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Chapter 8

Protein–Protein Interaction Assays with Effector–GFP Fusions in *Nicotiana benthamiana*

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Abstract

Plant parasites secrete proteins known as effectors into host tissues to manipulate host cell structures and functions. One of the major goals in effector biology is to determine the host cell compartments and the protein complexes in which effectors accumulate. Here, we describe a five-step pipeline that we routinely use in our lab to achieve this goal, which consists of (1) Golden Gate assembly of pathogen effector–green fluorescent protein (GFP) fusions into binary vectors, (2) *Agrobacterium*-mediated heterologous protein expression in *Nicotiana benthamiana* leaf cells, (3) laser-scanning confocal microscopy assay, (4) anti-GFP coimmunoprecipitation–liquid chromatography–tandem mass spectrometry (coIP/MS) assay, and (5) anti-GFP western blotting. This pipeline is suitable for rapid, cost-effective, and medium-throughput screening of pathogen effectors *in planta*.

Key words Agroinfiltration, Live-cell imaging, Affinity chromatography, DNA assembly, Proteomics

1 Introduction

Over the last decade, postgenomic analyses of eukaryotic plant pathogens—such as nematodes, fungi, oomycetes, and aphids—revealed hundreds of effector proteins [1–4]. Although effectors are encoded by pathogen genomes, they are operationally plant proteins, i.e., they function and have a phenotypic expression in plant tissues [5]. One challenge in effector biology is to study effectors *in planta* [6]. In many plant species this task is challenging due to the lack of transient transformation method. To circumvent this obstacle, scientists often use the model plant *Nicotiana benthamiana* primarily because it enables transient expression of effectors in leaf cells [3].

N. benthamiana is a dicot plant used as a model in plant biology. Notably, the infiltration of leaves with solutions of *Agrobacterium tumefaciens* carrying a binary vector—the so-called *Agrobacterium*-mediated transient expression or agroinfiltration assay—allows rapid expression of proteins in leaf cells [7, 8].

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If the protein expressed is fused to a fluorescent protein tag, such as the green fluorescent protein (GFP) for instance, it is then possible to combine two different assays. Firstly, a live-cell imaging assay—by using a laser-scanning confocal microscope—can be carried out to identify the subcellular compartment in which the effector–GFP fusion accumulates [9]. Secondly, a protein–protein interaction assay—by using anti-GFP coimmunoprecipitation–liquid chromatography–tandem mass spectrometry (coIP-LC/MS-MS or coIP/MS)—can be performed to identify the plant protein complexes with which the effector–GFP fusion associates [10–12].

Here, we detail the five-step pipeline that we routinely use in our lab to identify the leaf cell compartments and protein complexes in which effectors accumulate. This pipeline is aimed at achieving fast-forward screening of medium-sized effector sets in a cost-effective manner.

2 Materials

2.1 Golden Gate DNA Assembly

1. *Escherichia coli* subcloning efficiency DH5α competent cells. Store 50 μL aliquots at −80 °C.
2. Digestion/Ligation mix 1: 1 μL BbsI restriction enzyme, 1 μL T4 DNA ligase, 2 μL Bovine Serum Albumin (1 mg/mL stock solution), 4 μL T4 Ligase buffer, 1 μL Golden Gate Level 0 acceptor vector pICSL01005 (50 ng/μL in ddHOH stock solution), 6 μL ddHOH. Prepare right before use.
3. Digestion/Ligation mix 2: 1 μL BsaI-High fidelity restriction enzyme, 1 μL T4 DNA ligase, 2 μL BSA, 4 μL T4 Ligase buffer, 1 μL Golden Gate Level 1 binary acceptor vector (CaMV 35S promoter, OCS terminator, 50 ng/μL in ddHOH stock solution), 1 μL Golden Gate C-terminal GFP tag module vector pICSL50008 (50 ng/μL in ddHOH stock solution), 9 μL ddHOH. Prepare right before use.
4. Blue/White selection mix: 1/1 (v/v) 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)/20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside (X-Gal) in DiMethyl-Formamide (DMF). Prepare right before use.
5. Luria–Bertani (LB) growth medium: 10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl. Adjust pH to 7.0 with NaOH. Add 20 g/L Agar for solid medium. Autoclave and store at room temperature up to a month. Supplement with appropriate combination of antibiotics at the following final concentration: 100 μg/mL Carbenicillin (1000× stock solution), 50 μg/mL Kanamycin (1000× stock solution), 100 μg/mL Spectinomycin (1000× stock solution in 50% Dimethyl sulfoxide [DMSO]), 100 μg/mL Rifampicin (100× stock solution in Methanol).

2.2 Vector Insertion into A. tumefaciens and Infiltration of N. benthamiana Leaves

1. *A. tumefaciens* electrocompetent strain GV3101 (pMP90). Store 50 µL aliquots at −80 °C (*see* **Note 1**).
2. *N. benthamiana* 3- to 5-week-old plants grown at 22 °C in a glasshouse with 16 h day and 8 h night cycles.
3. MicroPulser Electroporator.
4. 2 mm gap electroporation cuvette.
5. Agroinfiltration buffer: 10 mM $MgCl_2$ (100× stock solution), 150 µm Acetosyringone (3333× stock solution in DMSO).

2.3 Laser-Scanning Confocal Microscopy

1. Leica DM6000B/TCS SP5 laser-scanning confocal microscope equipped with a 488 nm laser line, a 10× air, and a 63× water-immersion objectives.
2. Super Premium 1.2 mm microscope slides.
3. 17 µm-thick 22 × 50 mm cover glass.

2.4 Protein Isolation and Anti-GFP Immunoprecipitation

1. Ultrasonic cleaner.
2. Miracloth.
3. 0.22 µm syringe filter.
4. 5 mL syringe.
5. 30 mL ultracentrifuge tube.
6. GFP_Trap_A beads. Store at 4 °C.
7. Immunoprecipitation buffer: 10% (v/v) glycerol, 25 mM Tris–HCl pH 7.5 (40× stock solution), 1 mM Ethylenediaminetetraacetic (EDTA, 500× stock solution), 150 mM sodium chloride (NaCl, 33× stock solution), 0.1% Tween 20. Store at 4 °C up to a week.
8. Protein isolation buffer: Immunoprecipitation buffer, 2% (w/v) Polyvinylpyrrolidone (PVPP), 10 mM dithiothreitol (DTT, 100× stock solution), protease inhibitor cocktail (100× stock solution). Prepare right before use.
9. Laemmli buffer: 0.5 M Tris–HCl pH 6.8, 50 mM DTT, 2% [w/v] sodium dodecylsulfate [SDS], 20% glycerol, 0.0001% [w/v] bromophenol blue. Store at 4 °C up to a month (*see* **Note 2**).

2.5 Western Blotting

1. ImageQuant LAS 4000 luminescent imager.
2. Trans-Blot Turbo transfer machine.
3. Trans-Blot Turbo Mini PVDF Transfer Pack.
4. A4 transparency film.
5. Single step GFP (B2): sc-9996 horseradish peroxidase (HRP)-conjugated antibody.

6. Tris Buffer Saline (TBS): 24.2 g/L Tris, 80 g/L NaCl, adjust pH to 7.6 with HCl (10×).
7. TBS-T: TBS (1×), 0.1% Tween 20.
8. Blocking buffer: 3% [w/v] BSA in TBS-T.
9. Probing solution: 1/5000 single step GFP (B2): sc-9996 horseradish peroxidase (HRP)-conjugated antibody in TBS-T. Prepare right before use (*see* **Note 3**).
10. Pierce ECL Western Blotting substrate: 1/1 [v/v] Luminol/Peroxidase.
11. SuperSignal West Femto Maximum Sensitivity Substrate: 1/1 [v/v] Luminol/Peroxidase.
12. Revelation buffer: 19/1 [v/v] Pierce ECL Western Blotting substrate/SuperSignal West Femto Maximum Sensitivity Substrate.
13. Ponceau S solution: 0.1% [w/v] Ponceau S, 5% [v/v] Acetic Acid.

2.6 Protein Separation by SDS-PAGE, Gel Excision, and Trypsin Digestion

1. Mini-Protean electrophoresis system.
2. EZ-2 Genevac evaporator.
3. TGX Precast polyacrylamide gels.
4. Prestained Plus protein ladder.
5. Instant Blue.
6. Protein low binding 1.5 mL centrifuge tubes.
7. 10× SDS-PAGE running buffer: 30 g/L Tris base, 144 g/L glycine, 10 g/L SDS.
8. ABC buffer: 50 mM Ammonium Bicarbonate in ultrapure water.
9. Gel destaining solution: 1/1 [v/v] ABC buffer/100% Acetonitrile (ACN).
10. Reduction solution: ABC buffer, 10 mM DDT.
11. Alkylation solution: ABC buffer, 55 mM Chloroacetamide (CAM).
12. Trypsin buffer: 100 ng/μL Trypsin, 5% [v/v] ACN, 50% [v/v] ABC buffer.
13. Peptide extraction buffer: 5% [v/v] formic acid (FA), 45% [v/v] ultrapure water, 50% [v/v] ACN.

2.7 LC-MS/MS

1. Orbitrap Fusion trihybrid mass spectrometer in positive ion mode.
2. Nanoflow-UHPLC system Dionex Ultimate 3000.

3. Reverse phase trap column Acclaim PepMap, C18 5 μm, 100 μm × 2 cm connected to an analytical column Acclaim PepMap 100, C18 3 μm, 75 μm × 50 cm.
4. Nano-electrospray ion source with ID 0.02 mm fused silica emitter.
5. Mobile phase A: 3% ACN, 0.1% FA.
6. Mobile phase B: 80% ACN, 0.1% FA.

2.8 In Silico Data Analysis

1. Fiji (https://fiji.sc/).
2. Excel.
3. Scaffold v.4 (Proteome Software).
4. Mascot (Matrix Science).
5. Perl (https://www.perl.org/).
6. Blastclust program from Blast standalone program (http://blast.ncbi.nlm.nih.gov/).
7. TextWrangler (Bare Bones Softwares).
8. MSConvert package (Matrix Science).

3 Methods

We obtain the coding sequence of effectors by PCR cloning or by gene synthesis with codon optimization for expression in *N. benthamiana* and removal of BbsI and/or BsaI restriction sites if necessary. During this process, we replace the sequence coding the predicted signal peptide by the following nucleotides: CACCGAAGACACAATG. GAAGAC is the BbsI restriction site, AATG is the overhang for Golden Gate assembly with a promoter, the last three nucleotides (ATG) are the start codon. We also replace the stop codon by the following nucleotides: ggTTCGCCGTCTTCGTAG. GTCTTC is the BbsI restriction site, TTCG is the overhang for Golden Gate assembly with the coding sequence of a C-terminal tag. For more information about the Golden Gate DNA assembly method in plant biology, we refer readers to these recent reviews [13, 14].

3.1 DNA Assembly into Golden Gate Vectors

1. Mix 5 μL of purified PCR products or 1 μL of plasmid with a synthesized DNA fragment with the Digestion/Ligation mix 1. Incubate in a thermocycler with the following program: 20 × (4 min at 37 °C, 4 min at 16 °C), 10 min at 50 °C, 10 min at 80 °C.
2. Thaw a 50 μL-aliquot of *E. coli* DH5 α thermocompetent cells on ice for 5 min and add 5 μL of digestion/ligation reaction product from **step 1**. Keep on ice for 30 min.

3. Insert plasmids into *E. coli* cells by incubating at 42 °C for 20–30 s in a waterbath.
4. Keep the bacteria on ice for 1 min, then add 500 µL of LB liquid medium preheated at 37 °C and incubate at 37 °C for 90 min.
5. Spread 100 µL of Blue/White selection mix on a plate with LB-Agar supplemented with Spectinomycin. Keep for 1 h at 37 °C.
6. Plate 200 µL of bacteria and keep at 37 °C for 16–24 h.
7. Select 3–5 white bacterial colonies and verify the presence of the recombinant vector by colony PCR (*see* **Note 4**).
8. Grow bacteria from one colony PCR-positive colony in 10 mL liquid LB supplemented with Spectinomycin at 37 °C in a shaking incubator for 16–24 h.
9. Purify the plasmid, adjust it to 50 ng/µL in ddHOH, and store at −20 °C for further use or proceed directly to next step.
10. Mix 1 µL of plasmid with the Digestion/Ligation mix 2. Incubate in a thermocycler with the program described at **step 1**.
11. Repeat **steps 2–9** but replace Spectinomycin by Kanamycin.

3.2 Vector Insertion into A. tumefaciens Strains and Infiltration of N. benthamiana Leaves

1. Thaw a 50 µL-aliquot of *A. tumefaciens* competent cells on ice.
2. Add 0.2 µL of plasmid (*see* Subheading 3.1, **step 11**) to the cells into a 2 mm-gap electroporation cuvette.
3. Electroporate with a micropulser with the following setting: capacitance of 25 µF, voltage of 2.4 kV, resistance of 200 Ω (*see* **Note 5**).
4. Add 0.5 mL of liquid LB medium preheated at 28 °C and incubate at 28 °C for 1 h.
5. Plate 200 µL of bacteria on LB-Agar medium supplemented with Rifampicin and Kanamycin. Incubate at 28 °C for 36–48 h.
6. Select 3–5 colonies and verify the presence of the recombinant vector by colony PCR (*see* **Note 5**).
7. Grow bacteria from one PCR-positive colony in 10 mL liquid LB medium supplemented with Rifampicin and Kanamycin at 37 °C in a shaking incubator for 16–24 h.
8. Mix 1 mL of bacteria with 500 µL of 60% glycerol in a 2 mL centrifuge tube. Invert five times and store at −80 °C. Centrifuge the remaining 9 mL of culture at 4000 × *g* for 10 min (*see* **Note 6**).
9. Resuspend the bacterial pellet in 10 mL Agroinfiltration buffer and adjust to OD_{600} of 0.1–0.4. Keep on ice for 1 h to activate the bacteria.

10. Infiltrate 2–4 leaves from ranks 3–5 (starting from top) of three to five week-old *N. benthamiana* plants with a 1-mL syringe without needle (*see* **Note 7**).
11. Harvest the leaves 2–3 days after infiltration and transport/keep them in a plate with high humidity (*see* **Note 8**).
12. Cut 2–3 leaf stripes of approximately 2 mm by 10 mm per leaf for immediate use for confocal microscopy (*see* Subheading 3.3).
13. Snap-freeze the leaf in liquid nitrogen, and store at −80 °C or proceed further immediately (*see* Subheadings 3.4 and 3.5) (*see* **Note 9**).

3.3 Laser-Scanning Confocal Microscopy

1. Mount a leaf stripe (*see* Subheading 3.2, **step 12**) in ddHOH between slide and cover glass, with the lower epidermis toward the objective. Remove air bubbles from the mounting by gently tapping the cover glass.
2. Place the mounting on the microscope and set up the microscope with the following parameters: laser line: 488 nm at 15% power; receptor 1: collection from 505 to 530 nm, 100% gain (GFP fluorescence); receptor 2: collection from 680 to 700 nm, 50% gain (chlorophyll autofluorescence); receptor 3: bright field; scanning frequency: 400 Hz; image resolution: 1024 × 512 pixels; line average: 4 (*see* **Note 10**).
3. Adjust the focus to epidermal cells, and screen the leaf using the 10× objective to select a region of interest with pavement cells that show detectable accumulation of the GFP signal and no sign of stress (*see* **Note 11**).
4. Use the 63× water-immersion objective to perform high magnification imaging (*see* **Note 12**).
5. Repeat **steps 1–4** with leaf stripes from other leaves.
6. Save data in .lif format.
7. Use the software Fiji to read the .lif file, perform post-treatment as needed, and export final images as .png or .tif files.

3.4 Anti-GFP Immunoblotting

1. Grind leaf tissues (*see* Subheading 3.2, **step 13**) into powder in liquid nitrogen using mortar and pestle. Transfer the leaf powder into a 50-mL centrifuge tube.
2. Use a 1 mL pipet tip with the narrow extremity bent on approx. 0.5 cm to pick up a small amount of leaf powder. Resuspend the powder in 100 μL Laemmli buffer in a 1.5 mL centrifuge tube and immediately incubate at 95 °C under vigorous agitation for 15 min. Keep the 50 mL tube with the remaining powder at −80 °C up to a week or proceed further directly (*see* Subheading 3.5).
3. During **step 2**, set up a precast gel in the Mini Protean device following manufacturer's instructions. Poor 1 L of SDS-PAGE

running buffer in the upper and lower chamber up to the indicated levels 5 min before starting the electrophoresis.

4. Centrifuge at 15,000 × *g* for 5 min.
5. Transfer the supernatant in a new 1.5 mL centrifuge tube. Add 100 µL of Laemmli and incubate at 95 °C for 10 min.
6. Centrifuge at 15,000 × *g* for 5 min. Transfer the supernatant in a new tube.
7. Load 10 µL of protein mixture on the gel. Load 5 µL of Prestained Page ruler in the wells at each extremity of the sample(s) (*see* **Note 13**).
8. Start the electrophoresis at 120 V for 5 min, then increase to 160 V. Stop the electrophoresis when the migration front reaches the bottom of the gel.
9. Disassemble the cassette and incubate the gel two min in ddHOH.
10. Transfer the proteins to the PVDF membrane using the Trans-Blot Turbo machine and kit following manufacturer's instructions (*see* **Note 14**).
11. Incubate the membrane in TBS-T for 2 min.
12. Block the membrane in 15 mL of Blocking Buffer for 1 h under gentle rotating agitation.
13. Incubate the membrane in 15 mL of Probing Solution 1 h under gentle rotating agitation.
14. Wash the membrane five times with 15 mL of TBS-T for 1 min. Then wash the membrane with 15 mL of TBS for 1 min.
15. Cover the membrane with the 2 mL of Revelation Solution for 3 min.
16. Remove the excess of solution and place the membrane between two transparency films. Position the montage in the ImageQuant LAS 4000 luminescent imager, with the side of the membrane facing the camera objective.
17. Expose the membrane for 30 s using the following settings: chemiluminescence, tray position 1, precision, high sensitivity (*see* **Note 15**).
18. Save data as a .gel file. Adjust image brightness/contrast and save the image as an 8-bit .tif file.
19. Wash the membrane one minute with ddHOH. Stain the proteins by incubating the membrane in Ponceau S solution for 30 min. Wash the membrane with ddHOH for 1 min. Place the membrane between two transparency films and scan it with a standard scanner (*see* **Note 16**).

3.5 Protein Isolation and Anti-GFP Immunoprecipitation (IP)

1. Grind leaf tissues (*see* Subheading 3.2, **step 13**) into powder in liquid nitrogen using a mortar and a pestle. Transfer the leaf powder into a 50-mL centrifuge tube.
2. Weight leaf powder and resuspend it into 300% [v/w] ice-cold Protein Isolation Buffer. Vortex and shake vigorously for 30 s or until the powder is completely thawed and heterogeneously in solution (*see* **Note 17**).
3. Sonicate the samples at 4 °C for 15 min in a waterbath sonicator, using maximal sonication parameters.
4. Centrifuge at 5000 × *g* at 4 °C for 20 min. Filter the supernatant through a four-layered piece of Miracloth and transfer it into a 30-mL ultra-centrifuge tube.
5. Centrifuge at 50,000 × *g* at 4 °C for 90 min. Collect 10 mL of the filtered solution into a 15-mL centrifuge tube.
6. During step 5, pipet GFP_trap beads with a 1 mL tip with a cut extremity into a 1.5 mL tube. Use 30 μL of beads per sample.
7. Equilibrate the GFP_trap beads into IP buffer by adding 1 mL of IP Buffer, inverting for 1 min, centrifugating at 800 × *g* for 1 min, and discarding the supernatant. Repeat two more times. Keep the beads into 1 mL of IP buffer before use.
8. Mix the equivalent of the initial 30 μL of beads from **step 6** with the 10 mL of protein solution from **step 5**. Incubate at 4 °C with gentle inversion for 30 min (*see* **Note 18**).
9. Centrifuge at 800 × *g* at 4 °C for 5 min. Discard the supernatant without disturbing the pellet, resuspend the pellet into 1 mL of IP Buffer and transfer to a new 1.5 mL centrifuge tube.
10. Centrifuge at 800 × *g* for 30 s. Discard supernatant without disturbing the pellet and resuspend the pellet into 1 mL of IP Buffer.
11. Repeat **step 10** four more times.
12. Centrifuge at 800 × *g* for 30 s. Discard supernatant without disturbing the pellet and resuspend the pellet into 200 μL of IP buffer.
13. Centrifuge at 800 × *g* for 30 s. Discard the supernatant. Add 50 μL of Laemmli buffer to the beads and incubate at 70 °C for 15 min in a heating block under vigorous agitation.
14. Centrifuge at 800 × *g* for 1 min. Transfer the supernatant into a new 1.5 mL centrifuge tube.
15. Centrifuge at 15,000 × *g* for 5 min. Transfer the supernatant into a 1.5 mL centrifuge tube and proceed further directly (*see* Subheading 3.6) or keep at −20 °C.

3.6 Protein Separation by SDS-PAGE, Gel Excision, and Trypsin Digestion

1. Set up a precast gel in the Mini Protean device following manufacturer's instructions.
2. Poor 1 L of SDS-PAGE running buffer in the upper and lower chamber up to the indicated levels 5 min before starting the electrophoresis.
3. Load 15 µL of protein solution (*see* Subheading 3.5, **step 15**) in one well. Load 5 µL of Prestained Page ruler in the wells at each extremity of the sample(s).
4. Start the electrophoresis at 120 V for 5 min, then increase to 160 V. Stop the electrophoresis when the migrating front is approximately 3 cm away from the wells.
5. Disassemble the cassette and incubate the gel in ddHOH for 2 min.
6. Stain the gel in 20 mL of Instant Blue for 30 min under gentle rotating agitation.
7. Wash the gel with ddHOH for 1 min, then incubate the gel in 20 mL of 10% EtOH overnight to ensure destaining.
8. Transfer the gel to a fresh 10% EtOH solution. Cut up to five gel slices and store them in 10% EtOH in 1.5 mL protein low binding centrifuge tubes. Cut small gel slices for each major protein band, and larger slices for gel sections that do not show a band signal. Gel slices can be stored at −20 °C.
9. Incubate gel slices in destaining Solution for 2 × 30 min.
10. Destain gel slices in the Destaining Solution at 25 °C for 30 min under vigorous shaking. Repeat until gel pieces are completely colorless (*see* **Note 19**).
11. Dry the gel pieces in 100% ACN for 10 min and remove supernatant.
12. Reduce Cysteine residues by incubating the gel pieces in the Reducing Solution at 25 °C for 30 min with gentle agitation. Make sure that gel pieces are well-covered by the solution. Then remove supernatant.
13. Alkylate Cysteine residues by incubating the gel pieces in the Alkylation Solution in the dark at room temperature for 30 min. Make sure gel pieces are well-covered by the solution.
14. Wash the gel pieces in the destaining Solution for 2 × 10 min.
15. Dry the gel pieces in 100% ACN for 10 min.
16. Cover the gel pieces with the Trypsin Solution. When the gel pieces are fully rehydrated (i.e., they are transparent), cover them with ABC Buffer and incubate overnight at 37 °C.
17. Add one volume of Peptide Extraction Buffer, vortex for 10 s and sonicate for 10 min.

18. Transfer the supernatant to a 1.5 mL protein low binding centrifuge tube.
19. Cover the gel pieces from **step 17** with Peptide Extraction Buffer, vortex for 10 s and sonicate for 10 min.
20. Transfer supernatant to the 1.5 mL tube from **step 18**.
21. Evaporate the peptide solution at 30 °C in an evaporator with the HPLC setting until all the liquid is evaporated.

3.7 LC-MS/MS and Peptide Search

1. Trap peptides to the reverse phase trap column.
2. Elute peptides using a 3–30% ACN gradient over 50 min, followed by a 6 min gradient of 30–80% ACN at a flow rate of 300 nL/min at 40 °C.
3. Operate the mass spectrometer in positive ion mode, apply a spray voltage of +2200 V, with transfer capillary temperature set to 275 °C. Use a scan resolution of 120,000 at 400 *m/z*, range 300–1800 *m/z*, automatic gain control set 2e5, and maximum inject time to 50 ms. In the linear ion trap, use data dependent acquisition method with "top speed" and "most intense ion" settings to trigger MS/MS spectra. Use the Universal Method (above 100 counts, rapid scan rate, maximum inject time to 500 ms) to set the threshold for collision induced dissociation (CID) and HCD. Set dynamic exclusion to 30 s. Allow charge state between 2+ and 7+ to be selected for MS/MS fragmentation.
4. Prepare peak lists in .mgf format from raw data using the MSConvert package.
5. Search peak lists on Mascot server against a in-house *N. benthamiana* database and a separate in-house constructs database and an in-house contaminants database. Allow in the search tryptic peptides with up to two possible miscleavage and charge states +2, +3, +4. Include the following modifications in the search: oxidized methionine as variable modification and carbamidomethylated cysteine as static modification. Search data with a monoisotopic precursor and fragment ions mass tolerance of 10 ppm and 0.6 Da, respectively.
6. Combine Mascot results in Scaffold and export in Excel.

3.8 Protein Merging and Removal of Contaminants

1. Extract the sequences of putative interactors ("Accession Number" column) reported in the Excel spreadsheet exported from Scaffold (*see* Subheading 3.7, **step 6**) into a .fasta file.
2. Cluster sequences that have at least 80% identity over 80% of their length using the blastclust program. The command line to use is as follows: blastclust -i infile -o outfile -L 0.8 -S 80 -e F. The output of this program is a text file containing each cluster per line of protein identifiers separated by spaces.

3. Select a representative sequence (usually the longest sequence) for each cluster and create a "lookup table" in the spreadsheet to be used in next step.
4. Replace all the interactors belonging to a cluster with just one representative sequence and its description using "vlookup" function of the spreadsheet program and the lookup table created above.
5. Consolidate the interactors by adding the peptide hits for sequences in the same group.
6. Remove any rows containing the word "Decoy" in the spreadsheet. These are added by spectral search programs as internal controls.

4 Notes

1. To prepare competent cells, keep a liquid culture (OD_{600} at 0.5–0.7) 30 min on ice, then wash twice with ice-cold 10% glycerol by centrifugation (3000 × *g* for 15 min). Prepare 50 μL aliquots in 10% glycerol and store at −80 °C.
2. Do not try to weight the bromophenol blue powder. Rather, scratch it with a 1 mL pipet tip and dip it in the Laemmli buffer, which should instantly become blue.
3. Spin the antibody solution before using it to pellet precipitates. Also, it is possible to collect the antibody solution after use and keep it at −20 °C, to be reused up to two times.
4. Standard reactions result in 10–200 colonies, 80–100% being white. A lower rate of white colonies indicates a low efficiency of the digestion/ligation reaction. Over 95% of the white colonies are usually positive for the colony PCR screening. Primers for the colony PCR are plCSL01005_For: GTCTCA TGAGCGGATACATATTTGAATG and plCSL01005_Rev: CGTTATCCCCTGATTCTGTGGATAAC (primers amplify 350 nt in addition of the effector coding sequence), and pICH86988_For: GGACACGCTCGAGTATAAGAGCTC and pICH86988_Rev: GGATCTGAGCTACACATGCT-CAGG (primers amplify 190 nt in addition of the effector-GFP coding sequence).
5. Electroporation time is usually between 5 and 6 ms. Smaller time will decrease the electroporation efficiency.
6. Use the glycerol stock to start a fresh liquid culture or plate when needed. If using a plate culture, it is recommended to wash once the bacteria by resuspension–centrifugation in Agroinfiltration buffer before proceeding to leaf infiltration, in order to remove the excess of antibiotics.

7. Perform this task during the light cycle to ensure maximal opening of the stomata and optimal infiltration.
8. Achieve high humidity by placing a humid piece of paper roll at the bottom of the plate.
9. When using large leaves, cut out petiole and main nerves before snap-freezing.
10. All settings must be fine-tuned for each sample. As a general recommendation, keep the gain and the laser power as low as possible, the scanning frequency as fast as possible, and the windows for fluorescence collection as narrow as possible.
11. Sign of stress includes autofluorescence and packing of chloroplasts, irregular cell shapes, and bright artefactual light signal. Avoid regions near the edge of the sample. Guard cells are not transformed in agroinfiltration assays and should therefore show no fluorescence.
12. We recommend performing z-stack imaging as often as possible as they allow to better appreciate the tridimensional context of the sample.
13. Always keep the loaded volume as low as possible to avoid migration artifacts and cross-contamination between wells.
14. Make sure to select the transfer mode according to the size of the protein of interest. Disassemble the stack as soon as the transfer finishes.
15. Adjust exposure time from 10 s to 1 h according to the intensity of the signal.
16. Use the intensity of the band signal at 55 kDa to control the equal loading and transfer of the proteins.
17. The more powder you use the longer it takes to thaw it. Multiple steps of 30 s vortexing might be necessary. Keep the tubes on ice 1 min between each vortexing.
18. Resuspend the beads to homogeneity by gently tapping the tube before use.
19. If gel pieces still show some coloration, increase temperature to 55 °C.

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