Chapter 14

Dynamic Proximity Tagging in Living Plant Cells with Pupylation-Based Interaction Tagging

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Abstract

Identification of protein–protein interactions (PPIs) and protein kinase substrates is fundamental for understanding how proteins exert biological functions with their partners and targets. However, it is still technically challenging, especially for transient and weak interactions involved in most cellular processes. The proximity-tagging systems enable capturing snapshots of both stable and transient PPIs. In this chapter, we describe in detail the methodology of a novel proximity-based labeling approach, PUP-IT (pupylation-based interaction tagging), to identify PPIs using a protoplast transient expression system. We have successfully identified potential kinase substrates by targeted screening and tandem mass spectrometry analysis.

Key words Protein interactions, Proximity-tagging system, PUP-IT, Mesophyll protoplast, Signal transduction

1 Introduction

Protein–protein interactions (PPIs) contribute to the regulation and execution of most cellular pathways and processes. The majority of PPIs are highly dynamic and temporary in nature, or conditional that rely on post-translational modifications or conformational changes [1, 2]. Although multiple approaches have been developed to map PPIs, identifying the transient and unstable interactions remains a challenge [3–5].

Proximity-tagging systems have emerged as reliable techniques that enable the discovery of neighboring proteins in cells, capturing both transient or weak and stable interactions [4, 5]. Generally, these methods use proximity labeling enzymes, such as biotin ligase or peroxidase [6–9], to introduce biotin tags to proximal proteins close to the protein of interest (bait) in living cells. The biotinylated proteins can then be isolated with streptavidin beads [7], Tamavidin 2-REV [6], or antibiotin antibody [8], followed by

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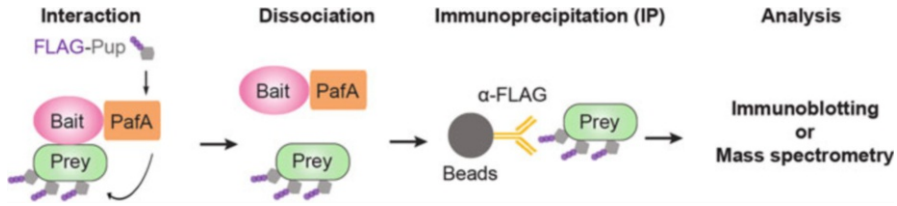

Fig. 1 Schematic presentation of the proximity-tagging system PUP-IT. The bait protein is fused with a bacterial Pup ligase (PafA), which conjugates the tagged Pup to a lysine residue of interacting proteins (prey). The Pup is triple-FLAG tagged at the N terminus. The 3XFLAG-Pup-labelled proteins could be immunoprecipitated with anti-FLAG antibody conjugated beads for immunoblotting or mass spectrometry analysis

immunoblotting and mass spectrometry analyses. However, living plant cells contain naturally biotinylated proteins [10, 11] and high endogenous peroxidases [12], which contribute to high background in proximity labeling by biotin ligase or peroxidase commonly used in mammalian cells.

Here, we introduce a newly developed proximity tagging method termed PUP-IT (pupylation-based interaction tagging) [13] to the plant research community. In this method, bait protein is fused with a bacterial Pup ligase (PafA), which conjugates the tagged Pup (64 amino acids) to a lysin residue in the prey protein in the presence of ATP (Fig. 1). This process resembles the ubiquitination process in plant and animal cells. The N terminus of Pup can be tagged to facilitate the purification of Pup-modified proteins (*see* **Note 1**). Compared to other proximity-tagging systems [14], the PUP-IT possesses additional advantages for plant research. First, unlike the biotinylated proteins, there are no preexisting Pup-modified proteins in the plant system, which provides a lower background of protein contaminants. Second, there is no need for extra biotin or H_2O_2 treatment, making it more suitable for in vivo and physiology studies. Third, as a relatively large molecule modification, Pup tagged proteins exhibit a significant size shift and retain a unique GGE motif on lysine, making it more specific for immunoblot and mass spectrometry analysis. However, the larger size of the enzyme and labeling tag may also affect the functions of the bait and prey protein. This needs to be considered when using PUP-IT or any other proximity-tagging/affinity-tagging systems. Additionally, the distribution of Pup should also be considered for studies of PPIs in organelles.

In this method, we perform the PUP-IT analysis in a protoplast transient expression system. The cell-based assay provides a versatile and quick approach to explore the function or substrate of a candidate protein [15]. The isolated plant cells retain almost all the cellular activities and processes, including transcription, translation, DNA repair, chromatin remodeling, cell wall biosynthesis, and other metabolic processes, as well as the ability to respond specifically to various signals, such as nutrient, hormone, stress, and

elicitors [16, 17]. Thus, the protoplast assay can be employed to analyze the PPIs in these signaling and metabolic processes. The PUP-IT assay works very well in *Arabidopsis* protoplast system. We have successfully identified a substrate of Target of Rapamycin (TOR) by targeted screening [18] and more potential interactors by tandem mass spectrometry using the protocols described in this chapter. The drawback of cell-based assays is that they may lose some cell-type-specific responses and cause an ectopic effect of part of prey genes. As there is no need to deliver substrates as in the other proximity-tagging systems [6–9], the PUP-IT system can be easily transformed into plants with universal, inducible, or cell-type-specific promoters to investigate the spatiotemporal resolution of PPIs (*see* **Note 2**).

2 Materials

2.1 Plant Material and Growth Conditions

Arabidopsis plants (Col-0) grown in soil (Metro-Mix 360, SunGro Horticulture, Burton, OH, USA) or Jiffy-7 (Jiffy Products Ltd., Canada) in a growth chamber at 23/20 °C, 12 h/12 h light/dark, 60% relative humidity, and 75 μmol m^{-2} s^{-1} of light for 4~5 weeks. Leaves 5–7 are used for optimal mesophyll protoplast isolation [15, 19].

2.2 Construction of the Plant Expression Plasmids

1. Bait protein in the PUP-IT vector: Clone the coding DNA sequence of desired protein into the SpeI site of *pCambia-PUP-IT* vector (Addgene plasmid #186478) by Gibson assembly (NEB #E2621).
2. Candidate prey protein for targeted screening: Clone the coding DNA sequence of desired protein into the *pHBT-MYC* plant expression vector (available from the authors upon request).

2.3 Protoplast Isolation and Transfection

1. Enzyme solution: 1% cellulase R10, 0.25% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7. Heat the enzyme solution at 55 °C for 10 min to inactivate proteases and enhance enzyme solubility. Cool the solution to room temperature before adding 10 mM $CaCl_2$, 1 mM ß-mercaptoethanol, and 0.1% BSA. Filter the solution through a 0.45 μm syringe filter.
2. Razor blades.
3. Petri dish (100 × 20 mm or 150 × 25 mm).
4. Desiccator.
5. Nylon mesh (35–70 μm, Lab-Line Instruments, #190-158-00).
6. 30 mL Round-bottom polypropylene tube
7. Hemocytometer.

8. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7.
9. WI solution: 0.5 M mannitol, 4 mM MES, pH 5.7, 20 mM KCl.
10. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7.
11. 40% (w/v) polyethylene glycol (PEG) solution: To make 10 mL of PEG solution, dissolve 4 g of PEG 4000 (Sigma-Aldrich) into 3 mL of H_2O, 2.5 ml of 0.8 M mannitol, and 1 ml of 1 M $CaCl_2$.
12. 15 mL or 50 mL round-bottomed microcentrifuge tubes.

2.4 Immunoprecipitation

1. Extraction buffer: 25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5 mM DTT, and fresh protease inhibitor cocktail.
2. Anti-FLAG M2 Agarose Beads (Sigma, A2220).
3. 3xFLAG peptide (Sigma, F4799).
4. Trichloroacetic acid (TCA).
5. Acetone.

2.5 Immunoblotting or Mass Spectrometry Analysis

1. Tris–glycine SDS–polyacrylamide gel.
2. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad, CA).
3. Tris-glycine running buffer: 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS.
4. Transfer buffer: 25 mM Tris, 190 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS.
5. TBST buffer: 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween®20.
6. Blocking and antibody incubation buffer: 1× TBST, 5% w/v nonfat dry milk.
7. HA-HRP (Sigma, 12,013,819,001, 1:5000), FLAG-HRP (Sigma, A8592, 1:5000), and MYC-HRP (Roche, 1-814-150, 1:1000).
8. Coomassie staining buffer: 0.2% Brilliant Blue G250 in 20% methanol, and 0.5% acetic acid.
9. Destaining buffer: 20% methanol, 0.5% acetic acid.
10. An ultraperformance liquid chromatography (UPLC) tandem mass spectrometry system (e.g., nanoEasy UPLC-Orbitrap Tribrid) and software for data analysis (e.g., Proteome Discoverer or MaxQuant, a freeware available at https://www.maxquant.org/).

3 Methods

3.1 Protoplast Isolation

1. Prepare fresh enzyme solution into a 100 × 20 mm or 150 × 25 mm petri dish (*see* **Note 3**).
2. Cut the middle part of well-expanded leaves (true leaf numbers five to seven) into 0.5–1 mm strips with a fresh sharp razor blade (*see* **Note 4**).
3. Immediately and gently transfer leaf strips into the petri dish and completely submerge them with enzyme solution with an inoculating loop (BD).
4. Cover the Petri dish with aluminum foil and apply vacuum infiltration for 30 min by using a desiccator connected to a building vacuum system.
5. Continue the digestion without vacuum and shaking for another 2.5–3 h at room temperature.
6. Gently shake the Petri dish by hand or use a shaker at 50 rpm for 1 min to release the protoplasts. Ideally, most leaf strips turn transparent, and the enzyme solution becomes green after this step.
7. Add an equal volume of W5 solution and filter the slurry with a wet 35–70 μm nylon mesh to remove undigested leaf tissues.
8. Centrifuge the flow-through at 100× *g* in a 30 mL round-bottomed tube for 1–2 min to pellet the protoplasts.
9. Remove as much supernatant as possible and gently resuspend the protoplast pellet with 10 mL of cold W5 solution.
10. Keep the protoplasts on ice for at least 30 min for recovery from isolation stress. Intact and healthy protoplasts should settle at the bottom of the tube by gravity.
11. Remove the W5 solution as much as possible and resuspend the protoplasts with MMg solution.
12. Count protoplasts using a hemocytometer under the light microscope and adjust the protoplasts in the MMg solution to a density of 2×10^5 cells/mL.

3.2 Protoplast Transfection

1. Prepare fresh 40% (w/v) PEG solution and high-quality plasmid DNA at 2 μg/μL (*see* **Note 5**).
2. For targeted screening of the prey protein, mix 100 μg of PUP-IT vectors with bait protein or control plasmids and 100 μg of candidate gene–MYC tag constructs in a 15 mL round-bottom microcentrifuge tube. For tandem mass spectrometry analysis, add 1000 μg of PUP-IT vector with bait protein into a 50ml round-bottom microcentrifuge tube (*see* **Note 6**).

3. Add 1 mL or 5 mL of protoplasts (100 μL protoplasts per 10–20 μg plasmid DNA) in MMg solution into the tube.
4. Add an equal volume (1.1 mL or 5.5 mL) of PEG solution and then mix thoroughly by gently tapping the tube.
5. Incubate the mixtures at room temperature for 5 min.
6. Dilute the transfection mixture with at least 3 volumes of W5 solution and mix well by gently rocking or inverting to quench the transfection.
7. Centrifuge at 100× *g* for 2 min at room temperature using a swinging-bucket centrifuge and remove the supernatant.
8. Resuspend the protoplasts gently with 5 mL or 20 mL of WI solution and transfer the cells into 100 × 20 mm or two 150 × 25 mm Petri dishes (*see* **Note 7**).
9. Incubate the protoplasts for 12 h at room temperature (22–25 °C) under low light (30–35 μmol m^{-2} s^{-1}) (*see* **Note 8**).
10. Harvest the protoplasts by centrifugation at 100× *g* for 2 min and remove the supernatant.
11. Freeze the samples immediately with liquid nitrogen and store them at −80 °C until further analysis.

3.3 Immuno-precipitation

1. Resuspend the frozen protoplasts in the extraction buffer (500 μL per 2 × 10^5 cells) on ice.
2. Vortex vigorously for 10 s and incubate on ice for 5–10 min to lysate the cells.
3. Distribute the extracts into 1.5 mL reaction tubes and sonicate them in an ice bath four times, 30 s on and 30 s off each time, at high setting using a Bioruptor UCD-200 (Diagenode).
4. Centrifuge at the maximum speed for 10 min at 4 °C and transfer all supernatant to the new tube.
5. Aliquot 30 μL of supernatant into a new tube as input sample, add 10 μL of 4 × sample buffer and boil for 5 min at 95 °C.
6. Add prewashed anti-FLAG M2 Agarose Beads to the rest supernatant (10 μL per 500 μL extracts), and incubate at 4 °C for 2 h.
7. Wash the beads four times with 1 mL extraction buffer on the rotor wheel for 5 min (transfer the beads into a new tube the first time).
8. Elute the proteins three times with an equal volume of 500 mg/mL of 3 × FLAG peptide at room temperature for 10 min with shaking at 1000 rpm.
9. For immunoblot analysis, add 10 μL of 4 × sample buffer to the eluted samples (30 μL) and boil for 5 min at 95 °C.

10. For mass spectrometry analysis, precipitate the elution with an equal volume of 20% TCA/acetone with 5 mM DTT on ice for 2 h and wash with acetone twice. Add 20 μL of 1 × sample buffer to the pellet and boil for 5 min at 95 °C.

3.4 Immunoblotting Analysis

1. Prepare 6–15% Tris-glycine SDS-polyacrylamide gel according to the size of the protein of interest.
2. Resolve 10–20 μL of input and eluted samples on the gel until the dye runs out.
3. Electrotransfer the proteins from the gel to a PVDF membrane.
4. Incubate the membrane in the blocking buffer for 1 h at room temperature under gentle shaking.
5. Incubate the membrane in the blocking buffer containing HRP-conjugated HA, FLAG, or MYC antibodies for 1–2 h at room temperature under gentle shaking.
6. Wash the membrane three times with TBST buffer for 5 min each at room temperature.
7. Proceed detection with ECL reagent. An example result is shown in Fig. 2a (*see* **Note 9**).

3.5 Prepare Samples for Mass Spectrometry Analysis

1. Prepare 4–12% gradient Tris–glycine SDS–polyacrylamide gel.
2. Load all the immunopurified samples (20 μL) to one well. Stop the electrophoresis when the bromophenol blue dye migrates approximately 1–2 cm into the gel.

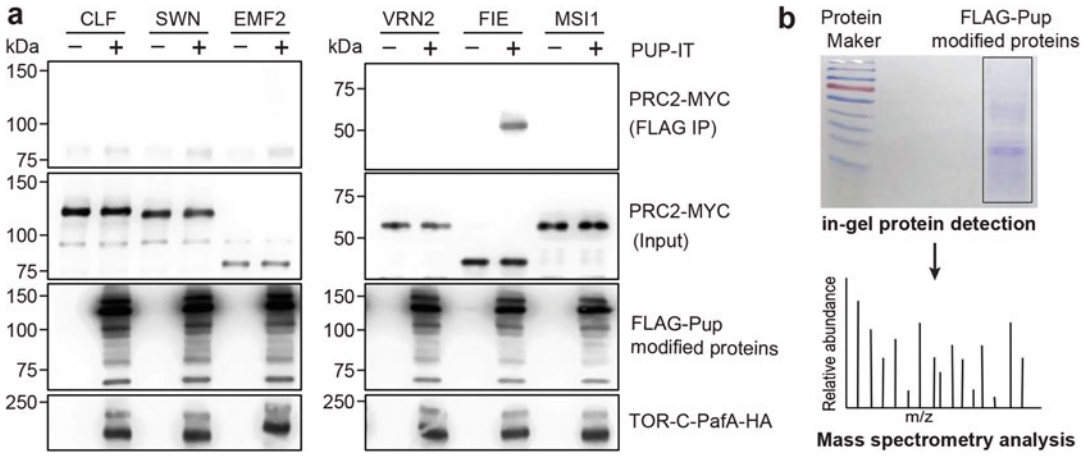

Fig. 2 Immunoblotting and mass spectrometry analyses of the PUP-IT labelling assay. (**a**) Immunoblot analysis of PUP-IT screening of MYC-tagged PRC2 components with TOR-C-PafA-HA. Only FIE interacts with TOR. (**b**) An example of FLAG-Pup modified proteins for mass spectrometry analysis. Proteins obtained from immunoprecipitation by anti-FLAG beads were separated by SDS-PAGE and stained with Coomassie Blue. Proteins excised from the gel were subjected to in-gel digestion with trypsin and analyzed by UPLC-tandem mass spectrometry

3. Rinse the gel 3 times with Milli-Q water to remove SDS and buffer salts.
4. Incubate the gel with Coomassie Brilliant Blue G250 staining buffer for 1 h with a gentle shake.
5. Gently shake the gel in the destaining buffer for at least 2 h with changes of this solvent every half hour until the desired background is achieved. An example result is shown in Fig. 2b.
6. Rinse the gel three times for 5 min with Milli-Q water.
7. Excise the gel containing protein into a clean tube. Freeze and store at −80 °C for further in-gel trypsin digestion and mass spectrometry analysis (*see* **Note 10**).
8. For UPLC-tandem mass spectrometry, please refer to the published method [20].

4 Notes

1. In the original version, the *E. coli* Pup protein was tagged with a bacteria-derived carboxylase domain, which can be biotin-labeled in vivo and further pulled down by streptavidin. The isolation of biotinylated proteins can be done under denaturing conditions to reduce the background. However, efficient labeling requires exogenous biotin treatment. The free biotin has the potential to interfere with streptavidin pull-down of pup-labeled proteins and needs extra steps to be removed. In this protocol, we fused the Pup protein with a commonly used triple FLAG tag, facilitating easy and efficient capture of Pup-modified proteins.
2. The transient expression PUP-IT vector is in the binary *pCambia1300* backbone, which can be directly used for *Agrobacterium*-mediated transformation. However, we found that the FLAG-Pup driven by the 35S promoter may be silenced after T2 generation. Endogenous or inducible promoters would be ideal options.
3. The volume of enzyme solutions should be calculated based on the purpose of the experiment. Usually, 10 mL of the enzyme solution can digest up to 40 leaves with a yield of 2×10^6 protoplasts.

   The 100 × 20 mm petri dish can hold 10–15 mL solution, while the 150 × 25 mm one can hold 30–50 mL solution. A workshop movie for protoplast isolation and transfection can be downloaded on the Sheen Lab Web site (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html). Be gentle with the protoplasts during all the steps!

4. The selection of healthy and fully expended leaves is critical for the success and efficient transfection of protoplasts. Leaves five to eight from 4-week-old Arabidopsis plants are normally used. However, well-expended leaves from younger plants, such as leaves three to four from 3-week-old plants and leaves one to two from 2-week-old plants, may also be used.
5. The quality of the plasmid DNA is very important for high transfection efficiency. We routinely use the CsCl gradient for maxi-plasmid DNA preparation. The protocol is available on the Sheen Lab Web site (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html). However, DNA preparation by commercial DNA maxiprep kits is acceptable but may lower protoplast transfection efficiency due to chemical carryover.
6. Protoplast transfection can be scaled up or down following the recommended DNA/protoplast ratio. Usually, 1 mL of cells (2×10^5) are sufficient for targeted screening, and at least 10 mL of cells (2×10^6) are required for mass spectrometry analysis.
7. The petri dish can be precoated with 5% (vol/vol) calf serum for 1–2 s to prevent the sticking of protoplasts to the plastic surface. The depth of the WI solution is approximately 0.1 mm to avoid hypoxia stress during protoplast incubation. The 100 × 20 mm petri dish is recommended for incubation of 5 mL of protoplasts/WI solution, and the 150 × 25 mm dish is recommended for incubation of 10–15 mL of protoplasts/WI solution.
8. Normally, 10–12 h is enough for PUP-IT labeling. However, the incubation time can still be optimized for a specific bait protein. The protoplasts could be treated with different stimuli during the incubation. For example, the flg22 elicitor can be added 1 h before harvest to trigger the innate immune response.
9. The Pup labeled prey protein would exhibit at least 11 kDa (one modification) size shift. The band shift could be detected in both input and immunoprecipitated samples for strong interaction but may only be detected after immunoprecipitation for weak interaction.
10. The mass spectrometry analysis can be performed as per the protocol described by Perron et al. [20]. There is an extra 243.10-Da mass (GGE modifications) on the lysine side-chain of Pup-modified peptides.

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