Purification of effector complexes from plants: *In planta* co-immunoprecipitation of effectors.

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1. Materials

1.1. Protein extraction and co-immunoprecipitation

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, 1X protease inhibitor cocktail (Sigma), 0.1% Tween 20 (Sigma) (or NP40)
3. Anti-FLAG M2 affinity Gel (Sigma)
4. 3X FLAG peptide (Sigma) stock solution at 5 mg ml^{-1}
5. Immunoprecipitation (IP) buffer: GTEN, 0.1% Tween 20 (NP40)
6. Elution buffer: IP buffer containing 150 ng μl^{-1} 3X FLAG peptides

1.4. Gel excision

1. Clean razor blades and spatulas to cut and handle gel slices.
2. 100% EtOH

2. Methods

We overexpress effectors using the high-expression vector pJL-TRBO, a binary plasmid containing a modified *Tobacco mosaic virus* with its coat protein gene substituted with cDNAs coding for FLAG-tagged mature effectors. Effector constructs were delivered into the leaves of *Nicotiana benthamiana* by agroinfiltration and expressed under the viral coat protein promoter. Leaves were harvested 2-3 days post-infiltration and total proteins were extracted. In this session we will co-immunoprecipitate (co-IP) effectors and their host interactors with anti-FLAG resins under non-denaturing conditions. Bound proteins will be specifically eluted using 3X FLAG peptides, separated by SDS-PAGE and visualized by colloidal Coomassie blue staining. Finally, protein bands are excised, digested with trypsin and identified by LC-MS/MS peptide ion spectrum matching.

2.1. 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 3000x *g* for 10 min at 4 °C and transfer the supernatant to a 2 ml micro centrifuge tube.
4. Centrifuge at full speed in a micro centrifuge for 10 minutes at 4 °C. Transfer the supernatant to a new tube.
5. To a new 2 ml micro centrifuge 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 800x *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 5x volumes of IP buffer.
9. Centrifuge at 800x *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 one hour at 4 °C.
13. Centrifuge at 800x *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^{-1} 3X 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).
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.

3.7 Gel excision

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 ºC, ensure that the gel slices are totally covered and change the buffer as required until completely destained.

3. References

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