Chapter 19

TurboID-Based Proximity Labeling: A Method to Decipher Protein–Protein Interactions in Plants

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Abstract

Proteins form complex networks through interaction to drive biological processes. Thus, dissecting protein–protein interactions (PPIs) is essential for interpreting cellular processes. To overcome the drawbacks of traditional approaches for analyzing PPIs, enzyme-catalyzed proximity labeling (PL) techniques based on peroxidases or biotin ligases have been developed and successfully utilized in mammalian systems. However, the use of toxic H_2O_2 in peroxidase-based PL, the requirement of long incubation time (16–24 h), and higher incubation temperature (37 °C) with biotin in BioID-based PL significantly restricted their applications in plants. TurboID-based PL, a recently developed approach, circumvents the limitations of these methods by providing rapid PL of proteins under room temperature. We recently optimized the use of TurboID-based PL in plants and demonstrated that it performs better than BioID in labeling endogenous proteins. Here, we describe a step-by-step protocol for TurboID-based PL in studying PPIs *in planta*, including *Agrobacterium*-based transient expression of proteins, biotin treatment, protein extraction, removal of free biotin, quantification, and enrichment of the biotinylated proteins by affinity purification. We describe the PL using plant viral immune receptor N, which belongs to the nucleotide-binding leucine-rich repeat (NLR) class of immune receptors, as a model. The method described could be easily adapted to study PPI networks of other proteins in *Nicotiana benthamiana* and provides valuable information for future application of TurboID-based PL in other plant species.

Key words Proximity labeling, TurboID, Biotinylation, Protein–protein interactions, NLR immune receptor

1 Introduction

Plant viruses are simple, obligate, and intracellular parasites. Due to their small genome size, plant viruses usually encode limited numbers of proteins. To successfully establish a viral infection cycle in host plants, plant viruses rely on various host machineries consisting of proteins, nucleic acids, membranes, and metabolites [1]. Therefore, dissecting molecular networks of plant–virus interactions is important for understanding the mechanism of viral infection and the defense responses against viruses by the host plant. Among

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them, protein–protein interactions (PPIs) have attracted the most interest because PPIs are intrinsically involved in almost all cellular functions and biological processes.

Traditional approaches, including yeast two-hybrid (Y2H) screening and antibody-based affinity purification coupled with mass spectrometry (AP-MS), have been widely applied to identify PPIs during plant–virus interactions [2]. However, both approaches suffer from some disadvantages. For example, although Y2H screening is a high throughput method, it is a heterologous system and may have false positives due to overexpression of proteins. Those PPIs with cell and organelle type restrictions may not take place in the heterologous yeast system [3]. Furthermore, Y2H screening requires available high-quality libraries of the target plant species, constructions of which are labor-intensive and costly. The affinity purification approach allows the enrichment of stable interactions between the protein of interest and its partners in plant cells, but it suffers from capturing the transient or weak PPIs due to stringent cell lysis conditions and the subsequent washing steps typically used in AP [4]. Affinity purification is also unsuitable for insoluble proteins or targets with low abundance. These limitations, therefore, greatly hamper our understanding of the protein interaction networks in plant–virus interactions.

Enzyme-catalyzed proximity labeling (PL) is an approach developed for mapping molecular interaction networks [5]. Generally, an engineered enzyme fused to the protein of interest can produce short-lived reactive biotin species, such as radical and activated ester, to preferentially label proximal proteins within a certain radius (<20 nm) [6] (*see* Fig. 1). Because the proximate proteins are covalently tagged with biotin, the cells/tissue expressing the fusion protein can be lysed under harsh conditions, resulting in efficient disruption and solubilization of membranes and protein complexes. Subsequently, labeled proteins are enriched by streptavidin-based affinity purification and analyzed by mass spectrometry (MS) (*see* Fig. 2). The high affinity of streptavidin–biotin interaction also allows stringent washing steps to remove false positives. PL compensates for the drawbacks of the traditional approaches for identifying PPIs as it enables the capture of weak and transient interactions and insoluble protein complexes in their native cellular environment. Two major groups of enzymes commonly utilized for proximity-dependent biotinylation are peroxidases and biotin ligases. Peroxidases [such as ascorbate peroxidase 2 (APEX2), horseradish peroxidase (HRP)] generate biotin-phenoxyl radicals in the presence of hydrogen peroxide (H_2O_2) [7, 8] (*see* Fig. 1a). Wild-type biotin ligases convert biotin to the reactive biotin-adenosine-5′-monophosphoester (biotinoyl-AMP) intermediate and catalyze the direct transfer of activated biotin to a specific lysine residues of the target protein. Promiscuous biotin ligases (BioID, BioID2, BASU, TurboID, miniTurboID, and

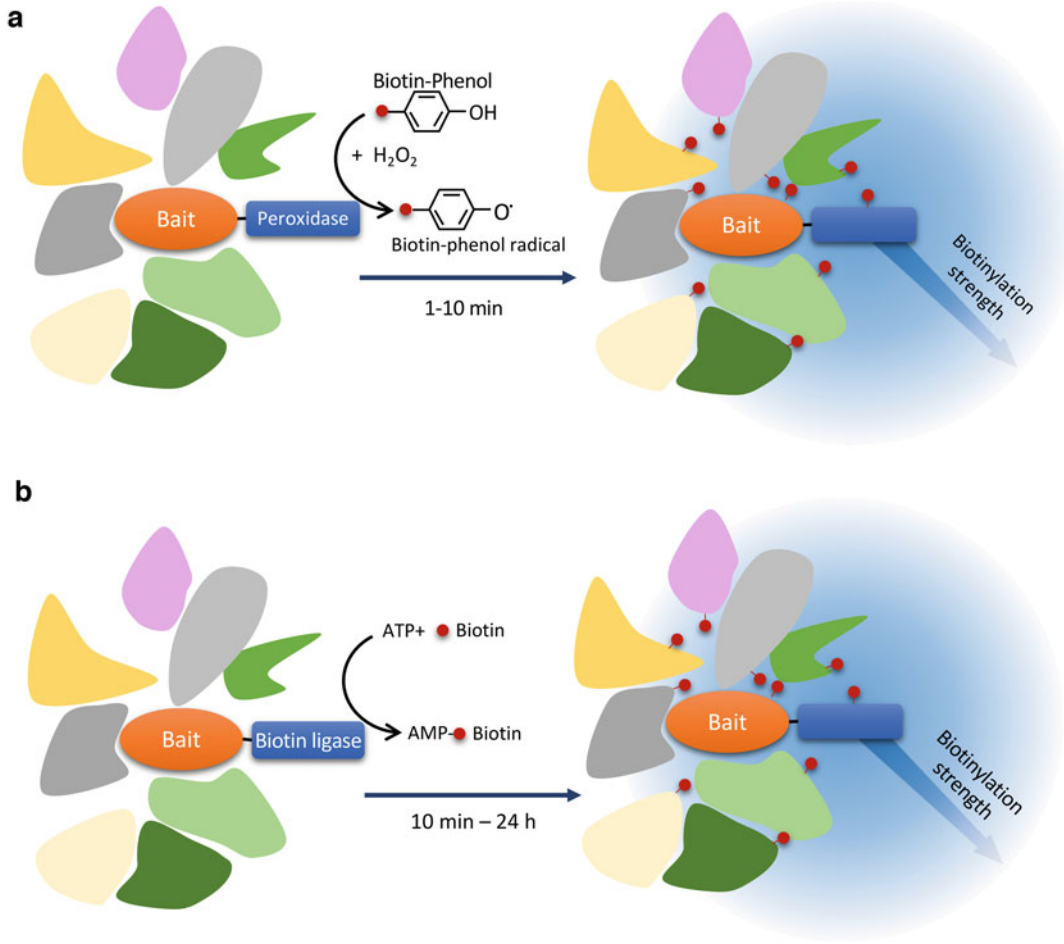

Fig. 1 Schematic of peroxidase- and biotin ligase-based proximity labeling. (**a**) Peroxidases, such as APEX or HRP, oxidize biotin–phenol into reactive radicals in the presence of H_2O_2, which labels proximal endogenous proteins. (**b**) Biotin ligases, such as BioID or TurboID, catalyze the formation of reactive biotin-AMP from biotin, which diffuses and labels proximal endogenous proteins

UltraID) modified from the wild-type enzymes have low affinity for the intermediate, allowing the generated intermediates diffuse out of the enzyme active site, and covalently biotinylate proximal proteins [9–12] (*see* Fig. 1b).

Although APEX offers rapid labeling kinetics (within minutes), the requirement of toxic H_2O_2 during labeling and high endogenous plant peroxidase activity make it unsuitable for PL studies in plants [7, 8]. In contrast, the biotin ligases provide simple and nontoxic labeling. BioID, a mutant of *Escherichia coli* biotin ligase BirA, represents the first generation of biotin ligase developed for PL. Compared to peroxidase-based PL, BioID requires 18–24 h for efficient labeling, making it difficult to capture transient PPIs. Moreover, the higher incubation temperature (37 °C) required by

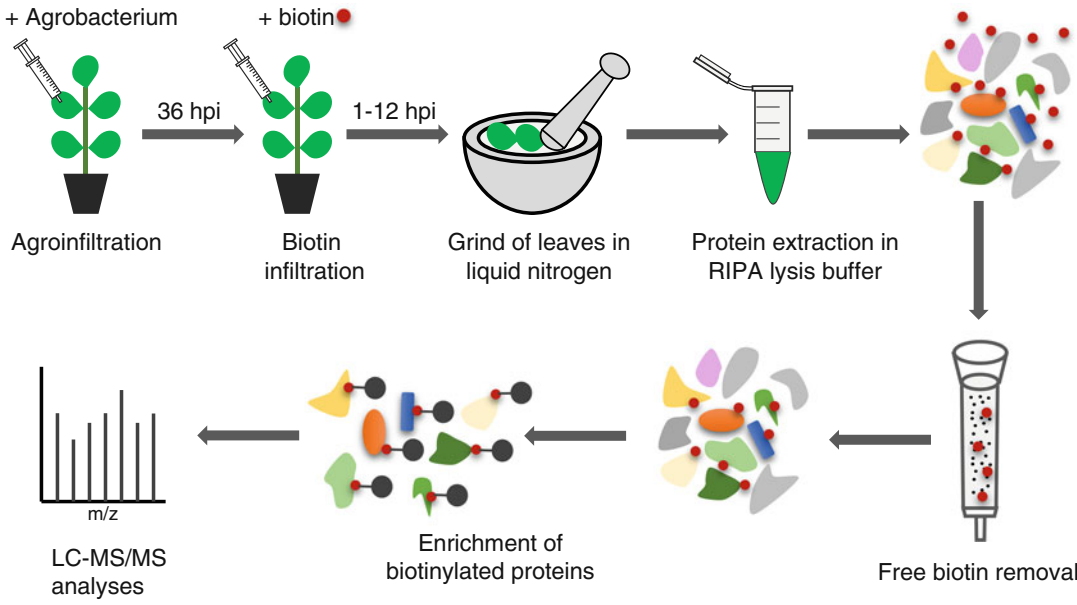

Fig. 2 Example workflow for mapping PPIs using TurboID-based proximity labeling method in *N. benthamiana*. TurboID fused to the protein of interest and a reference control are expressed in separate plants by agroinfiltration. 36 h post-infiltration, 200 μM biotin is infiltrated into the same leaves to initiate the proximity labeling of endogenous proteins. The infiltrated leaves were harvested after 1–12 h of incubation at RT and followed by grinding in liquid nitrogen. Proteins were extracted using a lysis buffer, such as RIPA lysis buffer, and a desalting column was employed to remove the free biotin in the extract. The biotinylated proteins were then affinity-purified with streptavidin-conjugated beads and identified via quantitative mass spectrometry. LC-MS/MS, liquid chromatography, and tandem mass spectrometry. (Reproduced from ref. [28] with permission from Nature Springer)

BioID is not optimal for *in vivo* PL in plants [11]. To reduce the interference with trafficking and function of the fusion protein, BioID2, a smaller biotin ligase derived from the *Aquifex aeolicus* was developed [10]. However, BioID2 requires the same labeling conditions as BioID, resulting in limited utilities of BioID/BioID2-based PL in plants [13–17]. Recently, new promiscuous biotin ligase variants of *E. coli* BirA, TurboID, and miniTurboID have been generated using yeast display-based directed evolution [9]. TurboID and miniTurboID have much higher catalytic activity than BioID or BioID2, allowing rapid nontoxic PL within 10 min under room temperature.

TurboID-based PL has been successfully conducted in a variety of cell types and organisms [18–25]. We and others recently optimized the utility of TurboID-based PL for PPIs studies in different plant systems such as *N. benthamiana*, *Arabidopsis*, and tomato [26–28]. TurboID showed a better PL performance than BioID in plants under room temperature [27, 28]. We used this method to identify interactors of plant viral immune receptor N that belong to the nucleotide-binding leucine-rich repeat (NLR) class of immune

receptors [28]. N contains toll/interleukin-1 receptor (TIR) homology domain at the N-terminus [29]. In this chapter, the detailed procedures for TurboID-based investigation of PPIs *in planta* are described, using the interactome analysis of the TIR domain of N NLR immune receptor in *N. benthamiana* as an example [28, 30]. With some recently published studies [26, 27, 31, 32], it is expected that the application of this approach can be extended to other plant species and will serve as a powerful tool for PPIs studies *in planta*.

2 Materials

Prepare solutions using distilled–deionized water (ddH_2O) and analytical-grade reagents. Prepare and store reagents at room temperature (RT) unless indicated otherwise. Diligently follow all waste disposal regulations when disposing of waste materials. Use autoclaved DNase/RNase-free low-binding tubes for all work with proteins.

2.1 Agroinfiltration and Biotin Treatment

1. *N. benthamiana* seeds.
2. Soil (Sunshine® Mix #1, SunGro Horticulture), 3.5′ × 3.5′ pots, and a 23–25 °C growth chamber.
3. *Agrobacterium tumefaciens* strain (GV3101) (*see* **Note 1**).
4. TurboID plasmids (p35S:Citrine-TurboID-3xHA, Addgene #199243; pUBQ:Citrine-TurboID-3xMyc, Addgene #199244).
5. Luria-Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl are dissolved in ddH_2O. Adjust pH to 7.0 and autoclave to sterilize.
6. LB agar plates with appropriate antibiotics.
7. 50 mg/mL kanamycin: Dissolve in ddH_2O, filter-sterilize (0.22 μm filter) and store at −20 °C.
8. 50 mg/mL gentamicin: Dissolve in ddH_2O, filter-sterilize (0.22 μm filter) and store at −20 °C.
9. 50 mg/mL rifampicin: Dissolve in DMSO, filter-sterilize (0.22 μm filter) and store at −20 °C.
10. 1 M 2-(N-morpholine)-ethanesulfonic acid (MES): Dissolve in ddH_2O, adjust pH to 5.6 with NaOH, filter-sterilize (0.22 μm filter), and store at −20 °C.
11. 250 mM acetosyringone (AS): Dissolve in DMSO and store at −20 °C.
12. 1 M $MgCl_2$: Dissolve in ddH_2O. Autoclave to sterilize.

13. Agroinfiltration medium: 10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 250 μM AS.
14. 1-mL Disposable syringe.
15. 50 mM Biotin (Sigma): Dissolve in 100 mM Na_2HPO_4. Adjust pH to 7.2 with 100 mM NaH_2PO_4. Bring to desired volume with 100 mM sodium phosphate buffer (pH 7.2). Filter-sterilize (0.22 μm filter) and store at −20 °C (*see* **Note 2**).

2.2 Protein Extraction and Desalting

1. Liquid nitrogen.
2. Mortar and pestle.
3. 2.0-ml Eppendorf tubes.
4. 15-ml or 50-ml Falcon tubes.
5. 1 M Tris–HCl buffer: Dissolve in ddH_2O. Adjust pH to 7.5. Autoclave to sterilize.
6. 5 M NaCl: Dissolve in ddH_2O. Autoclave to sterilize.
7. 0.5 M stock EDTA: Dissolve in ddH_2O. Adjust pH to 8.0. Autoclave to sterilize.
8. IGEPAL® CA-630.
9. 10% (w/v) SDS in ddH_2O.
10. 10% (w/v) Sodium deoxycholate in ddH_2O. Store in the dark.
11. 100 mM DTT: Dissolve in ddH_2O and store at −20 °C.
12. Complete EDTA-free protease inhibitor cocktail (Roche) or equivalent protease inhibitor cocktail.
13. RIPA lysis buffer: 50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA (pH 8.0), 1% IGEPAL® CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM DTT, 1 × complete EDTA-free protease inhibitor cocktail. Prepare fresh before use.
14. Zeba™ Spin Desalting Columns, 7 K MWCO, 10 mL (Thermo Scientific™): Store at 4 °C (*see* **Note 3**).

2.3 Quantification of Protein

1. 2 mg/mL Bovine Serum Albumin (BSA) Standard Ampules (Pierce™).
2. Coomassie brilliant blue G250.
3. Methanol.
4. 85% phosphoric acid.
5. 5× Bradford regent: 100 mg Coomassie brilliant blue G250, 47 mL of methanol, 100 mL of 85% phosphoric acid, 53 mL of ddH_2O.
6. Test tubes.
7. ELISA plate.

2.4 Enrichment of Biotinylated Proteins

1. Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen™): Store at 4 °C.
2. Magnetic separation rack.
3. 1.5-mL Eppendorf tube.
4. 1 M HEPES (pH 7.5): Dissolve in ddH_2O, adjust pH to 7.5 with NaOH, filter-sterilize (0.22 μm filter), and store at 4 °C.
5. Triton X-100.
6. 8 M LiCl.
7. Wash buffer I: 2% SDS in water.
8. Wash buffer II: 50 mM HEPES (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.1% deoxycholic acid (w/v), 1% triton X-100. Prepare fresh before use.
9. Wash buffer III: 10 mM Tris–HCl 7.5, 250 mM LiCl, 1 mM EDTA, 0.1% deoxycholic acid (w/v), 1% IGEPAL® CA-630. Prepare fresh before use.
10. 50 mM Tris–HCl (pH 7.5): Prepare fresh before use and store at 4 °C.
11. 50 mM Ammonium bicarbonate: Dissolve in ddH_2O, filter-sterilize (0.22 μm filter). Prepare fresh before use and store at 4 °C.

3 Methods

3.1 Growth of Plant

1. Sow *N. benthamiana* seeds at high density (about 40 seeds) into a pot filled with wet soil and place it in a plastic growing tray covered with a transparent humidity dome. Maintain them in a growth chamber with a photoperiod of 16 h light/8 h dark (75 μmol/m^2/s) at 23–25 °C and 40–60% humidity.
2. After 1 week, carefully transfer each germinated seedling to a 3.5′ × 3.5′ pot filled with wet soil and maintain plants in the same growth chamber for 3–4 weeks.

3.2 Plasmid Construction

1. Use a standard molecular cloning method to fuse the gene of interest in-frame with TurboID vectors (Addgene plasmids #199243; #199244). Here, we made a construct expressing TurboID-fused to TIR domain of N NLR immune receptor driven by cauliflower mosaic virus 35S promoter (p35S::TIR-TurboID-3xHA) [28] (*see* **Note 4**).
2. Generate a construct expressing TurboID-fused to citrine driven by the same promoter (p35S::Citrine-TurboID-3xHA) to serve as the control for subsequent quantitative analysis (*see* **Note 5**).

3. Confirm the sequence of all the constructs by Sanger sequencing.

3.3 Agroinfiltration and Biotin Treatment

3.3.1 Agroinfiltration

1. Transform the plasmids from Subheading 3.2 into *A. tumefaciens* strains GV3101 separately [33] (*see* **Note 1**).
2. Pick and streak *Agrobacterium* colony harboring the construct onto a fresh LB agar plate supplemented with appropriate antibiotics to select the plasmid, as well as for the *Agrobacterium* (for GV3101: 50 mg/L gentamicin and 50 mg/L rifampicin) and grow at 28 °C overnight (*see* **Note 6**).
3. Inoculate *Agrobacterium* harboring the construct into 3 mL of LB medium with appropriate antibiotics (*see* **step 2**) and incubate by shaking at 28 °C in a shaker overnight until the OD_{600} reaches approximately 2.0.
4. Collect *Agrobacterium* cells by centrifugation at 3000×*g*, 10 min, and resuspend the pellet in agroinfiltration media to $OD_{600} = 1.0$ (*see* **Note 7**).
5. Use a 1 mL needleless syringe to infiltrate the suspensions into the fully mature leaves of 3- to 4-week-old *N. benthamiana* through the (abaxial) epidermis (*see* **Note 8**).
6. Maintain plants in the growth chamber (*see* Subheading 3.1, **step 1**).

3.3.2 Biotin Treatment

1. At 36 h post-infiltration (hpi) (*see* **Note 9**), infiltrate 200 μM biotin (in 10 mM $MgCl_2$ solution) into the leaves pre-infiltrated with TurboID constructs (*see* **Note 10**).
2. Maintain the plant for an additional 1–12 h in the growth chamber (*see* Subheading 3.1, **step 1**) before sample collection (*see* **Note 11**).

3.4 Protein Extraction

To minimize keratin contamination, the subsequent procedure should be processed in keratin-free condition, wear powder-free sterile gloves, and keep all reagents keratin-free when possible.

1. Cut off the infiltrated leaves from the base of the petiole, remove the leaf vein, and then flash-freeze the tissue in liquid nitrogen (*see* **Note 12**).
2. Grind the collected tissue in liquid nitrogen with a pestle and mortar into a fine powder, and store in 15-mL or 50-mL Falcon tubes at −80 °C for subsequent use. Verify expression and biotinylation of proteins by western blot (*see* **Note 13**). Typical western blot results are shown in Fig. 3.
3. Transfer 0.35 g of tissue powder into a 2.0-mL Eppendorf tube. Prepare two tubes for each sample (0.7 g/sample in total).

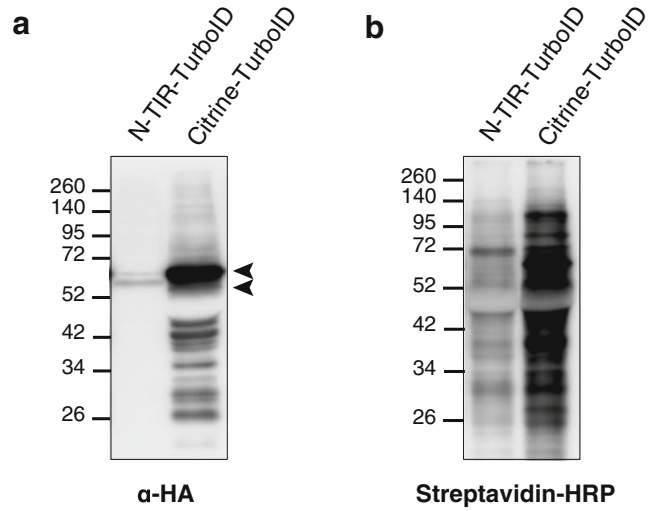

Fig. 3 Immunoblot detection of HA-tagged TurboID-fused proteins (**a**) and biotinylated proteins (**b**) from the sample collected in Subheading 3.4, **step 9**. Arrows indicate the specific band of different HA fusion proteins. (Reproduced from ref. [28] with permission from Nature Springer)

4. Add 700 μL of RIPA lysis buffer to each tube containing 0.35 g tissue powder.
5. Vortex for 10 min to mix thoroughly.
6. Leave the mixture on ice for 30 min. Mix the contents every 4–5 min by turning the tubes upside down several times.
7. Centrifuge at 16,500×*g* for 10 min at 4 °C.
8. Combine the supernatant from two tubes of the same sample and transfer it into a new 2.0-mL Eppendorf tube.
9. Take out 50 μL aliquot and store at −80 °C for subsequent immunoblot analysis of target proteins in the leaf extracts. Keep the rest samples on ice temporarily.

3.5 Removal of Free Biotin Using a Desalting Column

This usually takes 30–50 min. To save time, column equilibration can be done during the protein extraction (Subheading 3.4, **steps 6–9**). The protocol given here is for Zeba™ Spin Desalting Columns.

3.5.1 Equilibrate the Desalting Column

1. Remove the desalting column's bottom sealing material, loosen cap (do not remove cap).
2. Place the column into a collection tube (50-mL Falcon tube) and centrifuge at 1000×*g* for 2 min at 4 °C to remove the storage solution (*see* **Note 14**).
3. Discard flowthrough and replace the column back into the collection tube. Add 5 mL of RIPA lysis buffer on top of the

resin. Centrifuge at 1000×*g* for 2 min at 4 °C and discard the flowthrough.

4. Repeat **step 3** two additional times.
5. Blot the bottom of the column or plate to remove excess liquid. Transfer the column to a new collection 50-mL Falcon tube.
6. Store temporarily at 4 °C.

3.5.2 Desalting the Protein Extracts

1. Apply 1400–1500 μL of protein extract on top of the resin of the equilibrated desalting column (from Subheading 3.4, **step 9**) (*see* **Note 15**).
2. Add a stacker (another 100 μL of RIPA lysis buffer) on top of the resin as soon as the added sample has completely entered the resin (*see* **Note 16**).
3. Centrifuge at 1000×*g* for 2 min at 4 °C.
4. Keep the flowthrough (desalted protein extracts) on ice temporarily. Discard the column.

3.6 Quantification of the Desalted Protein Extracts Using a Bradford Assay (See Note 17)

1. Prepare a set of diluted BSA standards (50 μL each): 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1 mg/mL.
2. Dilute the desalted protein extract by mixing 5 μL of the sample with 45 μL of ddH_2O.
3. Prepare 1 × Bradford regent by diluting the 5 × Bradford regent with ddH_2O.
4. Pipette 50 μL of each BSA standard and diluted protein extract into an appropriately labeled test tube.
5. Add 2.5 mL of 1 × Bradford regent into each tube and mix well.
6. Incubate at RT for 10 min.
7. Add 200 μL of solution from each tube to a well of the ELISA plate (three technical replicates per sample).
8. Measure the 562 nm absorbance using a microplate reader (*see* **Note 18**).
9. Prepare the standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in μg/mL. Use the standard curve to calculate the concentration of each desalted protein sample (*see* **Note 19**).
10. Use 6–8 mg of desalted protein extract for subsequent biotinylated protein enrichment.

3.7 Enrichment of Biotinylated Proteins by Streptavidin Beads

3.7.1 Prewash Streptavidin-C1-Conjugated Magnetic Beads

1. Resuspend the streptavidin-C1-conjugated magnetic beads in the vial (i.e., vortex for >30 s, or tilt and rotate for 5 min).
2. For each sample, transfer 200 μL of streptavidin-C1-conjugated magnetic beads into a 2.0-mL Eppendorf tube.
3. Add 1 mL of RIPA lysis buffer to suspend the beads.
4. Place tubes on a magnetic separation rack for 3 min and gently remove the solution by pipette.
5. Remove the tube from the magnet and add 1 mL of RIPA lysis buffer to resuspend the beads.
6. Repeat **steps 4** and **5** for a total of three washes.

3.7.2 Enrichment of Biotinylated Proteins

1. Transfer each desalted protein extract to a 2.0-mL Eppendorf tube containing prewashed equilibrated streptavidin-C1-conjugated magnetic beads.
2. Incubate at 4 °C for 12–16 h (or overnight) with end-over-end rotation.
3. Separate the beads with a magnetic separation rack for 4 min at RT until the beads collect at one side of the tube, and then gently remove the supernatant by pipette (*see* **Note 20**).
4. Add 1.7 mL of wash buffer I into each tube and wash with end-over-end rotation at RT for 8 min. Repeat **step 3**.
5. Add 1.7 mL of wash buffer II into each tube and wash with end-over-end rotation at RT for 8 min. Repeat **step 3**.
6. Add 1.7 mL of wash buffer III into each tube and wash with end-over-end rotation at RT for 8 min. Repeat **step 3**.
7. Add 1.7 mL of 50 mM Tris–HCl (pH 7.5) into each tube and wash with end-over-end rotation at RT for 8 min. Repeat **step 3**.
8. Add 1 mL of 50 mM Tris–HCl (pH 7.5) into each tube, resuspend beads, and transfer to a new 1.5-mL Eppendorf tube. Wash with end-over-end rotation at RT for 8 min. Repeat **step 3**.
9. Add 1 mL of 50 mM ammonium bicarbonate buffer into each tube and wash with end-over-end rotation at 4 °C for 5 min.
10. Separate the beads with a magnetic separation rack for 4 min at 4 °C until the beads collect at one side of the tube, and then gently remove the supernatant by pipette.
11. Repeat **steps 9** and **10** five more times, for a total of six times wash with 50 mM ammonium bicarbonate buffer.
12. Resuspend beads with 1 mL of 50 mM ammonium bicarbonate buffer.

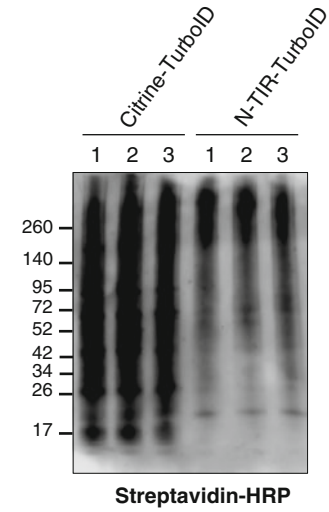

Fig. 4 Immunoblot analysis of the biotinylated proteins captured on Streptavidin beads in Subheading 3.7.2, **step 13** to confirm the enrichment of the biotinylated proteins. There are three independent replicates for each protein (reproduced from ref. [28] with permission from Nature Springer). Generally, citrine expresses at higher level, and hence, there is more cis-biotinylation in Citrine-TurboID samples

13. Take out 100 μL of beads for immunoblot analysis to confirm the enrichment of biotinylated proteins. A typical western blot is shown in Fig. 4.
14. Flash-freeze the rest of the protein samples and store them at −80 °C or send them immediately for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis on dry ice (*see* **Note 21**).

4 Notes

1. Other *Agrobacterium* strains, such as EHA105, GV2260, and C58CI, can also be used.
2. Biotin is a carboxylic acid, and as such it is less soluble under acidic conditions, but more soluble in DMSO and dilutes alkali. Biotin stock can also be prepared in DMSO.
3. PD-10 columns (GE Healthcare) can also be used for free biotin removal (*see* ref. [26]). It will be better to compare the efficiency of these columns.
4. The TurboID fusion should not interfere with the function or localization of the protein. Previously characterized functional fusion with fluorescent protein or epitope tag can be a promising indicator of whether TurboID should be fused to

the C- and N-terminus of protein. Usually, for cytoplasmic proteins, both termini should be acceptable. However, for membrane-localized proteins, the protein topology needs to be characterized in advance. If unsure, make and test both versions.

5. It is important to include a TurboID fusion control for validating the proximal proteins to the protein of interest. The control should be expressed at a level similar to that of the TurboID-fused target protein. This can be empirically determined by adjusting the *Agrobacterium* concentration during agroinfiltration. In addition, it is important that the control protein and the target protein of interest share a similar subcellular localization pattern or at least the control protein's subcellular distribution covers or encompasses the space that the TurboID fusion touches.
6. It is more optimal to confirm the presence of the specific binary construct in the *Agrobacterium* by colony PCR.
7. Although it is optimal to incubate the inoculum in the infiltration media for 2–3 h prior to agroinfiltration at RT, based on our experience with GV3101, there is no difference between the protein expression with and without preincubation. The preincubation requirement for other *Agrobacterium* strains needs to be verified.
8. To prepare enough leaf materials for three biological replicates, we suggest infiltrating three to four plants per construct. Each plant has three entire leaves infiltrated. For each leaf, 1.5–2.0 mL of resuspended *Agrobacterium* should be sufficient.
9. The reason for picking 36 hpi for biotin infiltration is that, according to our previous studies [28], the protein of interest reaches its peak expression at this time point. It is recommended to determine the time required for optimal expression of the protein of interest.
10. A comparable biotinylation level of TurboID-fused bait protein and its proximate proteins should be achieved with the addition of 50–200 μM biotin. It is reported that increasing the biotin concentration from 50 μM to 2 mM adversely affected the capture of proximate proteins, as only the bait itself could be identified. This is probably due to high residual free biotin accumulated in the protein extract even after desalting, thus occupying the streptavidin binding sites on the beads (*see* ref. [26]).
11. The incubation time post-biotin infiltration depends on the features of the bait protein and the experimental design. Usually, longer biotin incubation time clearly increases the robustness of PL, but 1–12 h of biotin treatment should be sufficient

for the labeling of most proteins proximal to the bait protein by the TurboID fusions.

12. Harvest three to four pieces of leaves for each biological replicate from different plants. Wear gloves when handling liquid nitrogen or operating any object cooled by liquid nitrogen.
13. The protocol can be paused here. Prior to subsequent steps, it is recommended to verify protein expression and biotinylation of the target protein by immunoblot analyses.
14. After each spin, the resin should appear white and free of liquid. If a liquid is present, make sure you are using the correct centrifugation speed and time. Some models of centrifuge will time down right after pushing the "Start" button, but here requires timed runs at speed. Incomplete centrifugation may result in poor sample recovery or sample dilution.
15. A combination of the samples from each group, as described in Subheading 3.4 can result in at least 1500 μL of protein extract per sample, as the total volume after protein extraction was invariably increased to some extent relative to the original volume of added RIPA lysis buffer.
16. Adding a stacker is optional but recommended for dilute protein solutions or small sample volumes to ensure maximum sample recovery.
17. Other protein quantification methods, such as BCA, can also be used.
18. A spectrometer with an appropriate wavelength range combined with the use of cuvettes can be an alternative for measuring 562 nm absorbance.
19. Usually, the total protein concentration obtained from 0.7 g of leaves ranges from 3 to 6 mg/mL.
20. Collect beads from the lid of the tubes by brief centrifugation each time before placing them on the magnetic separation rack.
21. Typical MS results can be found in our previous publication (*see* ref. [28] Fig. 2, Supplementary Data 1, and Supplementary Data 2 in ref. [28]).

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