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PROTOCOL

Co-Immunoprecipitation of Membrane-Bound Receptors

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The study of cell-surface receptor dynamics is critical for understanding how cells sense and respond to changing environments. Therefore, elucidating the mechanisms by which signals are perceived and communicated into the cell is necessary to understand immunity, development, and stress. Challenges in testing interactions of membrane-bound proteins include their dynamic nature, their abundance, and the complex dual environment (lipid/soluble) in which they reside. Co-Immunoprecipitation (Co-IP) of tagged membrane proteins is a widely used approach to test protein-protein interaction *in vivo*. In this protocol we present a method to perform Co-IP using enriched membrane proteins in isolated microsomal fractions. The different variations of this protocol are highlighted, including recommendations and troubleshooting guides in order to optimize its application. This Co-IP protocol has been developed to test the interaction of receptor-like kinases, their interacting partners, and peptide ligands in stable *Arabidopsis thaliana* lines, but can be modified to test interactions in transiently expressed proteins in tobacco, and potentially in other plant models, or scaled for large-scale protein-protein interactions at the membrane.

I. INTRODUCTION

Plasma-membranes are required to confine the reactions required for life. Membranes allow cells to interact with their surroundings, control the flow of molecules, and provide structural support and anchoring for signaling and energy-generating complexes. Approximately 25% of all Arabidopsis proteins are predicted to contain at least one transmembrane domain (Schwacke et al 2003), however this figure likely under-represents the actual number of proteins found in membranes since only half of the *Arabidopsis* proteins bound to plasma membranes in green tissues appear to have transmembrane domains (Alexandersson et al, 2004). The underestimation of proteins found at the membrane can be partially explained by the presence of proteins that, despite lacking transmembrane domains, establish strong non-covalent interactions with integral membrane proteins or undergo covalent attachment of fatty acids in processes such as S-acylation (like palmitoylation) or N-terminal myristoylation. For instance, in *Arabidopsis*, 319 proteins are myrisotylated (Podell and Gribskov, 2004), and approximately 600 are S-acylated (Hemsley et al., 2008). Membrane proteins and the different ways by which they bind the phospholipid bilayers offer a vast network of potential interaction mechanisms with varying biophysical properties. Streamlined strategies for identifying protein-protein dynamics at this interface are thus required in order to understand virtually any biological process.

Establishing interactions at the membrane level is challenging and usually requires multiple forms of validation. *In vitro* approaches that require purified proteins are particularly cumbersome as the membrane proteins in general require native chaperones and lipid-environments to fold properly, and usually undergo heavy posttranslational modifications, such as the glycosylation of ectodomains in receptor-like kinases (RLKs; van der Hoorn et al, 2005). Consequently, recombinant expression of transmembrane proteins often leads to the production of incorrectly folded forms limiting the biochemical study of native function (Jamshad et al, 2011). Membrane proteins such as receptor kinases can be dissected to remove the kinase domain and transmembrane domains to produce recombinant extracellular domains. This approach uses eukaryotic cell expression systems such as baculovirus-infected insect cells or tobacco BY-2 cells and has allowed for structural receptor-ligand binding analysis, such as the elucidation of the crystal structure of BRASSINOSTEROID INSENSITIVE 1 (BRI1: At4g39400) binding to brassi-

nolide (Hothorn et al., 2011, Sun et al, 2013). The expression of truncated forms lacking only the kinase domain *in planta* is also commonly used. The removal of the kinase domains increases the stability of some receptors that, with the presence of cytosolic domains, would otherwise undergo increased endocytosis and turnover. For example, the kinase-deleted version of the receptor kinase ERECTA (ER: At2g26330) accumulates at much higher levels than the full-length, endogenous ERECTA protein (Shpak et al, 2003), while the removal of the kinase domain in the CLAVATA1 (CLV1: At1g75820) receptor improves its expression without affecting the ligand-binding affinity of its ectodomain (Ogawa et al, 2008). These truncated proteins have been successfully used in binding assays such as pull down, gel filtration, fluorescence anisotropy, and surface plasmon resonance (Pollard, 2010, Luoni et al, 2006, Lee et al 2012). However, interpretation of this binding data has to include a thorough analysis of the potential biological effects caused by removing the kinase domains and the changes in stoichiometry caused by the over-accumulation of stabilized receptors.

Additional methods that have been implemented to test membrane-protein dynamics include methods based on fluorescence, yeast-two hybrid, and mass spectrometry. *In vivo* fluorescence microscopy is often conducted utilizing fluorescently-tagged proteins in order to test membrane protein interactions in techniques such as Föster resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC), which can be used with transient or stable protein expression (Reviewed in Kerppola, 2008). Recently, the mating-based split ubiquitin system (mbSUS) was used in testing the interaction of plant membrane proteins. This method relies on a modified yeast two-hybrid protocol and has been used to generate a membrane interactome in *Arabidopsis* (Lalonde et al, 2010). A highly sensitive detection of protein interaction is the mass spectrometric identification of co-immunoprecipitated proteins, which has been successfully used to dissect signaling networks involving membrane receptors (Fàbregas et al, 2013, Weis et al, 2013, Kadota et al, 2014, Li et al, 2014). All of these techniques suffer from limitations caused by: the low protein abundance at the plasma membrane; the absence of additional complex components; the transient nature and low affinity of some of the interactions tested; the non-specific interactions caused by the nature of membrane emulsification methods; and, since many approaches required tagged-proteins, the interference in protein function and stability caused by epitope tags. Thus, the need to provide multiple techniques to assess protein-protein interactions is fundamental to fully validate their physical interactions.

Here we present a protocol our lab has optimized to test the interaction of full-length membrane-bound receptor kinases that can be extended to testing the interaction of other membrane proteins (Figure 1). The protocol including the immune-detection of immunoprecipitated proteins takes 3 to 4 days depending on the immunoblotting method of choice (Figure 2). Plants either stably or transiently expressing epitope-tagged proteins are used to isolate the microsomal fraction, followed by membrane protein emulsification in the presence of a surfactant (Figure 3). The interaction of the solubilized proteins is then tested by co-immunoprecipitation (Co-IP) with antibodies specific to the tags used for each protein, followed by detection by immunoblotting. This approach is particularly powerful when studying low-abundant proteins that are either weakly expressed or exclusively found

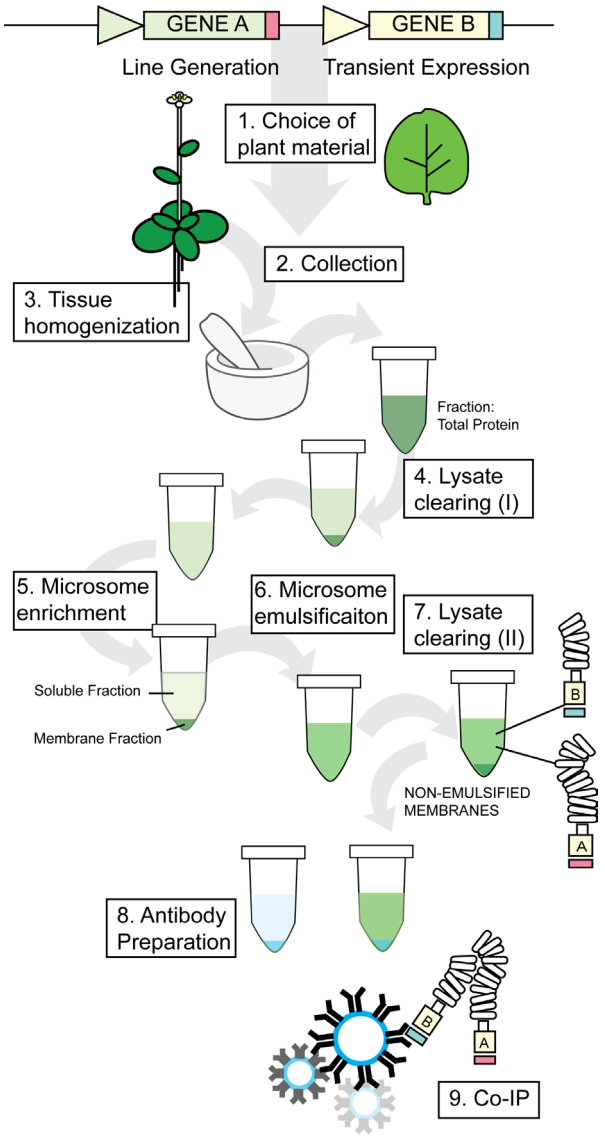

Figure 1. Illustrated summary of the Co-immunoprecipitation of membrane proteins protocol

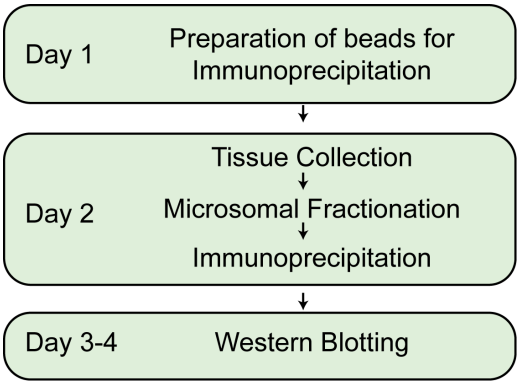

Figure 2. Flow chart for a typical Co-IP experiment

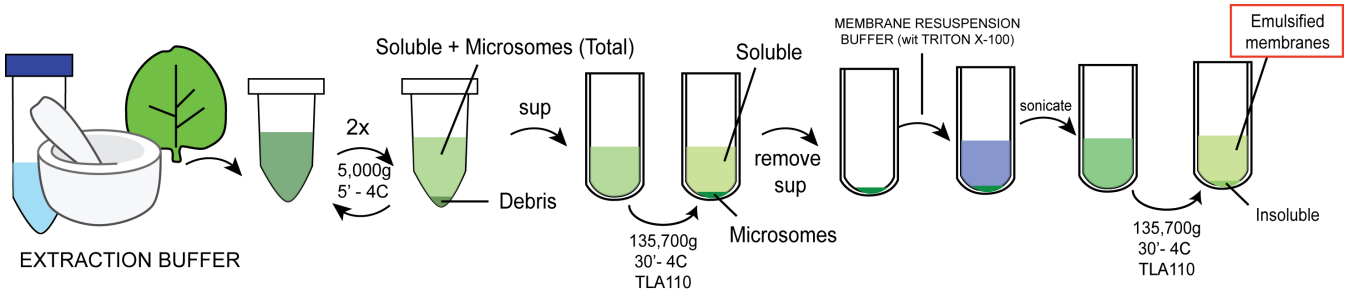

Figure 3. Illustrated protocol of microsomal fractionation.

in certain tissues or cell types, as well as proteins that cannot be overexpressed due to their stability or potential dominant negative effects. Throughout the protocol we highlight variations and possible solutions to challenges in the experimental design.

I-A. Choice of protein expression method

The expression method for the interacting proteins to be analyzed should be carefully considered for both stable and transient protein expression approaches due to undesired artifacts caused by overexpression, such as improper protein folding, spurious complex formation, and signaling disruption (Gibson et al, 2013). When possible, stable lines generated should express the proteins at endogenous expression levels. This can be accomplished by using each gene's native promoters and the gene coding region including intronic sequences. This is particularly critical for ERECTA, which requires the presence of introns in order to express properly as they are required for mRNA stability (Karve et al, 2011). Alternative expression methods include overexpression, such as the 35S Cauliflower Mosaic virus promoter (Odell et al, 1985), or inducible expression, such as the estradiol inducible system (Zuo et al, 2000). In some instances, the increase in protein expression can cause undesired phenotypic effects. With inducible expression systems, the optimal point for tissue collection can be determined by immunoblotting or fluorescence microscopy when the appropriate fluorescent tags are used for evaluating the expression. Similarly, this approach can test the expression at different developmental stages or stimulus-based time points. When using overexpression or inducible promoters, along with fluorescent tags, it is important to determine the correct cellular localization of the protein. For instance, the overexpression of some membrane associated proteins causes abnormal localization patterns (Cai et al, 2014, Uemura et al, 2004). The formation of protein aggregates or protein instability caused by overexpression can be detected by immunoblotting with the appearance of high molecular complexes under denaturing conditions or the cleavage products of the wrong molecular mass.

I-B. Choice of epitope tags

In addition to the choice of promoters, the impact of the epitope tags on protein function should be taken into account. The functionality of tagged proteins can be assessed by estimating their ability to complement their respective mutant phenotypes. To do so, prior knowledge about the phenotypic impact of mutations affecting the gene/protein of interest may be required. This prerequisite could hinder the analysis of membrane proteins with unknown function. In some instances however, the expression of membrane proteins carrying a tag can manifest neomorphic phenotypes, indicating that the tag chosen should be avoided. For instance, the addition of a green fluorescent protein (GFP) tag to a vacuolar H^+ pyrophosphatase was reported to cause the formation of ectopic subcellular structures termed "bulbs". This is due to membrane adhesion triggered by GFP dimerization (Segami et al., 2014). In addition to the undesired phenotypic effects caused by the epitope tag of choice, tags should be chosen preferably the success rate at which they have been previously used in Co-IPs in the literature (Table 1).

Table 1. Commonly used antibody and antibody resins in co-immunoprecipitation in *A. thaliana* expressed seedlings.

Co-IP Proteins	Antibody / Beads	Reference
BRI1-FLAG BAK1-GFP	α-FLAG M_2 Affinity Gel (Sigma-Aldrich, St. Louis, MO) α-GFP mouse antibody (Molecular Probes) + Protein A beads (Pierce, Rockford, IL)	Wang et al, 2005
BAX-Inhibitor 1-GFP	GFP-Trap A® beads (ChromoTek, Munich, Germany)	Weis et al, 2013
TDP1-FLAG EMS1-cMyc	α-FLAG M_2 Affinity Gel (Sigma-Aldrich, St. Louis, MO) Profound c-Myc Tag IP/Co-IP kit (Pierce)	Jia et al, 2008
HA-RTE1 ETR1-5xMYC	anti-HA monoclonal antibody (Roche Applied Science) + Protein A sepharose (Unspecificed manufacturer)	Dong et al, 2010

Some receptor kinases play multifaceted roles in development and environmental responses, and in such a case, it is essential to confirm whether a tagged fusion protein rescues the specific biological process of investigations. The notable example is BAK1 (BRI1 ASSOCIATED KINASE1: AT4G33430), which act as a partner receptor for brassinosteroid (BR), innate immunity, and other developmental and stress signaling. It was reported that while C-terminal epitope-tagged fusions of BAK1 are mostly functional for BR signaling, they failed to fully complement the innate immunity response (Ntoukakis et al. 2011).

Established lines for Co-IPs should: (1) express the proteins to be tested for interaction at endogenous levels; (2) be in the appropriate mutant backgrounds which they are shown to fully complement (Figure 4 B); and (3) express the proteins to levels detectable in immunoblotting (see microsomal enrichment protocol Figure 4 A). The dual transgenic lines used in the Co-IP expressing both epitope-tagged versions of the genes can be generated by genetic crosses. Special attention has to be taken into consideration with double mutant backgrounds for corresponding transgenes (Figure 5). This procedure can be facilitated if the selection markers in the lines to be crossed are still functional and fluorescent tags are being used. Heterozygote F1 lines can be used to quickly test the interaction. It is recommended that homozygote lines are established in order to fully remove the endogenous untagged gene/proteins of interest as they may outcompete the tagged versions in the Co-IP (Figure 5).

II. MATERIALS

II-A. Reagents

Trizma® hydrochloride, Sigma T5941

NaCl

Ethylenediaminetetraacetic acid (EDTA), Sigma

Glycerol

NaF (Sigma 201154)

Phenylmethanesulfonyl fluoride (PMSF)

cOmplete protease inhibitor Cocktail tablets, Roche

Triton™ X-100, Sigma T8787

Antibodies

- anti-GFP (Abcam, ab290)
- anti-HA (Roche, 12CA5)
- Anti-C-MYC (Abcam, ab32)
- Anti-FLAG M2 (Sigma 9E10)

Protein G Agarose beads (Pierce, 20397)

II-B. Supplies and Equipment

Water bath sonicator pre-chilled to 4°C

Microcentrifuge pre-chilled to 4°C

Ultracentrifuge (Beckman Coulter Optima™ MAX-XP)

Ultracentrifuge rotor TLA110 (Move to 4°C fridge when starting extraction protocol)

Polycarbonate thick wall 3.2ml ultracentrifugation tubes (Beckman 362305)

Orbital shaker at 4°C

II-C. Buffers and Stock Solutions

Extraction buffer. 100mM Tris-HCl at pH 8.8, 150mM NaCl, 1mM EDTA, 10% glycerol, 20mM NaF, 1mM PMSF, cOmplete protease inhibitor cocktail, 1mM Na_2MoO_4, 50mM β-glycerophosphate, 10mM Na_3VO_4.

Membrane protein solubilization buffer. 100mMTris-HCl at pH 7.3, 150mM NaCl, 1mM EDTA, 10% glycerol, 20mM NaF, 1% Triton X-100, 1mM PMSF, cOmplete protease inhibitor cocktail 1X.

Phosphate Buffer Saline (PBS), pH 7.4: For 1L, add: 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4. Adjust the pH to 7.4. Sterilize by autoclaving; Store at room temperature.

0.1M Na_3VO_4. 180mg in 10ml ddH_2O, store aliquots at -20°C

0.1 Na_2MoO_4. 180mg in 10ml ddH_2O, store aliquots at -20°C

0.1M NaF 42mg in 10 ml, store aliquots at -20°C

Complete protease inhibitor cocktail 100X. Dissolve two tablets in 1ml ddH_2O, store at -20°C

CAUTION: NaF and PMSF are toxic compound.
PMSF stock solutions should be made using isopropanol.
Make buffers fresh using pre-made stock solutions.

III. PROCEDURE

III-A. Preparation of immobilized antibody resin for IP *Duration: 16 h*

1. Use 1µl of PolyGFP Antibody 1µl per IP, or 2 µl of HA Antibody per 25 µl of Dynabeads (Protein G). NOTE
2. Remove bead storage solution (20% Ethanol) and rinse beads with 500µl of PBS (Phosphate Buffered Saline).
3. Mix antibody with 200µl of PBST (PBS + Tween 20 [0.1% w/v]) and antibody. Incubate overnight with antibody at 4°C on nutator.

NOTES AND TROUBLESHOOTING

N1. In different IP experiments we have used magnetic as well as agarose-based antibody beads. Recently we have started using pre-immobilized beads (ChromoTek GFPTrap) which save time and effort as steps 1 through 3 in this protocol are no longer required. In our experience, however, the type and concentration of emulsifying agent affect the amount of non-specific binding. For instance, magnetic beads exhibit increased non-specific binding at high Triton X-100 (1% v/v) or 4-nonylphenyl-polyethylene glycol (NP40) (2% v/v) concentrations. Therefore the efficiency of IP in the beads as well as the amount of non-specific binding should be carefully standardized.

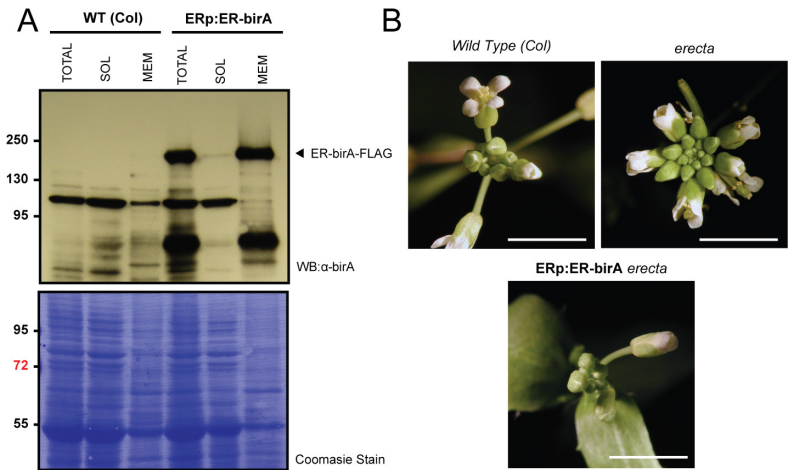

Figure 4. Complementation and expression analysis of tagged membrane proteins.

A. Immunoblotting on microsomal fractionation of ERECTA-birA stably expressed at endogenous levels. **B.** Flower phenotype of *erecta* mutant lines expressing ERECTA-birA at endogenous levels. The expression of ERECTA-birA rescues (complements) short, clustered inflorescence phenotype of *erecta*, indicating that the fusion protein is functional.

III-B. Preparation of microsomal fraction ***Duration: 3 hours***

4. Harvest tissue (approximately 500mg to 700 mg for 11 day old *Arabidopsis* seedlings half of a 150 mm 1/2MS-0 plate of densely sown seeds) and freeze immediately in liquid nitrogen. We grow seedlings at 21°C under a 16 hour light and 8 hour dark cycle. Each researcher should use the growth condition optimized for his/her research.

5. Grind tissue to a powder in liquid nitrogen in pre-chilled mortar and pestle to a fine powder. Do not let the tissue thaw.

STOPPING POINT:
The tissue can be stored at -80°C.

CAUTION
Use protective clothing when handling liquid nitrogen. When processing multiple samples we use cotton gloves to manipulate chilled mortar, pestles, and spatulas which, after continued use can reach extremely low temperatures.

6. Immediately transfer tissue with a pre-chilled spatula to a mortar containing three volumes of protein extraction buffer (for example 1.5ml of extraction buffer per 500 mg of tissue).[NOTE]

7. Immediately, grind tissues with a pestle in extraction buffer until no clumps remain. The solution should be homogenized to an even mixture that can be pipetted without clogs.[NOTE]

8. Transfer homogenate to 1.5ml microcentrifuge tubes and sonicate the homogenate for 10 seconds in pre-chilled water bath (or ice bucket).

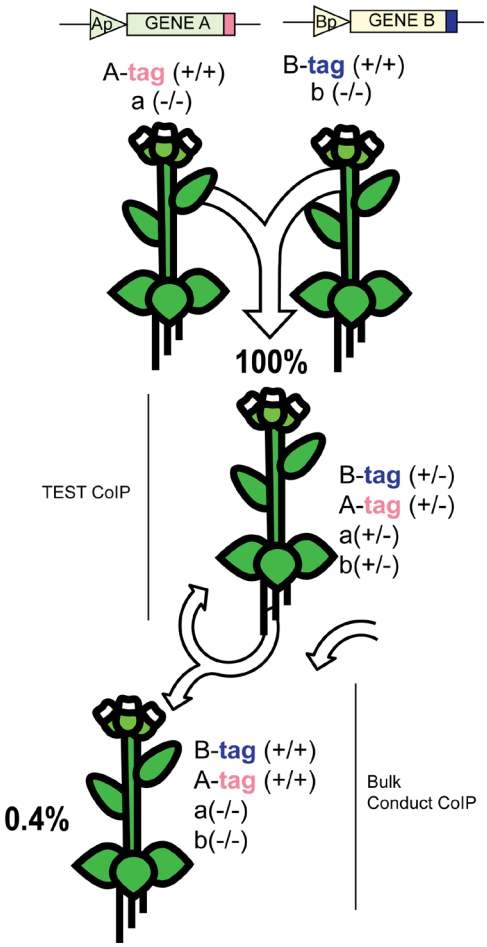

Figure 5. Crossing and selection of transgenic lines stably expressing tagged genes to be used for Co-IP analysis.

9. Centrifuge lysate at 5,000g for 5 minutes at 4ºC.

10. Transfer supernatant to new 1.5ml tubes.[NOTE]

11. Centrifuge lysates again at 5,000g for 5 minutes at 4ºC to remove cell walls and other debris.

12. Save a 30µl fraction of supernantant and estimate its protein concentration using Protein Assay Reagent (BioRad). Add 10µl of 4x SDS sample buffer reagent, boil at 95°C for 5 minutes and store at -20°C until ready to perform immunoblotting. This corresponds to the Total Fraction.[NOTE]

13. Transfer supernatant to an ultracentrifugation tube.

14. Spin at 135,700g for 30 minutes at 4ºC.

15. Transfer supernatant into new tube, quantitate concentration and collect a 30µl aliquot as done for the total fraction on Step 7. This is the Soluble Fraction.[NOTE]

16. Add 1 volume of *membrane solubilization* buffer to membrane pellet remaining in the ultracentrifuge tube (500µl if 1.5 ml used of protein extraction buffer). [NOTE]

17. Sonicate briefly to resuspend membrane pellets. [NOTE]

18. Centrifuge resuspended membranes at 135,700g for 30 minutes at 4ºC to remove any insoluble particles. [NOTE]

19. Transfer the supernatant into a clean microcentrifuge tube and estimate the protein concentration. Adjust the concentrations of all samples to 2.5 µg/µl with *membrane solubilization* buffer and collect a 30µl aliquot as done for the total fraction on Step 7. This corresponds to the membrane fraction that will be used for the IP and will be used as the input controls in imunoblotting. Make sure IP samples are resolved alongside in the same SDS-PAGE and immunoblot. [NOTE]

NOTES AND TROUBLESHOOTING

N6. Tissue can be stored frozen at -80°C Transfer tissue with a pre-chilled spatula back into a 50ml or 15ml conical tube for long-term storage. We have stored tissue for up to a month without any apparent protein degradation. Longer storage might require replication of experiment to test sample decay. The tissue should never be allowed to thaw. Thawing tissue will turn dark green and acquire a soggy appearance.

N7. Additionally the use of polyvinylpolypyrrolidone (PVPP) is recommended for tissues rich in phenolics, particularly important if subsequent mass spectrometric analysis of the samples is required (Discussed in detail in Isaacson et al, 2006). If used, however, PVPP should not be mixed with the extraction buffer before adding to the ground tissue as it is insoluble in it. It should rather be applied directly to the sample when or after grinding at concentrations of approximately 1% (w/v)

N10. The supernatant should contain microsomes and proteins bound to them (since there is no detergent in this buffer) and soluble proteins.

N11. For the amount of buffers used (1: 3 w/v) the concentration of proteins obtained from Arabidopsis *seedlings oscillates around 2.5 µg/µl. Increased amounts of tissue will lead to higher protein concentrations which can saturate the buffering capability of the extraction buffer, causing the pH to drop to levels that may activate acidic proteases (Martinez et al, 2007). In this protocol the pH of the initial lysis buffer used is high in order to prevent acidification if excessive tissue is used. It is advisable to monitor the pH and adjust the buffering capabilities for each application by spotting the prepared lysate on pH indicator paper strips. If the quantitated concentration falls below pH 7, additional extraction buffer should be added to dilute the lysate or higher concentrations of buffer used. Aliquots collected can sit on ice until all fractions have been collected and are ready to be heated at 95ºC in SDS sample buffer.*

N12. In our hands, typical protein concentrations of soluble fractions from 11-day-old seedlings are around 2.5 µg/µl.

N13. The membrane solubilization buffer in this protocol uses the non-ionic detergent Triton X-100 at a concentration of 1%. The stringency of the IP can be enhanced by increasing the concentration of detergents (see Optimization, IVb), which in the case of Triton X-100 has been used as high as 2%. Certain membrane proteins require testing additional concentrations and detergents. For instance, Qi and Katagiri (2009) screened different conditions to emulsify the RLK RPS2, which appears to be present in lipid rafts, and is therefore insoluble in non-ionic detergents. In this study the ionic detergent sodium deoxycholate was found to function for both the solubilization and subsequent pull down of the biotinylated receptor. However, the choice of ionic detergents should be carefully evaluated as they can denature the proteins studied and interfere with subsequent immunoprecipitation steps.

N14. Re-suspending pelleted membranes by pipetting is not advisable since membrane clumps are hard to resuspend and can stick to the tip body. Sonication will prevent this from happening and will fully emulsify membrane proteins within seconds.

N15. In this step non-emulsified membranes will precipitate. It is fundamental to remove insoluble material at this step. Failure to do so will increase the appearance of non-specific protein complexes and may suggest false interactions.

N16. The concentration of the membrane fraction oscillates around 3 µg/µl. The careful standardization of protein concentration across samples is fundamental to guarantee a uniform protein input among IPs, and this should be reflected in an even protein load in the immunoblotting of IP inputs.

III-C. Immunoprecipitation

17. Add 20µl of antibody immobilized to Dynabeads (Step 1) to 1.5 ml of each solubilized microsomal fraction prepared in step 19. Each IP should contain the same volume and same protein concentration in order to obtain consistent results when the experiment is replicated.

18. Incubate antibody with lysates for 1 hour at 4°C gently shaking on nutator.

19. Use a magnetic stand prechilled at 4ºC to immobilize the beads while removing the supernatant (unbound lysate). Wash the beads by resuspending in 500µl of IP buffer. Remove wash buffer each time using the magnetic stand and repeat the washes a total of three times. NOTE

20. After the last wash remove supernatant and add 50µl of 2xSample buffer

21. Boil the beads at 95ºC for 5 minutes.

22. Bring the magnetic beads back to magnetic stand, and transfer the supernatant to new tubes.

STOPPING POINT:
Store samples at -80°C until ready to conduct immunoblotting.

23. Proceed to immunoblotting detecting with the appropriate antibodies.

NOTES AND TROUBLESHOOTING

N22. A fraction of the "flow-through" lysate obtained after separating the non-bound lysate from the beads can be saved for assessing the amount of target protein that bound to the beads. We have observed during some washing steps protein aggregation and precipitation. This occasionally happened when using pre-immobilized antibody resins or when we immobilized antibodies to either magnetic or agarose-based beads as described in this protocol. Precipitation appears to be more likely if the wash buffer lacks emulsifying agents or glycerol, and when a high concentration and volume of protein in the IP lysate is incubated with the beads. Precipitation is observable as tiny clumps of greenish color that sometimes develop a stringy appearance. If any observable precipitation takes place, it is very likely that the protein being Co-IPed will be present in the negative controls. To standardize this step, the concentration of emulsifying agents and glycerol in the wash buffer composition should be optimized, as well as the concentration of the starting lysate to be incubated with the beads.

IV. OPTIMIZATION

IV-A. Antibody choice

A critical component to guarantee a successful IP relies on the choice of a robust antibody. Make sure that antibodies of your choice, whether custom made antibodies for proteins of your interest or commercial antibodies for epitope tags, would be suited for Co-IP and/or immunodetection in prior studies. A quick survey of the *Arabidopsis* literature should be conducted to identify commonly used antibodies and resins in the IP of membrane proteins (Table 1).

IV-B. Standardizing binding: avoiding false positives and false negatives

There is a delicate equilibrium in Co-IP and it relies on standardizing factors that will increase the appearance of false positives (such as non-specific binding to the resin, and low stringency in the washes) or conversely false negatives in which the washes are too stringent.

First, every resin will bind proteins non-specifically to some degree. In order to decrease the amount of non-specific binding, it is advisable to pre-clear the lysate by binding it to the Protein A or Protein G beads in the absence of the epitope-specific antibody. This step is usually not done when pre-immobilized antibody resins are being used. Instead, pre-blocking of the beads with BSA is sometimes done, however, this step tends to work only with beads with a hydrophobic surface. Dynabeads®, for instance, have a hydrophilic surface that makes the BSA blocking step unnecessary.

Second, the stringency of the washes can be adjusted by modifying: (1) the number and volume of washes; (2) the detergent concentration; and (3) the ionic strength of the buffer. An easy way to standardize the IP conditions is to conduct the immunoprecipitation in parallel with as many resin aliquots for each sample as conditions being tested. This way, one single experiment can address the questions of whether: the washes are stringent enough, there is specific interaction with the proteins tested, and whether there is full removal of the proteins IPed from the negative controls.

IV-C. Choosing the right controls

In a Co-IP testing the interactions of proteins A and B, there should be at least four experimental controls: a lysate obtained from an untransformed line; a lysate from a line expressing A; a lysate from a line expressing B; and a lysate from the plant expressing both A and B. If the IP is being done with antibodies specific to A, there should be no protein B detected in the IP with for the sample expressing B alone, the same is true if the reciprocal IP in which antibodies are used against B.

The use of a specificity control is highly desirable. There are concerns about whether hydrophobic domains of emulsified membrane proteins aggregate non-specifically after emulsification which could cause false positives (Angers et al, 2002), or whether partially emulsified membrane-domains pull down membrane proteins that are not directly interacting with the protein of interest, but reside in the vicinity of its membrane microenvironment. One approach to addressing this issue is to use unrelated membrane proteins that are unlikely interactors of the proteins being tested. For example, Kadota et al. (2014) used a membrane-anchored GFP as a control for Co-IP experiments to isolate EFR-GFP receptor complex. It could be easily implemented in transient expression systems such as protoplasts or tobacco. Another approach is to express the receptors separately and after the proteins are extracted and emulsified, they are mixed together. No interaction is usually found indicating that the proteins can only interact when expressed together and indicating that the interaction is not the outcome of non-specific association (Hall, 2005). This approach has been tested in mammalian cells, but to our knowledge has never been implemented in *Arabidopsis*.

Finally, experimental and control Co-IP should be ran alongside on the same SDS-PAGE and blotting membrane. Failure to do this will make band intensity comparisons impossible.

V. ANTICIPATED RESULTS

An example of a typical result for a Co-IP experiment is shown on Figure 6. In general, three biological replicates are conducted to fully validate an interaction. However, Co-IP experiments are performed multiple times in order to standardize the right conditions that demonstrate the interaction or absence thereof. Co-IP is an important tool to demonstrate the association of membrane-proteins, but careful interpretation of these associations should be drawn as the direct interaction observed might be the outcome of multi-protein complexes rather than direct, individual interactions.

ACKNOWLEDGEMENTS

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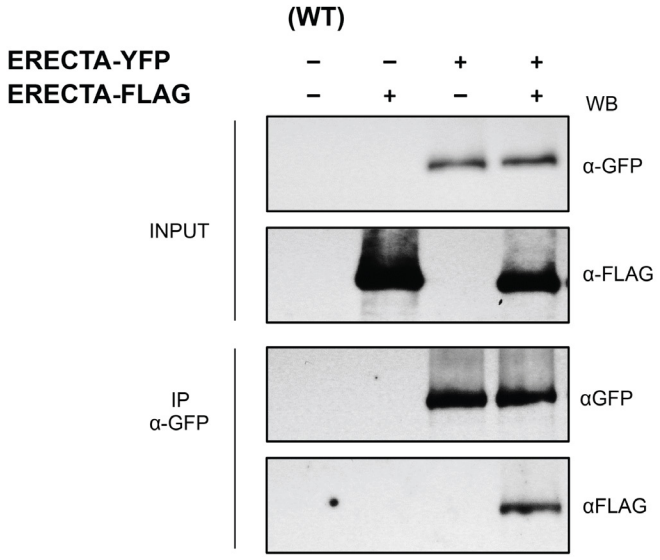

Figure 6. Co-Immunoprecipitation of ERECTA complexes.

Arabidopsis seedlings from wild-type (wt), *erecta* null mutant expressing ERECTA-FLAG, ERECTA-YFP, or both epitope-tagged constructes were subjected to IP using anti-GFP antibody. Both inputs and co-IP fractions (IP α-GFP) were subjected to Immunoblotting (WB) with anti-GFP (α-GFP) or anti-Flag (α-Flag) antibodies. The ERECTA-FLAG can be Co-IPed with anti-GFP antibodies when co-expressed with ERECTA-GFP, showing the protein-protein interactions. This blot is a biological replicate for Lee et al. (2012).

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