Chapter 15

Purification of Effector–Target Protein Complexes via Transient Expression in *Nicotiana benthamiana*

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Abstract

Effectors of plant pathogens play important roles in not only pathogenesis but also plant immunity. Plant pathogens use these effectors to manipulate host cells for colonization, and their activities likely influence the evolution of plant immune responses. Analyses of genome sequences revealed that oomycete pathogens, such as *Phytophthora* spp., possess hundreds of RXLR effectors that are thought to be delivered into the host cells and hence function inside the cells by interacting with the host protein complexes. This article describes a co-immunoprecipitation protocol aimed at identifying putative target complexes of the effectors by transiently overexpressing the tagged effectors *in planta*. The identification of the eluted protein complexes was achieved by LC-MS/MS mass spectrometry and peptide spectrum matching.

Key words: Effectors, Co-immunoprecipitation, Oomycetes, *Phytophthora*, Mass spectrometry, Target complexes, Plant–microbe interaction

1. Introduction

The surveillance system of plant immunity relies on detection of molecules generated by an invading pathogen to mount defence responses. Perturbations caused by these pathogen molecules are perceived by resistance gene products in plants and defence procedures against the pathogen are initiated through downstream signalling cascades (1, 2). Although much knowledge has been accumulating in molecular events of recognition and signalling, little is known about pathogen molecules and their cognate functions except for a few cases in prokaryotes. Molecules, usually proteinaceous, secreted by pathogens to manipulate their hosts are known as "effectors", and their activities likely influence the evolution of plant immune responses. It is therefore pertinent to

John M. McDowell (ed.), *Plant Immunity: Methods and Protocols*, Methods in Molecular Biology, vol. 712,
DOI 10.1007/978-1-61737-998-7_15,

study the functions of these effectors *in planta* to gain deeper understanding of plant immunity.

In addition to validated effectors (3, 4), repertoires of effectors can be predicted for eukaryotic pathogens and studied in high-throughput manner from the available genome sequences. For example, by performing motif searches, complete sets of RXLR effectors are identified from the genome sequences of *Phytophthora sojae* and *Phytophthora ramorum*, oomycetes that cause devastating diseases on soybean and oak trees, respectively (5). A set of RXLR effectors have also been identified bioinformatically in potato late blight pathogen *Phytophthora infestans* and other oomycetes (6). RXLR effectors are pathogen-secreted proteins that are thought to translocate and function inside plant cells. It is most likely that effectors interact with plant proteins to condition the host plant for colonization.

Significant insights into how an effector carries out its function can be achieved by examining its interacting partners, including other proteins, inside the host cell (7, 8). An effector of interest can be tagged, expressed *in planta*, and purified in its native form by immunoprecipitation. In doing so, associated proteins are co-immunoprecipitated and these can be identified by mass spectrometry. Here, we describe a medium-throughput co-immunoprecipitation procedure adapted from ref. 7 to identify plant protein complexes interacting with pathogen effectors.

2. Materials

2.1. Preparation of Electro-Competent Agrobacterium Cells

1. *Agrobacterium tumefaciens* strain GV3101::pMP90::pSoup (see Note 1).
2. Luria–Bertani (LB) medium: Dissolve 10 g Bacto Tryptone (Difco, Voigt Global Distribution Inc., Lawrence, KS), 5 g yeast extract (Difco), 10 g NaCl in 1 L water, and adjust pH to 7.00 with NaOH. Autoclave at 121°C and 103.42 kPa for 15 min.
3. 10% (v/v) Glycerol.

2.2. Expression Vector and Transformation of Agrobacterium by Electroporation

1. TRBO high efficiency over expression vector (9).
2. Antibiotic stocks: Tetracycline (5 mg/mL in ethanol (EtOH)), rifampicin (10 mg/mL in methanol), kanamycin (10 mg/mL).
3. Gene Pulser Xcell Electroporation System (Bio-Rad) or similar electroporator.
4. 60% (v/v) Glycerol.

2.3. Agro-Infiltration

1. Plants: *Nicotiana benthamiana*, 4- to 6-week-old.
2. Greenhouse: Average temperature of 20°C and 16/8 h (light/dark) cycle supplemented with sodium vapour lamps.
3. Acetosyringone: Dissolve powder in DMSO to make a stock solution of 500 mM. Store at –20°C.
4. Agrobacterium Infiltration medium: 10 mM $MgCl_2$, 10 mM MES, pH 5.6, 150 mM acetosyringone (Sigma-Aldrich, St. Louis, MO). This should be made fresh from stock solutions.

2.4. Protein Extraction and Co-immuno-precipitation

1. GTEN: 10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl.
2. Extraction buffer: GTEN, 2% w/v PVPP, 10 mM DTT, 1× protease inhibitor cocktail (Sigma), 0.1% Tween 20 (Sigma).
3. Anti-FLAG M2 affinity Gel (Sigma).
4. 3× FLAG peptide (Sigma) stock solution at 5 mg/mL.
5. Immunoprecipitation (IP) buffer: GTEN, 0.1% Tween 20 (see Note 2).
6. Elution buffer: IP buffer containing 150 ng/μL 3× FLAG peptides.

2.5. SDS–Polyacrylamide Gel Electrophoresis

1. Separating buffer (4×): 1.5 M Tris–HCl, pH 8.8.
2. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8.
3. 10% (w/v) SDS (see Note 3).
4. Forty percent acrylamide/bis solution (37.5:1) (Bio-Rad, Hercules, CA) (see Note 4).
5. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).
6. Ammonium persulfate (APS, Bio-Rad): prepare 10% solution in water just before use. The solution can be stored at 4°C for up to 5 days.
7. Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad).
8. Running buffer (10×): Dissolve 30.3 g Tris base, 144 g glycine and 10 g SDS in 700 mL water and make to 1 L with water after everything is dissolved.
9. Sample loading dye (4×): 200 mM Tris–HCl (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 50 mM EDTA, 0.08% bromophenol blue.
10. 1 M Dithiothreitol (DTT). Divide into 1 mL aliquots and store at –20°C.
11. Prestained Protein Marker, Broad Range (New England BioLabs, Ipswich, MA).
12. Colloidal Coomassie Blue solution, e.g. SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA).

2.6. Western Blotting

1. Transfer buffer: 25 mM Tris base, 190 mM glycine, 20% (v/v) methanol.
2. Polyvinylidine fluoride membrane (PVDF, Bio-Rad) and 3MM chromatography paper (Whatman, Maidstone, UK).
3. Mini Trans-Blot Cell (Bio-Rad).
4. Tris-buffered saline with Tween (TBS-T) (10×): 1.37 M NaCl, 27 mM KCl, 250 mM Tris–HCl, pH 7.4, 1% (v/v) Tween 20.
5. Blocking solution: 5% (w/v) non-fat milk powder in 1× TBS-T.
6. Primary antibody: anti-FLAG monoclonal antibody (Sigma).
7. Secondary antibody: anti-mouse IgG conjugated to alkaline phosphatase (AP) (Sigma).
8. Colour development reagent: AP Conjugate Substrate Kit (Bio-Rad) (see Note 5).

2.7. Gel Excision and Tryptic Digestion

1. Clean razor blades and spatulas to cut and handle gel slices.
2. 20 mM Ammonium bicarbonate (ABC; NH_4HCO_3, Sigma, Cat. No. A6141).
3. 100% EtOH.
4. 50 mM ABC/100% EtOH (1:1).
5. 10 mM Dithiothreitol (DTT, Sigma, Cat. No. D0632) in 50 mM ABC.
6. 55 mM Iodoacetamide (IAA, Sigma, Cat. No. I6125) in 20 mM ABC.
7. Trypsin (12.5 µg/mL Promega Gold, Cat. No. V5113).
8. 100% TFA (trifluoroacetic acid).
9. 100% FA, formic acid.
10. 100% ACN, acetonitrile.

2.8. In-Solution Digestion

1. Rapigest SF (Waters Corp., #186001861).
2. Reduction buffer: 1 M dithiothreitol (DTT, Sigma, Cat. No. D0632) in 50 mM ammonium bicarbonate (Sigma, Cat. No. A6141).
3. Alkylation buffer: 55 mM iodoacetamide (Sigma, Cat. No. I6125) in 50 mM ABC.
4. Trypsin, sequencing grade, modified (Promega, Cat. No. V5113).

2.9. Mass Spectrometry

1. Nano-flow liquid chromatography system (nanoAcquity, Waters Corp.).
2. LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany).
3. Pre-column (Symmetry C18 5 µm beads, 180 µm × 20 mm column, Waters Corp., #186003514).

4. Analytical column: (BEH 130 C18 1.7 μm beads, 75 μm × 250 mm column, Waters Corp., #186003545).
5. Nano-spray source (Proxeon).
6. HPLC buffer A: MS-grade water with 0.1% formic acid (Water LiChrosolv, VWR International Ltd # 1 15333 2500).
7. HPLC buffer B: MS-grade acetonitrile with 0.1% formic acid (Acetonitrile UV grade, Fisher Scientific UK Ltd, # A0627B17).

2.10. Data Analysis

1. Bioworks/extract.msn (version 3.3.1, Thermo Scientific, San Jose, USA).
2. merge.pl (Matrix Science, UK).
3. Mascot (v 2.2 Matrix Science, UK).
4. Scaffold (v 2.2.03, Proteome Software).

3. Methods

We designed effector proteins for expression by replacing the N-terminal secretory signal peptide with FLAG epitope (MDYDDDDK) which can be used for affinity purification of the tagged protein with anti-FLAG resin. We made use of a gene synthesis service for our effector constructs: coding sequences of the constructs were codon-optimized for *in planta* expression, synthesized, and cloned by GenScript Corp. (Piscataway, NJ) into TRBO.

To prepare material for co-immunoprecipitation, *A. tumefaciens* cells are transformed with TRBO-effector constructs by electroporation. Agro-infiltration enables over expression of FLAG-tagged effector proteins in *N. benthamiana* leaves after T-DNA transfer by agrobacteria. Effector proteins are allowed to accumulate in the leaves for 2–3 days post-infiltration before harvesting. Total proteins are extracted from the leaves and FLAG-tagged effectors are co-immunoprecipitated with anti-FLAG M2 affinity resins. Bound proteins are specifically eluted with 3× FLAG peptides and separated by SDS–PAGE. Proteins in the gel are stained with colloidal Coomassie blue, excised, digested with trypsin, and identified by mass spectrometry.

Samples from co-IP experiments may be prepared for mass spectrometry in two ways; by excision from SDS–PAGE gel or the eluates can be analysed directly with in-solution digestion. Preparing samples in 1D gels offers the advantages of visualizing the proteins before analysis, allowing an estimate of quantity and complexity. Additionally, 1D gels provide a simple and reliable method to pre-fractionate complex protein mixtures, concentrating proteins according to their size and allowing more abundant proteins

to be cut away from less abundant proteins. The disadvantages of gel preparation are that it provides ample opportunity for the introduction of significant amounts of keratin and some smaller proteins (<10 kDa) are typically lost from standard gels. Analysing eluates directly can retain the smaller proteins and reduce contamination from skin proteins, but complex mixtures may require further fractionation (for example, by STAGE tips (10)) before analysis.

3.1. Preparation of Electro-Competent GV3101 Cells and Transformation

1. Plate the original culture of GV3101 cells on LB agar plate supplemented with tetracycline (2.5 μg/mL) and grow 2–3 days at 28°C.
2. Pick one colony and grow in 5 mL LB supplemented with tetracycline (5 μg/mL) medium, overnight at 28°C in a shaking incubator.
3. Take 1 mL start culture and grow in 200 mL LB + tetracycline (5 μg/mL) overnight at 28°C in a shaking incubator.
4. Measure the OD_{600}. When OD_{600} reaches 0.5–0.7, divide the culture into two pre-chilled 250-mL centrifuge bottles and keep on ice at least 30 min.
5. Centrifuge the cultures for 15 min at 3,500 × *g* at 4°C.
6. Pour off the supernatant and resuspend the pellets in 50 mL ice cold 10% glycerol.
7. Centrifuge the suspension for 15 min at 3,500 × *g* at 4°C.
8. Discard the supernatant and wash pellets with 50 mL ice cold 10% glycerol again.
9. Discard the supernatant and resuspend each pellet in 200 μL 10% glycerol.
10. Aliquot 50 μL of the resuspended competent cells into pre-chilled 1.5-mL Expender tubes.
11. Snap-freeze the cells in liquid nitrogen and store at –80°C for up to 1 year.

3.2. Transformation of Agrobacterium by Electroporation

1. Thaw competent cells on ice (50 μL per transformation).
2. Add TRBO plasmid DNA (1–2 μL) to the cells, and mix them together on ice by tapping the side of the tube.
3. Transfer the mixture to a pre-chilled electroporation cuvette with 2 mm gap.
4. Carry out electroporation using a Bio-Rad electroporator set to following conditions: capacitance: 25 μF, voltage: 2.4 kV, resistance: 200 Ω. This should yield a time constant (pulse length) of 5 ms upon electroporation.
5. Immediately after electroporation, add 950 μL LB to the cuvette, and transfer the bacterial suspension to a 15-mL culture tube. Incubate for 4 h at 28°C with gentle agitation.

6. Collect the cells by centrifuging briefly, and spread them on an LB agar plate containing kanamycin (50 μg/mL) and rifampicin (100 μg/mL).
7. Incubate the cells for 3–4 days at 28°C.
8. Screen colonies by polymerase chain reaction (PCR) for the presence of TRBO plasmid constructs and pick 2–3 colonies with inserts.
9. Confirm the integrity of inserts by sequencing the PCR products.
10. Grow validated clones in 5 mL LB medium supplemented with kanamycin (50 μg/mL) and rifampicin (100 μg/mL) overnight in a shaking incubator at 28°C.
11. Add 0.5 mL 60% glycerol to 1 mL overnight cultures in a 2-mL cryotube, mix by vortexing, and store at −80°C until use.

3.3. Agro-Infiltration

1. Streak out glycerol stock of Agrobacterium containing the TRBO construct onto LB plates containing 50 μg/mL kanamycin and 100 μg/mL rifampicin and incubate at 28°C for 24 h.
2. Inoculate 10 mL LB broth containing 50 μg/mL kanamycin and 100 μg/mL rifampicin with a colony from the streak and grow overnight at 28°C in a shaking incubator.
3. Centrifuge the cells at 3,500 × *g* and resuspend in Agrobacterium infiltration medium and incubate at room temperature for at least 2 h (overnight incubation also works).
4. Take an OD_{600} of each Agrobacterium culture to be infiltrated. Dilute the culture with the Agrobacterium infiltration medium to achieve OD_{600} 0.3–0.5.
5. Infiltrate middle leaves of 4- to 6-week-old *N. benthamiana* plants using a 1-mL syringe without the needle making sure the whole leaf area is infiltrated.
6. Harvest the leaves 3–4 days post-infiltration for protein extraction.

3.4. Protein Extraction and Co-immunoprecipitation

1. Freeze 3–4 leaves in liquid nitrogen and grind into powder using a mortar and pestle making sure the samples are constantly frozen with liquid nitrogen during grinding.
2. Weigh out 1 g of leaf powder on a pre-chilled aluminium foil and add the powder to 2.0 mL of ice-cold extraction buffer in a 15-mL centrifuge tube. Vortex to mix so that all the powder comes in contact with the extraction buffer. Keep the tube on ice until the powder is thawed completely in the extraction buffer. Vortex to mix thoroughly for 20 s.
3. Centrifuge at 3,000 × *g* for 10 min at 4°C and transfer the supernatant to a 2-mL microcentrifuge tube.

4. Centrifuge at full speed in a microcentrifuge for 10 min at 4°C. Transfer the supernatant to a new tube.
5. To a new 2-mL microcentrifuge tube, add 250 µL of extract (freeze the left-over samples in liquid nitrogen and store at –80°C until use). Bring up the total volume to 2.0 mL with IP buffer. Keep this solution on ice until use.
6. Resuspend the resin well by tapping the side of the vial several times and pipetting the resin up and down using a 1-mL pipette with a cut tip (so that the opening is wide enough to let the resin move through without too much damage).
7. Pipette enough resin (50 µL per sample, e.g. prepare 200 µL resin for four samples) into a 2.0-mL Eppendorf tube and centrifuge at 800×*g* for 1 min and remove the supernatant using a needle attached to a syringe (take care not to aspirate the resin).
8. Resuspend the resin in 5× volumes of IP buffer.
9. Centrifuge at 800×*g* for 1 min and remove the supernatant as above.
10. Repeat above two steps twice more.
11. Resuspend the resin to the original volume with the IP buffer and add 50 µL of resin to the leaf extract prepared above (step 5).
12. Mix the resin and the extract well by turning end-over-end for at least 1 h at 4°C.
13. Centrifuge at 800×*g* for 30 s. Discard supernatant and add 1 mL of fresh IP buffer. Repeat four more times but always leave about 50 µL at the bottom of the tube to avoid aspirating the beads. After the last wash, centrifuge to spin down any liquid on the sides of the tube and aspirate the remaining liquid with a needle attached to a 1-mL syringe.
14. Elute the bound proteins by adding 100 µL IP buffer containing 150 ng/µL 3× FLAG peptide and incubating with gentle shaking for 30 min at 4°C.
15. Transfer the supernatant containing the eluted proteins to a fresh tube using a syringe and needle (take care not to aspirate any resin) (see Note 6).
16. Load 10–20 µL of the sample onto an SDS–PAGE gel for colloidal Coomassie blue staining followed by protein identification using mass spectrometry and western blotting.

3.5. SDS–Polyacrylamide Gel Electrophoresis

1. Set up the gel cassettes on casting stand of Mini PROTEAN electrophoresis system using the glass plates with 1-mm spacers following the manufacturer's instructions.
2. Prepare a 15% separating gel by mixing 2.5 mL 4× separating buffer, 3.8 mL 40% acrylamide/bis solution, 3.5 mL water,

100 µL 10% SDS, 50 µL 10% APS, and 5 µL TEMED. Immediately pour 3.75 mL gel mixture between the glass plates seated on the gel casting stand and overlay with 1 mL water. There should be enough solution for two gels. The gels should polymerize within 30 min.

3. Once the gels have polymerized, pour off the water and rinse the top of the gel with water.
4. Prepare the stacking gel by mixing 1.25 mL 4× stacking gel buffer, 0.5 mL 40% acrylamide/bis solution, 3.2 mL water, 50 µL 10% SDS, 50 µL 10% APS, and 5 µL TEMED. Immediately pour the stack on top of both gels and insert the 10-well combs. The stacking gel should polymerize within 30 min. Allow at least one additional hour for gels to set completely.
5. Prepare 1 L running buffer by mixing 100 mL 10× running buffer and 900 mL water.
6. Carefully remove the combs from the gel and rinse the wells with running buffer using a Pasteur pipette. Remove the gel plates from the casting frames and place in electrode module and buffer tank.
7. Add the running buffer to upper and lower chambers of the electrophoresis unit to the indicated level and load the wells with up to 35 µL sample containing 4× sample loading dye and 100 mM DTT. Samples should be loaded on one gel for western blotting and duplicate sample on the other gel for Coomassie blue staining and subsequent analysis by mass spectrometry.
8. Place the lid on the buffer tank and assembly making sure to align the colour coded banana plugs and jacks. The lid should be securely and tightly positioned on the tank.
9. Connect the cables to the power supply and run at a constant voltage of 200 V until the dye front reaches to the bottom of the gel.

3.6. Western Blotting

Proteins separated by SDS–PAGE are transferred using Mini Trans-Blot Cell (Bio-Rad) to PVDF membrane for detection by anti-FLAG antibody and anti-mouse secondary antibody conjugated to alkaline phosphatase catalysing a colorimetric reaction. Fill the Bio-Ice cooling unit with water and store it at –20°C until ready to use.

1. Remove the gel from the cassette and rinse with water and place in transfer buffer and equilibrate for 15 min on a shaking platform.
2. Cut the PVDF membrane and the 3MM paper to the dimensions of the gel.

3. Soak the membrane, 3MM paper, and fibre pads in transfer buffer for 10 min.
4. Prepare the gel cassette following the manufacturer's instructions.
5. Place the cassette in module and add the frozen Bio-Ice cooling unit. Place in tank and completely fill the tank with buffer.
6. Add a stir bar to the tank to distribute buffer temperature and ion in the tank. Set the speed as fast as possible.
7. Put on the lid, plug the cables into the power supply, and run the blot at constant current of 250 mA for 1 h.
8. Upon completion of the run, disassemble the blotting sandwich and carefully remove the membrane.
9. Wash the membrane in water for 2–3 min with mild agitation, then with TBS-T.
10. Incubate the membrane with blocking solution for 1 h at room temperature.
11. Rinse the membrane with TBS-T.
12. Dilute anti-flag M2 antibody (usually 1:3,000) in 10 mL blocking solution and add to the membrane. Incubate for 1 h on a rocking platform.
13. Rinse twice with TBS-T and then wash three times with TBS-T, each time for 10 min.
14. Add anti-mouse antibody (diluted 1:40,000 in blocking solution), incubate for 1 h.
15. Rinse twice with TBS-T and then wash three times with TBS-T, each time for 10 min.
16. Prepare AP substrate solution by mixing 400 μL 25× development buffer, 9.4 mL water, 100 μL AP colour reagent A, 100 μL AP colour reagent B immediately before use.
17. Immerse the membrane in the AP substrate solution and incubate at room temperature with gentle agitation until colour development reaches desired intensity.
18. Stop the development by washing the membrane in ddH_2O for 10 min with gentle agitation. Air dry the membrane and digitally record the image of the membrane.

3.7. Gel Excision and Trypsin Digestion

At all stages strive to avoid keratin contaminations of gels; wear gloves at all times and use clean trays that have never been used for processes, such as Western blotting.

1. Cut gel slices into 1-mm cubes so that they site at the base of the tube. Do not mash or grind the gel.
2. Wash the gel pieces in 50% EtOH with shaking at 40°C, ensure that the gel slices are totally covered and change the buffer as required until completely destained.

3. Remove destaining solution and cover gel pieces with 100% EtOH. Gel pieces will dehydrate, shrinking and becoming opaque, remove EtOH.
4. Add 50–150 µL of 10 mM DTT in 20 mM ABC (see Note 7).
5. Incubate for 30 min 57°C with shaking.
6. Cool samples to room temperature before adding 55 mM IAA in 20 mM ABC (same volume as above).
7. Incubate 15 min in the dark at room temperature.
8. 2 × 10 min wash in 20 mM ABC/EtOH 1:1, and 10 min dehydration in 100% EtOH.
9. Add 10–50 µL trypsin in 20 mM ABC to the dehydrated gel pieces.
10. Add 20–100 µL 20 mM ABC to keep gel wet (ensure that large amounts of gel are covered).
11. Incubate 4 h or overnight at 37°C.
12. Transfer supernatant to new tube. Add 20–200 µL 100% ACN to gel pieces, vortex and gently centrifuge. Combine ACN with supernatant and speed-vac to remove ACN and reduce total volume to approximately 10 µL for MS analysis.

3.8. In-Solution Digestion

1. Prepare fresh Rapigest to 0.1% (w/v) in 50 mM ABC.
2. Dissolve the protein pellet (often invisible) in 0.1% Rapigest solution, using a cycle of sonication, vortexing and heating to 95°C (5 min) to fully dissolve the pellet.
3. Add DTT to a final concentration of 5 mM.
4. Incubate at 57°C for 30 min with shaking.
5. Cool to room temperature before adding IAA to a final concentration of 15 mM.
6. Incubate 15 min in the dark at room temperature.
7. Add trypsin (1:100 w/w). Incubate samples at 37°C for 4 h or overnight.
8. Acidify samples (TFA to final concentration of 0.5%) to halt digestion and hydrolyse Rapigest. Incubate samples for 30 min at 37°C.
9. Centrifuge acid-treated samples at maximum speed in a bench top centrifuge for 15 min. A whitish pellet may be visible (see Note 8).
10. Immediately after centrifugation take the supernatant to a clean, labelled tube. Do not disturb the pellet.

3.9. Mass Spectrometry

LC-MS/MS analysis is performed using an LTQ-Orbitrap XL mass spectrometer, a UPLC system adapted for nano-flow and a nano-electrospray source. The MS is operated in positive ion

mode with a capillary temperature of 200°C, no sheath gas is employed and the source and focusing voltages are optimized for the transmission of angiotensin (see Note 9).

1. Apply peptides to a pre-column connected to a self-packed C18 8-cm analytical column.
2. Elute peptides using a gradient of 2–40% acetonitrile in 0.1% formic acid over 60 min at 250 nL/min (see Note 10).
3. Acquire full scan MS in profile mode from 300 to 2,000 m/z at 60,000 resolution using an internal lock mass. Maximal accumulation time for MS1 is set to 1 s to a target fill of 1×10^6.
4. A minimum signal of 3,000 counts is required to trigger data dependent acquisition of MS2 spectra. MS2 are acquired in a data dependent mode consisting of selection of the six most abundant ions in each cycle. Fragmentation is typically collision induced disassociation (CID) with collision energy at 35%, in MS2 ions are accumulated to 5×10^4 and spectra are centroided at acquisition by the MS.
5. Dynamic exclusion parameters allow one repeat hits before the precursor m/z is added to an exclusion list for 300 s.

3.10. Data Analysis

1. Extract MS/MS spectra from the raw file using BioWorks (v 3.3.1). We typically use the following parameters to generate dta files; MW range 300–4,500, a threshold of 100 ion counts, precursor ion tolerance of 5 ppm, one group scan and a minimum ion count of 10. MS level is set to automatic and we do not use any of the filtering options such as ZSA.
2. Use the merge.pl script (Matrix Science) to concatenate the dta files into generic mascot format (an mgf file).
3. Search the appropriate database with Mascot and compile the results in Scaffold to combine data from gel bands and to facilitate comparison between samples.

4. Notes

1. We use *A. tumefaciens* strain GV3101 for its ability to transfer T-DNA efficiently to *N. benthamiana* and mainly for its high transformation efficiency in our lab. For more information on use of *A. tumefaciens* and binary vectors, see ref. 11.
2. We use non-denaturing non-ionic detergent Tween 20 to extract the proteins from the leaves. NP-40 is also commonly used but discontinued by the manufacturer. Denaturing ionic detergents may disrupt the protein complexes.

3. SDS powder should be weighed out in a safety cabinet. SDS causes irritation to eyes, respiratory system, and skin on contact.
4. Always wear protective clothing, such as gloves, lab coat, and safety glasses, when handling Acrylamide. Acrylamide is highly toxic and may cause cancer and heritable genetic damage.
5. An alternative to visualize the western blots is to use secondary antibodies conjugated to horseradish peroxidase and chemiluminescent substrate to expose X-ray films. This procedure may offer better sensitivity if the protein expression is very low.
6. Repeat this step if there is contamination with IgG fragments in the eluted samples. Smallest number of resin beads could contribute to the IgG contamination.
7. Alter the volumes used to ensure that the gel pieces are entirely covered. Adjust subsequent volumes to compensate.
8. We have occasionally observed a white residue that floats on the surface of the preparation rather than forming a pellet, we think this is Rapigest and recommend transferring the supernatant to a clean tube and repeating the centrifugation at 4°C. If the solution is not entirely clear, we filter the peptides though a 10-kDa membrane (e.g. microcon from Millipore) before mass spectrometry.
9. Other types of mass spectrometer are suitable for this analysis. Some steps of the data analysis differ accordingly.
10. The gradient used depends on the complexity of the peptide mixture and the HLPC available. We typically use a 60-min gradient for medium complexity mixtures (<100 proteins, with identification rather than coverage as the goal) and increase up to 4 h gradients for highly complex mixtures.

Acknowledgments

We thank John Lindbo (UC Davis) for providing us with the TRBO vector, and Liliya Serazetdinova for technical assistance. This work was supported by the Gatsby Charitable Foundation.

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