrib space between different samples to prevent cross-contamination.
17. Place plants in a growth cabinet under normal growth conditions.

3. Observation of the reconstituted YFP signal

18. Excise a 5mm x 5mm segment of leaf tissue within the infiltrated zone
19. Mount the sample, in water, on a glass microscope slide, cover the sample with a coverslip and examine the interactions using a confocal or fluorescence microscope.
20. Expression should be monitored every 24 hours from the time of infiltration up to 5 days later as over expression/trafficking can result in fluorescent fusion proteins displaying several different locations over a time course.

4. Representative Results:

An example of protein-protein interaction tested by BiFC assay is shown in Figure 1. AtTHP1 and AtSAC3A are believed to be components of the *Arabidopsis* homolog of yeast TREX-2 complex. They can interact with a nuclearporin AtNUP1 and facilitate mRNA export[10]. AtTHP1 and AtSAC3A were cloned into pEarlyGate201-YC while AtNUP1 was cloned into pEarlyGate202-YN. The constructs were subsequently transformed into GV3101. The three constructs were paired up with 3 possible combinations (AtTHP1+AtNUP1; AtTHP1+AtSAC3A; AtNUP1+AtSAC3A) for infiltration. The reconstituted YFP signal was observed under a LCSM 48 hours after infiltration. The signals were observed in the epidermal cells and localized in the nuclear periphery or nuclear plasma, which is consistent with the sub-cellular localization of these proteins. The negative controls did not show any YFP signal.

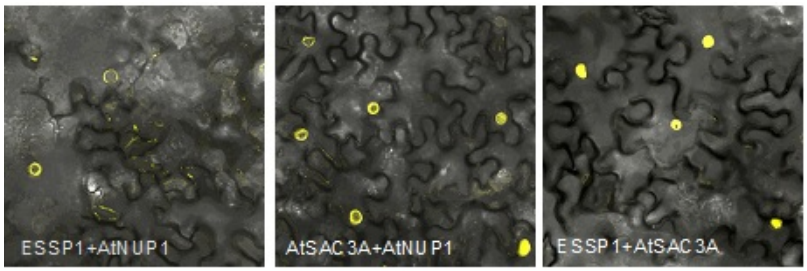

Figure 1. *Arabidopsis* TREX-2 mRNA export complex components interact with each other. *N. benthamiana* leaves, co-transformed with constructs of GOI fused to pEarleyGate201-YC and pEarleyGate202-YN (as indicated), were imaged 48-72 h after infiltration, using a Leica TCS SP2 confocol microscope. Images are shown as merged confocal YFP and bright-field images of epidermal *N. benthamiana* leaf cells

Discussion

BiFC assay is a powerful tool for studying protein interactions. Unlike the traditional Y2H assay, BiFC not only allows the visualization of protein-protein interactions, but also provides more information of the protein complex such as sub-cellular localization. Also, it is possible to detect indirect interactions as long as the two candidate proteins can be brought close enough by a third partner. Like any technology, BiFC has its limitations. Due to the requirement of molecular oxygen for fluorophore formation, BiFC cannot be used in obligate anaerobes, which cannot survive in the presence of oxygen. This limits the use of BiFC to aerobic organisms[6]. The formation of fluorophore complex is irreversible. This prevents proteins from interacting with others and may disrupt the association and disassociation of protein complexes in dynamic equilibrium[6]. However, this irreversibility also enables us to visualize weak or transit interactions. Fluorescent protein fragments have a limited ability to associate independent of the proteins to which they are fused. One must provide the necessary and numerous controls to distinguish between true and false-positive protein interactions[6]. Also, as fluorophore reconstitution can occur at a distance of 7nm or more, fluorescence complementation may indicate either a direct or indirect interaction. One needs to use other methods such as Y2H or Co-Immunoprecipitation to test the exact interaction relationship[7].

In order to generate reliable data, proper controls should be used for the interpretation of the results. The empty vectors should not be directly used as controls as they contain the typical gateway fragment between attR1 and attR2 cassettes. Thus, these empty vectors are not really "empty". To overcome this problem, we generally use a pair of unrelated proteins fused to the vectors as a control. Another potential problem is the auto-fluoresce from the plant cells, especially from old or stressed plants. Inexperienced investigators should first look at a sample from the uninfiltrated zone to get familiar with the background fluoresce.

Recently, more BiFC-based methods have been developed to further extend the applications of the BiFC assay to various aspects of proteins, RNA-protein interactions[12], and DNA hybridization[13]. For example, multicolor BiFC and BiFC-based FRET (BiFC-FRET) assay have been developed to identify and visualize ternary complexes in living cells[14].

The combination of Gateway and BiFC is a powerful tool for researchers seeking to understand protein interactions in intact cells. The constructs used in our BiFC system can also be used to generate stable transgenic plants to visualize more dynamic protein interactions in different tissues and at various developmental stages which may not be observed in transient expression system. We have incorporated a HA tag and a FLAG tag in our BiFC vectors (pEarleyGate201-YC and pEarleyGate202-YN), respectively. This modification makes it possible for various downstream applications such as western blotting, Co-Immunoprecipitation and affinity purification, etc., after the visualization of the interactions.

Disclosures

No conflicts of interest declared.

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