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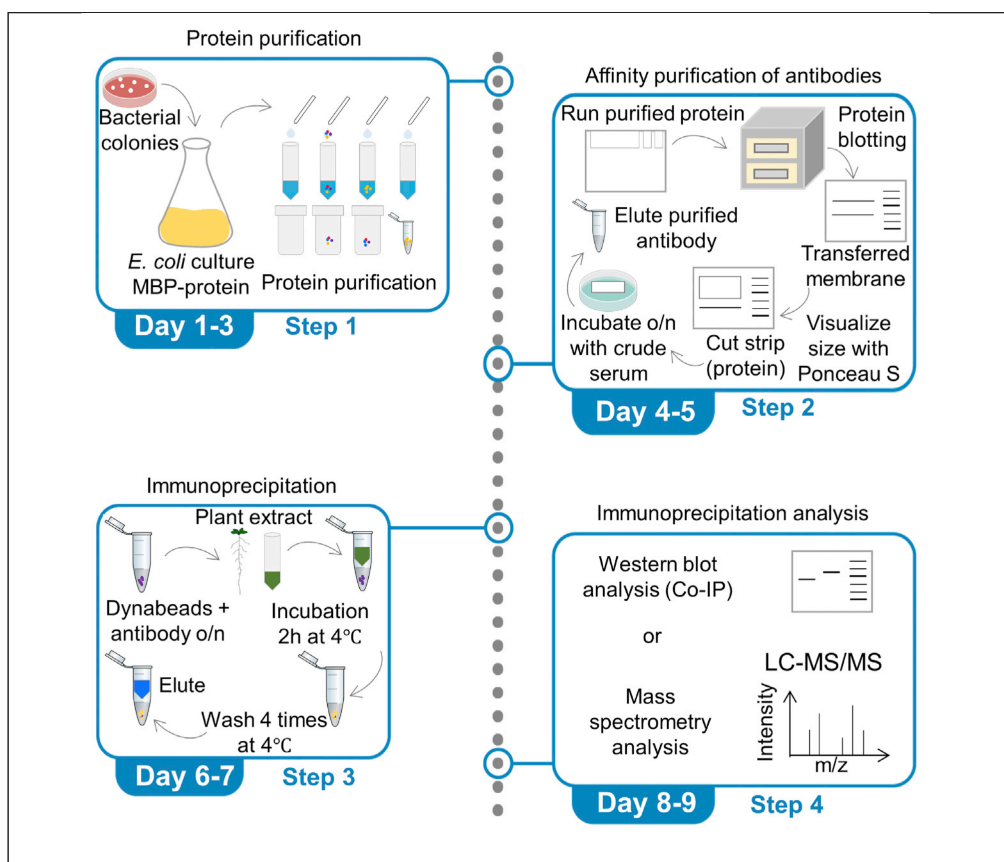
Protocol

Protein and antibody purification followed by immunoprecipitation of MYB and GATA zinc finger-type maize proteins with magnetic beads



Co-immunoprecipitation (Co-IP) is a widely used and powerful approach for studying protein-protein interactions *in vivo*. Here, we describe a protocol for antibody purification and immobilization followed by immunoprecipitation from plant tissue extracts using magnetic beads. The protocol has been used to detect regulators in the *Zea mays* phenylpropanoid pathway. The protocol is amenable to a variety of downstream assays, including western blotting and mass spectrometry.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protein expression and purification of MBP fusions

Affinity purification of antibodies

Immunoprecipitation from maize cell extracts with magnetic beads

Amenable to downstream assays like mass spec and western blot

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Protein and antibody purification followed by immunoprecipitation of MYB and GATA zinc finger-type maize proteins with magnetic beads

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SUMMARY

Co-immunoprecipitation (Co-IP) is a widely used and powerful approach for studying protein-protein interactions *in vivo*. Here, we describe a protocol for antibody purification and immobilization followed by immunoprecipitation from plant tissue extracts using magnetic beads. The protocol has been used to detect regulators in the *Zea mays* phenylpropanoid pathway. The protocol is amenable to a variety of downstream assays, including western blotting and mass spectrometry.
For complete details on the use and execution of this protocol, please refer to Vélez-Bermúdez et al. (2015).

BEFORE YOU BEGIN

Reagent preparation

Timing: 2–4 h

Note: This protocol has been successfully used for transiently expressed proteins in tobacco and Arabidopsis. Here, we describe the specific steps for maize.

The day before the start of the experiment (day 0):

1. Prepare LB, 50% glucose, column buffer, the rich broth, and autoclave.

Note: The glucose should not be autoclaved for more than 15 min to avoid degradation and release of toxic substances.

CRITICAL: We used the pMAL purification system (NEB #E8200S).

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Note: Inoculate 500 mL of rich broth for the purification of each protein.

2. Prepare stocks of antibiotics and IPTG.

    Note: For this protocol we prepared a stock of 50 mg/mL of Ampicillin, the antibiotics can vary according with the vectors used. The IPTG should be prepared to a stock of 0.1 M. Both reagents can be sterilized with a 0.22 μm filter.

    Note: The antibiotics depend on the selection of the plasmid-encode genes used for the protein expression.

3. Streak a single colony of the bacteria carrying the plasmid in a LB + antibiotic plate and incubate for 18 h at 37°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-ZmMYB11. Working dilution: 1:100	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Rabbit polyclonal anti-ZmZML2. Working dilution: 1:100	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
ECL Rabbit IgG, HRP-linked whole Ab from donkey	GE Healthcare	NA934
Bacterial and virus strains		
ECOS™ 21 Competent Cells	Yeastern Biotech	Cat#FYE207-40VL
Rosetta™(DE3) Competent Cells	Novagen	Cat#70954
Biological samples		
Maize (*Zea mays*) B73 leaves (9 days old)	USDA	https://npgsweb.ars-grin.gov/gringlobal/search
Chemicals, peptides, and recombinant proteins		
BD Difco™ Dehydrated Culture Media: LB Broth, Miller (Luria-Bertani)	BD Difco	Cat#BD 244620
BD Difco™ Dehydrated Culture Media: LB Agar, Miller (Luria-Bertani)	BD Difco	Cat#BD 244520
D-(+)-Glucose	Sigma-Aldrich	Cat#G8270
Bacto™ Tryptone	Thermo Fisher Scientific	Cat#211669
Yeast Extract	Merck	Cat#70161
Sodium chloride (NaCl)	Merck	Cat#7647-14-5
Ampicillin	Sigma-Aldrich	Cat# A9393
Phenylmethylsulfonyl fluoride (PMSF)	Merck	Cat#11359061001
Isopropyl β-D-thiogalactoside, Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Merck	Cat#I6758
cOmplete, EDTA-free protease inhibitor cocktail (1 unit/25×)	Roche	Cat# 11873580001
Bradford Reagent	Merck	Cat#B6916-500ML
Amylose Resin	Biolabs	Cat#E8021S
D-(+)-Maltose monohydrate	Sigma-Aldrich	Cat# M9171
Trizma® hydrochloride solution (pH 7.4)	Sigma-Aldrich	Cat#T2194-1L
UltraPure™ 1 M Tris-HCl, pH 8.0	Invitrogen	Cat#15568025
Tris-HCl (pH 6.8)	Abcam	Cat#ab286853
UltraPure™ 0.5 M EDTA, pH 8.0	Invitrogen	Cat#15575020
Nupage 4–12 Bis-Tris Gel	Thermo Fisher Scientific	Cat#NP0335B0X
Ponceau S Solution	Abcam	Cat#ab270042
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat# A2153
PBS - Phosphate-Buffered Saline (10×) pH 7.4, RNase-free	Invitrogen	Cat# AM9625

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TWEEN® 20	Sigma-Aldrich	Cat#P1379
Glycine	Sigma-Aldrich	Cat#G7126
Hydrochloric acid	Sigma-Aldrich	Cat#320331
Sodium hydroxide solution	Sigma-Aldrich	Cat#2105
Dynabeads Protein A	Invitrogen	Cat#10001D
MG-132, 10 mM (1 mL in DMSO)	Selleckchem	Cat#S2619
DL-Dithiothreitol solution	Sigma-Aldrich	Cat#43816
IGEPAL® CA-630	Sigma-Aldrich	Cat#I8896
2-Mercaptoethanol	Sigma-Aldrich	Cat#M6250
Glycerol	Sigma-Aldrich	Cat#G5516
Sodium dodecyl sulfate solution, Molecular Biology Grade (10% w/v)	Promega	Cat#V6551
Bromophenol Blue sodium salt	Sigma-Aldrich	Cat#B5525
PageRuler™ Prestained Protein Ladder, 10–180 kDa	Thermo Fisher Scientific	Cat#26616
NuPAGE™ MES SDS Running Buffer (20×)	Thermo Fisher Scientific	Cat# NP0002
Methanol	Merck	Cat#106009
Autoclaved distilled water	n/a	N/A
Liquid nitrogen	n/a	N/A
Recombinant ZmMYB11-MBP	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Recombinant ZmZML2-MBP	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Critical commercial assays		
pMAL™ Protein Fusion & Purification System	New England Biolabs	Cat#E8200S
Trans-Blot Turbo RTA Mini 0.2 µm PVDF Transfer Kit, for 40 blots	Bio-Rad	Cat#170-4272
Dynabeads™ Co-Immunoprecipitation Kit	Invitrogen	Cat#14321D
Deposited data		
Raw and analyzed data	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Recombinant DNA		
Plasmid: pDONR207	Invitrogen	https://www.snapgene.com/resources/plasmid-files/?set=gateway_cloning_vectors&plasmid=pDONR207
Plasmid: pDESTTH1	(Hammarström et al., 2002)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2373440/
Full-length cDNA encoding ZmMYB11	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Full-length cDNA encoding ZmZML2	(Vélez-Bermúdez et al., 2015)	https://academic.oup.com/plcell/article/27/11/3245/6096690?login=false
Other		
Safe lock tubes 2.0 mL	Eppendorf	Cat#30120094
Eppendorf® LoBind microcentrifuge tubes	Merck	Cat#EP0030108302-100EA
Amicon® Ultra-4 centrifugal filter unit	Millipore	Cat#UFC805024
Pipette filter tips, 10 µL	Labcon	Cat# 1051-800-000-9
Pipette filter tips, 200 µL	Labcon	Cat# 1059-800-000-9
Pipette filter tips, 1000 µL	Labcon	Cat# 1097-965-008-9
Eppendorf centrifuge	Eppendorf	Cat#5417R
Trans-Blot Turbo Transfer System	Bio-Rad	Cat#1704150
Econo-Pac chromatography columns	Bio-Rad	Cat#732-1010
Sonicator Ultrasonic Processor	Misonix, Inc.	Cat# S-4000
PowerWave XS2, microplate spectrophotometer	Agilent Technologies	N/A
MF-Millipore® membrane filter, pore size 0.22 µm	Millipore	Cat#GSWP04700
Mini gel tank	Invitrogen	Cat#A25977
BioVoltTM power supply	BioProducts	Cat#SBE 250
Cold room	N/A	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mini heating dry bath incubator, MD-MINI	Ms major science	N/A
DynaMag™-2 magnet	Invitrogen	Cat#12321D
Vortex-Genie 2	Scientific Industries, Inc.	Cat#SI-0236
ELMI Intelli-Mixer™ RM-2L	ELMI	N/A

MATERIALS AND EQUIPMENT

Note: We suggest using molecular biology grade reagents and to autoclave and filter all the following buffers.

Rich medium, glucose and ampicillin		
Reagent	Final concentration	Amount
Tryptone	1%	10 g
Yeast extract	0.5%	5 g
NaCl	0.5%	5 g
Glucose	0.2%	2 g
ddH2O	N/A	999 mL
Ampicillin (Stock 100 μg/mL)	100 μg/mL	1 mL
Total	N/A	1 L

Note: Adjust the pH to 5.8 using sodium hydroxide (NaOH). Autoclave for 15 min and add the ampicillin just before inoculation with 5 mL culture.

⚠ CRITICAL: Sodium hydroxide (NaOH) is a base and caustic substance. It is highly recommended to wear protecting gloves, clothing, eye, and face protection.

Note: Prepare immediately before use, cOmplete, EDTA-free protease inhibitor cocktail dilute to 1× and 1 M Maltose.

Column buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (Stock 1 M)	20 mM	20 mL
NaCl	200 mM	11.7 g
EDTA (Stock 0.5 M)	1 mM	2 mL
ddH2O sterile	N/A	Up to 1 L
Total	N/A	1 L

Note: Sterilize with a 0.22 μm filter.

Elution buffer		
Reagent	Final concentration	Amount
Maltose (Stock 1 M)	10 mM	1 mL
Column buffer	N/A	99 mL
Total	N/A	100 mL

Note: Prepare immediately before use.

NuPAGE™ MES SDS running buffer		
Reagent	Final concentration	Amount
NuPAGE™ MES SDS running buffer (20×)	1 ×	50 mL
ddH2O sterile	N/A	950 mL
Total	N/A	1 L

Note: Prepare immediately before use.

Transfer buffer BIO-RAD		
Reagent	Final concentration	Amount
Transfer buffer BIO-RAD (5×)	1 ×	100 mL
Methanol	N/A	100 mL
ddH2O sterile	N/A	300 mL
Total	N/A	500 mL

Note: Prepare immediately before use.

⚠ CRITICAL: Methanol is a wood alcohol highly flammable and toxic. Avoid contact with skin and eyes. Avoid inhalation of vapor and keep away from sources of ignition.

Note: For the affinity purification of antibodies, prepare PBS - Phosphate-buffered saline 1× + 0.1% TWEEN® 20 and a 0.2 M of glycine solution adjusted to pH 2.6 with HCl and sterilize with a 0.22 μm filter. The 5% bovine serum albumin solution should be freshly made and keeping at 4°C until use.

⚠ CRITICAL: Glycine is a proteinogenic amino acid that may cause irritation. Avoid contact with eyes, skin, and clothing.

⚠ CRITICAL: Hydrochloric acid also known as muriatic acid, is a corrosive substance and toxic if inhaled. Handle the reagent while under a fume hood, wear a chemical-resistant apron, gloves, and goggles.

Washing buffer Dynabeads		
Reagent	Final concentration	Amount
PBS - Phosphate-buffered saline (10×)	0.2 M	10 mL
Phenylmethylsulfonyl fluoride (PMSF, Stock 100 mM)	1 mM	1 mL
ddH2O sterile	N/A	Up to 89 mL
Total	N/A	100 mL

⚠ CRITICAL: Phenylmethanesulfonyl fluoride is a serine protease inhibitor that can cause acute oral toxicity, skin, and eye irritation. It is recommended to wear protective gloves, googles, clothing, and face protection.

NaCl Solution		
Reagent	Final concentration	Amount
NaCl	5 M	5.85 g
ddH2O sterile	N/A	Up to 100 mL
Total	N/A	100 mL

Note: Sterilize with a 0.22 μm filter.

Extraction buffer (Option A)		
Reagent	Final concentration	Amount
IP buffer (Invitrogen Co-IP kit, 10×)	1 ×	10 mL
NaCl	100 mM	0.2925 g
DL-dithiothreitol (Stock 1 M)	2 mM	0.1 mL
MG132 (10 mM)	50 μM	0.25 mL
cOmplete, EDTA-free protease inhibitor cocktail	2 ×	2 tablets
Phenylmethylsulfonyl fluoride (PMSF, Stock 100 mM)	1 mM	0.5 mL
ddH2O sterile	N/A	39.15 mL
Total	N/A	50 mL

⚠ CRITICAL: DL-dithiothreitol (DTT) is a reducing agent that can cause skin, eye and respiratory irritation. It is advised to wear appropriate protective equipment.

Extraction buffer (Option B)		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (Stock 1 M)	50 mM	2.5 mL
NaCl (Stock 5 M)	150 mM	1.5 mL
IGEPAL-CA-630	0.75%	0.375 mL
MG132 (10 mM)	50 μM	0.25 mL
cOmplete, EDTA-free protease inhibitor cocktail	2 ×	2 tablets
ddH2O sterile	N/A	45.375 mL
Total	N/A	50 mL

Washing buffer 1		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (Stock 1 M)	50 mM	2.5 mL
NaCl (Stock 5 M)	150 mM	1.5 mL
IGEPAL-CA-630	0.25%	0.125 mL
MG132 (10 mM)	50 μM	0.25 mL
cOmplete, EDTA-free protease inhibitor cocktail	2 ×	2 tablets
ddH2O sterile	N/A	43.125 mL
Total	N/A	50 mL

Washing buffer 2		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (Stock 1 M)	20 mM	1 mL
ddH_2O sterile	N/A	49 mL
Total	N/A	50 mL

1.5× Sample buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 6.8 (Stock 1 M)	62.5 mM	3.125 mL
2-Mercaptoethanol (14.3 M)	2 mM	0.00699 mL
Glycerol	10%	5 mL
Sodium dodecyl sulfate solution, molecular biology grade (10% w/v)	2%	10 mL
Bromophenol Blue sodium salt	0.01%	0.005 g
ddH_2O sterile	N/A	31.868 mL
Total	N/A	50 mL

⚠ CRITICAL: 2-Mercaptoethanol is very toxic; wear protective gloves, clothing, eye, and face protection, and manipulate the reagent as well the buffer under a chemical hood.

⚠ CRITICAL: Sodium dodecyl sulfate (SDS) is an organic compound that can cause skin, eye, and respiratory irritation. Avoid inhalation of dusts, substance contact, and keep away from heat and sources of ignition.

STEP-BY-STEP METHOD DETAILS

Protein expression and purification—3 days

This first part of the protocol is an optimization of the pMAL™ Protein Fusion & Purification System protocol (NEB #E8200S). pMAL is one of the most used systems for the purification of fusion proteins. An advantage of this method is the fact that maltose-binding protein (MBP) binds to a positively charged resin at pH 5.5, where most other proteins do not bind. Moreover, the MBP-fusion protein can be purified in a one-step MBP affinity purification with high quality. This step is critical since the resultant purified protein will be used for the second step of this protocol.

Pre-culture

⏲ Timing: 10 min; day 1

Note: For this protocol we used the pDESTH1 vector (Ampicillin resistant).

1. Add 5 mL LB medium, 0.2% glucose, and 10 μL Ampicillin (stock 50 mg/ mL) to a culture tube.
2. Inoculate with a single bacterial colony from the agar plate (from day 0).
3. Culture at 37°C and shake for 16 h.

Note: The LB medium can be stored at 20°C–25°C for 1 month. 50% glucose can be stored at 4°C for 6 months and Ampicillin should be store at -20 for up to 6 months.

Protein induction

⏲ Timing: 5–6 h; day 2

4. In an Erlenmeyer flask, inoculate 500 mL of rich medium glucose (0.2%) and Ampicillin (100 μg/ mL) with 5 mL of a culture of cells grown for 16 h containing the fusion plasmid. Mix well and measure the starting OD of the culture.

Note: The rich medium can be stored at 20°C–25°C for up to 1 month.

Note: Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the *E. coli* host, one of which is an amylase that can degrade the amylose on the affinity resin.

5. After 2 h, measure the optical density (OD) of the culture $A_{600} \sim 0.5$ (2 × 108 cells/mL), add IPTG to a final concentration of 1 mM, and incubate the culture at 37°C for 3 h.

   Note: IPTG can be stored at −20°C in dark for up to 9 months.

   Note: The time and temperature until the OD of the culture reach an optical density of 0.5 can vary with the host strain and the stability of the protein.

6. Divide the induced culture to two 500 mL polypropylene bottles (e.g., Beckman Coulter), centrifuge the culture at 4,000 × *g* for 20 min at 4°C, discard the supernatant, resuspend the pellets in 25 mL column buffer (500 mL of culture), and freeze the sample for 16 h at −20°C.

   ⚠ CRITICAL: To avoid degradation of the protein, add 0.1 mM PMSF (phenyl methylsulfonyl fluoride) and protease inhibitor cocktail (1×). Harvest the cells quickly and keep them chilled.

   Note: PMSF can be stored at −20°C in dark; it should be stable for at least two years. The column buffer can be stored at 4°C for up to 6 months.

   Pause point: The resuspended pellet can be stored at −20°C for up to one month.

Protein purification

Timing: 5–6 h; day 3

7. Thaw the sample in cold water.
8. Transfer the sample to a 50 mL Nalgene centrifuge tube, place in an ice bath, and sonicate in short pulses of 15 s.

   Note: The sonication program parameters used were: amplitude: 37, process time: 2:00 min, pulse on: 0:15 s, pulse off: 0:15 s, temperature monitoring and shutdown: 115°C.

   Note: Keep the samples on ice to avoid degradation of the protein.

9. Centrifuge the sample at 20,000 × *g* for 20 min at 4°C, keep the supernatant (crude extract), and dilute the crude extract 1:2 with column buffer (total = 50 mL).

   Note: It is important to add 0.1 mM of PMSF (phenyl methylsulfonyl fluoride) and protease inhibitor cocktail (1×) to avoid degradation of the protein.

10. Transfer 3 mL of amylose resin to a 1.5 × 12 cm polypropylene column.

    Note: The binding capacity of the amylose resin is 2–3 mg fusion protein/mL bed volume.

11. Wash the column five times with 15 mL column buffer.
12. Load the diluted crude extract at a flow rate of no more than 1.87 mL/min.

    Note: The flow rate should be 1.87 mL/minute for a 1.5 cm column ([50 × $(1.5)^2$] mL/hour).

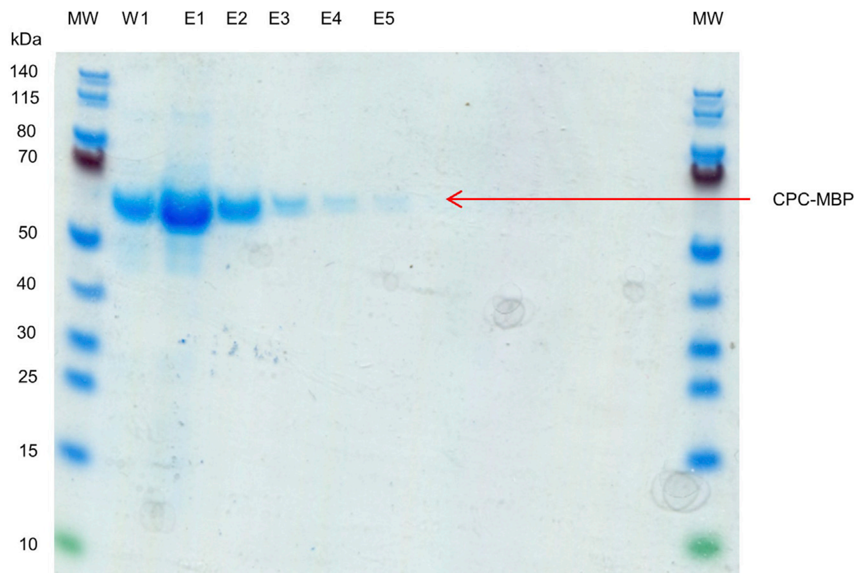

Figure 1. Recombinant CPC-MBP fusion protein purification
The figure shows an example of protein purification using the protocol described here. Fractions were analyzed by SDS–PAGE (MW molecular weight markers). E; elution fractions.

13. Wash the column with 36 mL of column buffer at a flow rate of no more than 3.75 mL/min.

    Note: The flow rate should be 3.75 mL/minute for a 1.5 cm column ([100 × $(1.5)^2$] mL/hour).

14. Elute the fusion protein with column buffer + 10 mM maltose and collect 15 fractions of 500 μL each.

    Note: The elution buffer (column buffer + 10 mM maltose) should be prepared fresh.

    Note: The maltose solution can be stored at 4°C for 3 days; for long-term storage (6 months), stock solutions should be stored at −20°C. It is recommended that the maltose solution is prepared and used at the same day.

15. Run 15 μL of each elution in an acrylamide gel and quantify using Bradford protein assay, the fractions containing the purified protein (Figure 1).
16. If the concentration is lower than desired, pool the protein-containing fractions and concentrate to about 1 mg/mL using an Amicon Ultra-0.5 centrifugal filter unit.

    Note: The pore size of the Amicon Ultra centrifugal filter should be selected according to the molecular weight of the purified protein.

17. Perform a western blot using 5 μg of purified protein to check the quality. A representative western blot is shown in Figure 2.

    Pause point: The purified protein can be stored for 1 day on ice at 4°C.

Affinity purification of antibodies—2 days

Timing: 10 min; day 4

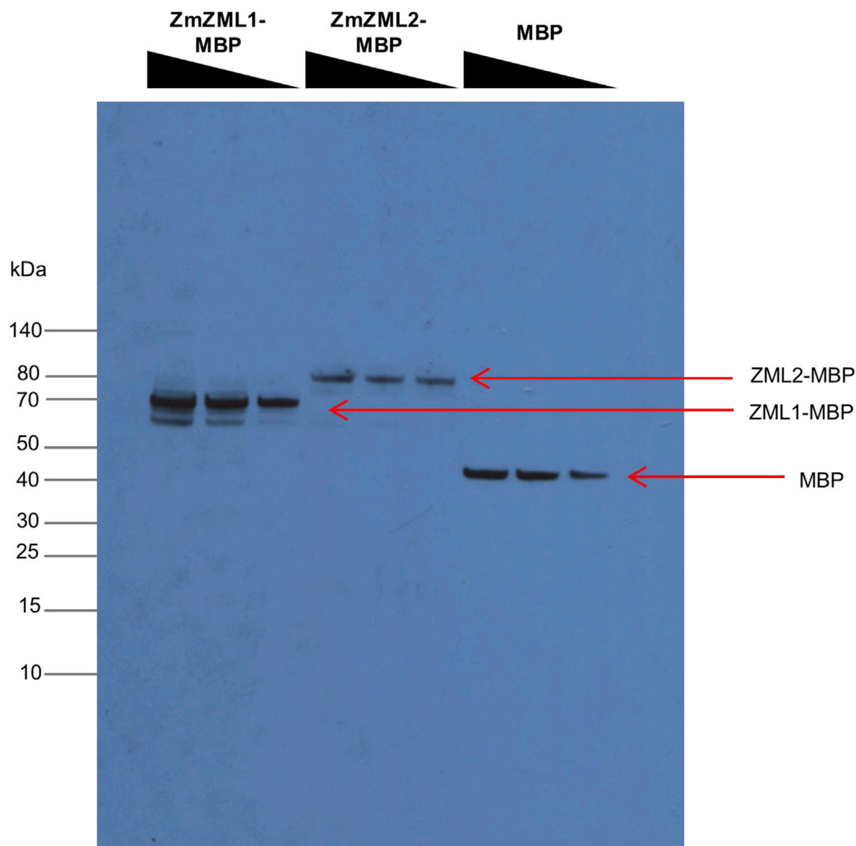

Figure 2. Immuno-detection of purified ZML2, ZML1-MBP, ZML2-MBP, and MBP proteins using purified antibodies raised against MBP

To validate the quality of the purified protein, three different dilutions (50, 40 and 30 ng) of two MBP fusion proteins (ZML1-MBP and ZML2-MBP) and MBP alone as a control were loaded in a gel and a western blot was performed using antibodies raised against MBP. The red arrows indicate the presence of the proteins. Adapted from Vélez-Bermúdez et al. (2015).

This second part of the protocol is critical for a successful immunoprecipitation assay. Currently, different protocols for affinity purification of antibodies are available. Here, we offer an inexpensive and accurate method to obtain a pure and intact antibody for the IP experiment.

18. Take 800 μg of the purified protein and run on an SDS-PAGE gel.

    Note: This protocol is an optimization of the protocol provided by Agrisera (https://www.agrisera.com/en/info/affinity-purification-of-antibodies-low-amount-of-antigen-available.html).

    Note: We suggest using Nupage 4–12 Bis-Tris gel 1.5 mm × 10 wells (Invitrogen, NP0335B0X) and a run time of 38 min. The sample can be loaded into the lanes 1–8. Keep two lanes empty and use the last lane for the protein marker (Figure 3A).

    Note: The BIO-RAD transfer buffer should be stored at 4°C before use for at least 30 min.

    Note: This protocol can be use when the amount of purified protein is at least 230 μg.

19. Transfer the protein onto a membrane.

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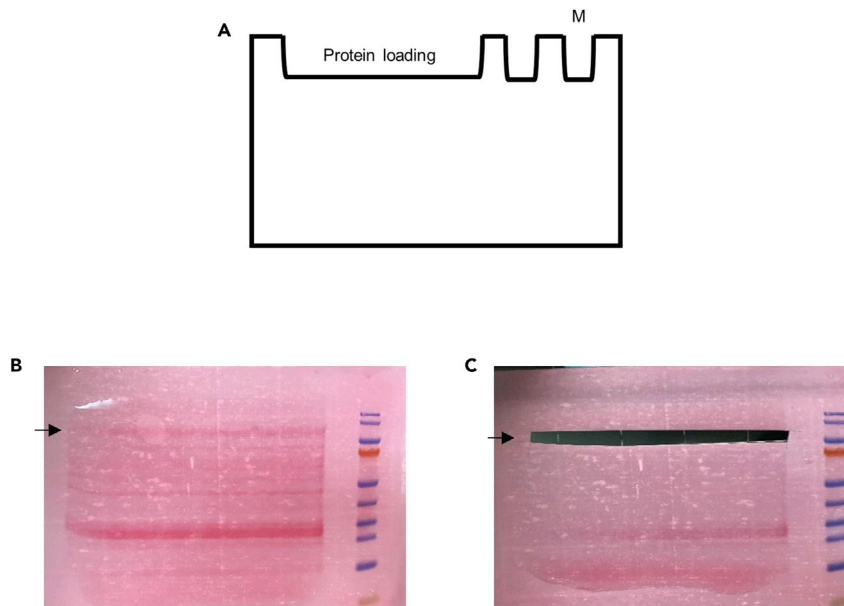

Figure 3. Representative example of affinity antibody purification
(A) Scheme of Bis-Tris gel showing how to load the protein; M indicates the line for the protein marker.
(B) Ponceau S staining of the membrane after transferring the protein from the gel to visualize the size of the corresponding antigen. Purified protein is indicated by an arrow.
(C) Membrane after removing the strip containing the protein of interest. The absence of the strip containing the purified protein is indicated by an arrow.

Note: We use the Bio-Rad Trans-Blot Turbo Transfer System. Standard SD protocol, 30 min, and up to 1.0 A; 25 V.

20. Stain the membrane with Ponceau S (Figure 3B).
21. Locate the size corresponding to your protein, cut out the band, and mark the protein side (Figure 3C).
22. De-stain the strip with 1× PBS-Tween 0.1% and block with 5% BSA (fatty acid-free) in 10 mL 1× PBS for 1 h at 25°C.

Note: PBS 1× can be stored at 20°C–25°C for up to 1 year. BSA should be prepared immediately before use and stored at 4°C.

23. Wash (by shaking) the strip 5 times with 10 mL 1× PBS-Tween 0.1%.
24. Incubate the strip with the antibody serum for 16 h at 4°C.

Note: Since in our case the specific antibody constitutes 1%–10% of the total antibody pool, we have used at least 5 mL of the antibody (if the concentration of the antibody is ~1 mg/mL) and 1 mg of the antigen (purified protein).

Note: 1 mg of purified protein can theoretically bind around 0.5–2 mg of the specific antibody.

Note: Since the antibodies have been produced in rabbit, this step is required to obtain a clean antibody for the immunoprecipitation.

25. Remove the serum.

Note: We suggest storing the antibody at −20°C until validation of the purified antibody.

26. Wash the strip twice with 10 mL 1× PBS-Tween 0.1% during 5 min each time.
27. Wash the strip twice with 10 mL 1× PBS during 5 min each time.

Note: Measure the last wash with 1× PBS spectrophotometrically at A_{280} to make sure that the optical density it is below 0.1.

28. Collect 11 elutions of the purified antibody bound to the strip with 0.2 M glycine pH 2.6 in 1.5 mL Eppendorf tubes.

Elution	0.2 M glycine pH 2.6	1 M Tris pH 8.0	Incubation time (minutes)
E1	200 μL	60 μL	15
E2	320 μL	96 μL	30
E3	200 μL	60 μL	10
E4	200 μL	60 μL	10
E5	290 μL	87 μL	10
E6	385 μL	115.5 μL	10
E7	400 μL	120 μL	10
E8	405 μL	121.5 μL	10
E9	1270 μL	381 μL	10
E10	990 μL	297 μL	10
E11	1000 μL	300 μL	10

Note: The glycine solution can be stored at 20°C–25°C for up to one month.

Note: 1 M Tris pH 8.0 buffer is used to neutralize the glycine.

Note: Wash the strip 3 times with 1× PBS for 5 min each time and store samples at −20°C for further use.

29. Store the elutions at −20°C until use.

Pause point: The purified antibody can be stored at −20°C for 1 month or at −80°C for up to 2 years.

30. Perform a western blot using recombinant protein and total plant protein extract to verify the quality of the different fractions of the purified antibody (Figure 4).

Immunoprecipitation of protein complexes—2 days

Timing: 10 min; day 6

Immunoprecipitation is one of the most widely used methods for studying protein-protein interactions. Here, an antibody against a specific target protein is used to detect an immune complex with that target under a particular condition. This part of the protocol details the associated procedures of co-immunoprecipitation and considerations associated with this experiment. The use of a clean and specific secondary antibody is critical for the successful detection of a specific putative partner through western blot analysis.

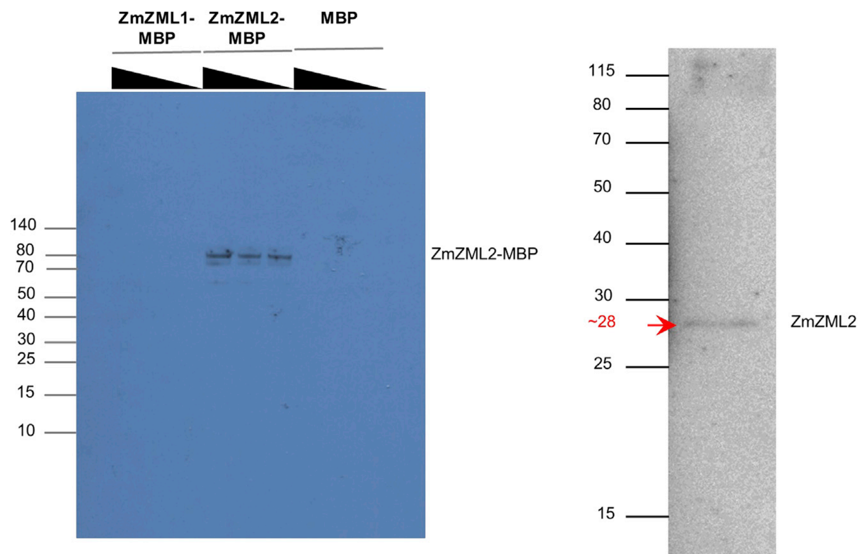

Figure 4. Immuno-detection of ZmZML2 using the purified antibody raised against this protein
(Left) Immunoblot using three different dilutions of the purified recombinant proteins ZML1-MBP, ZML2-MBP, and MBP (50, 40 and 30 ng). The membrane was hybridized with an antibody raised against ZmZML2, and a band of 80 KDa corresponding to the ZML2-MBP was detected. (Right) Western blot analysis using 50 μg of total protein extract from leaves of 9-day-old maize B73 plants hybridized with the purified antibody raised against ZmZML2. The native ZML2 is detected at 28 kDa. Adapted from Vélez-Bermúdez et al. (2015).

Antibody coupling

31. Resuspend the Dynabeads protein A in a vial.

    Note: Do not vortex the Dynabeads, mix gently by hand.

32. Transfer 50 μL (1.5 mg) of Dynabeads protein A into a 2 mL low-binding protein Eppendorf tube for each immunoprecipitation (antibody), place on the magnet and discard the supernatant.
33. Wash the Dynabeads three times with 200 μL 1× PBS+1 mM PMSF.

    Note: The washing buffer for Dynabeads should be prepared immediately before use and stored at 4°C until use.

    Note: During the IP protocol, the Dynabeads should be washed by turning the Eppendorf tubes 4 full turns without removing the tube from the magnet. Discard the supernatant each time, it is important to avoid that the Dynabeads dry out.

    Note: We suggest using low-binding protein Eppendorf tubes for all the steps involving Dynabeads.

34. Incubate the Dynabeads with the purified antibodies for 16 h at 60 rpm rotation at 4°C.

    Note: The final volume (antibody + 1× PBS + 1 mM PMSF) should be 200 μL per 50 μL of Dynabeads.

    Note: 50 μL of Dynabeads correspond to 1.5 Ab/mg beads for IgG.

Note: For an efficient immunoprecipitation the amount of purified antibody is typically between 5 to 7 μg/mL of Dynabeads.

Cell lysate and Co-IP

Timing: 10 min; day 7

35. Grind 1 g plant material in liquid nitrogen in a pre-chilled mortal to a fine powder, resuspend in 500 μL extraction buffer A or B, and incubate in ice during 30 min with 5 min vortex every 5 min.

CRITICAL: The extraction buffer (Option A) is supplied in the Dynabeads™ Co-Immunoprecipitation Kit (Invitrogen). In case of selecting this buffer, continue the process using the buffers included in the kit.

Note: The extraction buffer (Option A) should be prepared immediately before use and stored at 4°C. IP buffer (Invitrogen Co-IP kit, 10×) can be stored at 20°C–25°C according to the manufacturer's instructions. NaCl can be stored at 20°C–25°C for up to 28 days. DL-dithiothreitol (DTT) can be stored at 4°C for 1 year or indefinitely at −20°C in dark conditions. MG132 can be stored at −20°C for up to 1 year. cOmplete, EDTA-free protease inhibitor cocktail tablets should be stored dry at 4°C; stock solutions can be stored at 4°C for 1–2 weeks or at −20°C for at least 12 weeks. Phenylmethylsulfonyl fluoride can be stored at −20°C for at least two years.

Note: The extraction buffer B is described in Abraham-Juárez (2019). Buffer B is suitable for proteins that are dynamically transported between nucleus and cytosol in native conditions, and can be used as an alternative to the kit. If your protein has another subcellular localization or some specific conditions, the buffer should be optimized for this purpose.

Note: The extraction buffer (Option B) should be prepared immediately before use and stored at 4°C. Tris-HCl, pH 7.4 (Stock 1 M) may be stored at 2°C–8°C for at least 1 year. NaCl can be stored at 20°C–25°C for up to 28 days. IGEPAL-CA-630 can be stored at 8°C–25°C in a cool and dry area for up to 5 years. MG132 can be stored at −20°C for up to 1 year.

CRITICAL: Liquid nitrogen is a cryogenic that expands 695 times in volume when it vaporizes, has no warning properties such as odor or color, and may cause asphyxiation hazards. Use protective clothing and handle in well-ventilated areas.

36. Centrifuge at 11,200 × *g* at 4°C for 10 min.
37. Collect the supernatant and transfer to a fresh Eppendorf tube.
38. Quantify the total protein from the lysate using a protein assay.

Note: We use the Pierce 660 nm protein assay (Thermo Fisher).

39. Wash twice the Dynabeads-Ab complex with 1× PBS + 1 mM of PMSF during 5 min each time at 4°C.
40. Incubate the Dynabeads-Ab complex with 500 μL (1.5 mg) of the lysate for 3 h at 4°C in rotation. Keep 100 μL of the lysate for the input.

Note: Depending on the stability and abundance of the protein complex of interest, you can use 1–20 mg of total protein extract.

Note: The incubation time for the Dynabeads-Ab complex + lysate depends on the antibody. For purified antibodies, we suggest to incubate 3 h, commercial antibodies should be incubated for 1 h to avoid background.

41. Wash the Dynabeads-Ab complex three times with 200 μL washing buffer 1 for 5 min each time at 4°C.
42. Wash the Dynabeads-Ab-Ag complex once with 200 μL washing buffer 2 in rotation at 30 rpm for 5 min at 4°C.

Note: Washing buffers 1 and 2 should be prepared immediately before use and stored at 4°C.

43. Place the Dynabeads-Ab-Ag complex on the magnet and discard the supernatant, immediately resuspend in 35 μL 1.5× sample buffer, and boil at 95°C for 5 min.

Note: 1.5× Sample buffer can be stored at 4°C for at least 1 year. Tris-HCl, pH 6.8 (Stock 1 M) can be stored at 2°C–8°C for at least 1 year. 2-mercaptoethanol (14.3 M) can be stored at 2°C–8°C for at least 3 years. Glycerol 50% can be stored at −20°C for 2 weeks or indefinitely at −80°C.

Store stock solution of sodium dodecyl sulfate solution (10% w/v) at 25°C for use with a recommended minimum shelf life of 3 years. Bromophenol Blue sodium salt can be stored at 20°C–25°C for up to 2 years.

44. Place the Dynabeads-Ab-Ag complex on the magnet and recover the supernatant in a fresh Eppendorf tube.

Note: You can collect a second elution of 30 μL and recover in another Eppendorf tube (do not mix elutions).

45. Use the first elution (35 μL) for western blot or mass spectrometry analysis (LC-MS/MS) (Figure 5).

Pause point: The samples can be stored at −80°C until immunoblotting or mass spectrometry analysis.

Note: Keep 100 μL of lysis sample to use as a control for the experiment.

Note: To avoid background during western blot analysis, we suggest using Clean-Blot™ IP detection reagent (HRP), Thermo Scientific, cat# 21230 for polyclonal antibodies.

Note: For mass spectrometry analysis, you can also make the digestion directly in the beads.

EXPECTED OUTCOMES

Co-IP is a useful tool to demonstrate protein-protein interactions and interactions between protein complexes. Following this detailed protocol, we were able to identify a novel set of putative interaction partners in Arabidopsis through Co-IP followed by sequencing, and successfully confirmed direct interaction between two proteins in maize using Co-IP experiments followed by western blot analysis. It is highly recommended that the experiments are performed multiple times to standardize the conditions to study the proteins of interest.

LIMITATIONS

The main and critical limitation for the Co-IP experiment is the quality of the antibody. We have included here our method for purifying antibodies, which has been successfully employed to purify various custom-made antibodies raised against proteins in maize and Arabidopsis. For Co-IP experiments preceding sequencing, it is critical to standardize the experiment to avoid non-specific binding to the Dynabeads or a low amount or stringency in the washes, which may lead to false positives in the first and false negatives in the second case. In Co-IP experiments that are followed by western

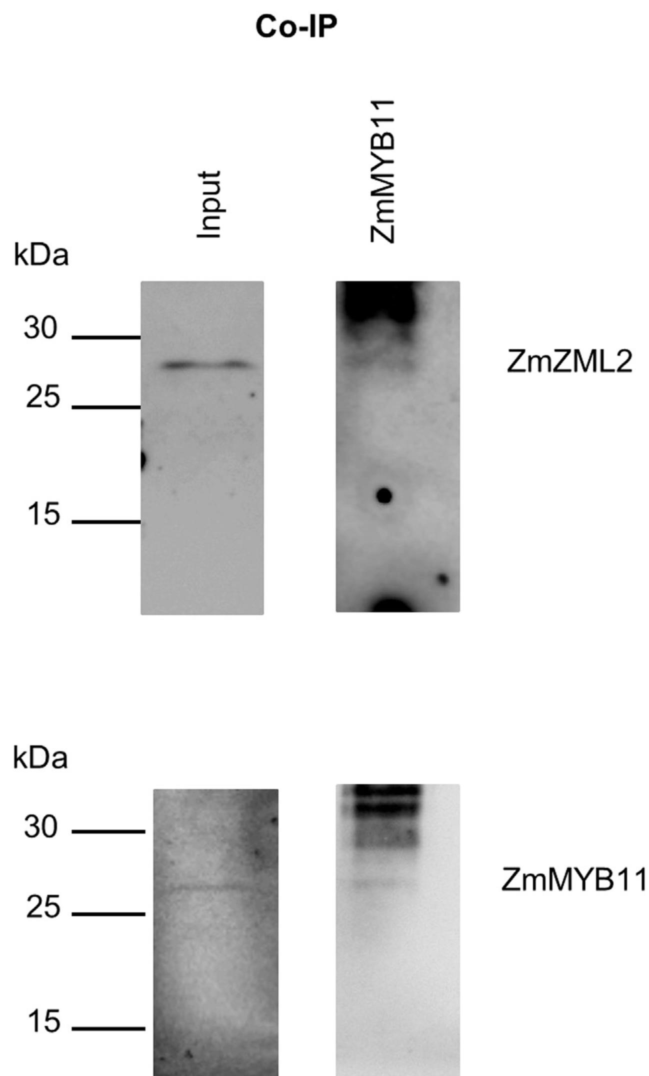

Figure 5. Co-immuno-precipitation of ZmMYB11 and ZmZML2
The presence of ZML2 and MYB11 was detected by immunoblot analyses using ZML2 and MYB11 antibodies. Input extracts from 9-d-old maize leaves were immunoprecipitated with MYB11 antibody, and ZML2 was detected by immunoblot. The presence of MYB11 in the immunoprecipitated samples was detected as a control. Adapted from Vélez-Bermúdez et al. (2015).

blot analysis, it is very important to use highly specific primary and secondary antibodies, since lack of specificity may result in unwanted background signal. If this is the case, we suggest using Clean-Blot™ IP detection reagent (HRP) as a secondary antibody for polyclonal antibodies to avoid the interference with both heavy and light chains of IgG, which can cause problems to resolve western blot with proteins with a molecular weight of 50 or 25 kDa. For mass spectrometry analysis, we suggest to use the transgenic plant expressing the empty vector as a negative control. If an antibody against the endogenous protein is used, Dynabeads without antibody, or the pre-immune bleed can be used as a negative control.

TROUBLESHOOTING

Problem 1

Low amount or degradation of the purified protein (steps 15 and 16).

Potential solutions

Try to use another host strain such as *E. coli* Rosetta (DE3).

Add glucose to the growth media to avoid degradation of the amylose on the affinity resin.

Optimize the time and the temperature during expression depending on the stability of the protein and host strain.

Harvest cells quickly and keep them chilled.

Add proteinase inhibitors and PMSF. Avoid that the column is running dry.

Problem 2

Poor quality of the purified protein (step 17).

Potential solutions

Avoid loading the column for more than 16 h, increase the number of washing steps, and wash the column at a maximum of 10 mL/min for a 2.5 cm column.

Problem 3

Low concentration and quality of the purified antibody (step 30).

Potential solutions

Use at least 222 μg of purified protein.

Use at least 10–15 mL of serum or IgY sample.

Adjust the elution conditions and time for each specific antibody.

Do not use glycine older than 1 month, since the pH may vary.

Neutralize the glycine with 1 M Tris pH 8.0. It is also possible to concentrate the antibody using an Amicon Ultra centrifugal filter or a similar device.

Problem 4

Weak or no signal in the Co-IP western blot (step 45).

Potential solutions

This problem may be caused by degradation of the proteins during either sampling or grinding.

To prevent degradation, the tissue should be stored immediately at −80°C.

To solve problems caused by grinding, avoid thawing the tissue before adding the extraction buffer.

Low concentration of proteins obtained from Arabidopsis or maize during lysis. To solve this problem, keep the samples and buffers at 4°C and increase the amount of protease inhibitors or MG132. We also suggest using 1 g of starting material and an amount 1:2 (w/v) of buffer.

Low affinity or concentration of the antibody. To solve this problem, optimize the required antibody.

Perform the Co-IPs in a cold room or at 4°C.

Problem 5

High background and nonspecific bands in the Co-IP western blot (step 45).

Potential solutions

Use less antibody to prevent binding of non-specific proteins to the beads.

Reduce the amount of sample loaded onto the Dynabeads to prevent non-specific binding of proteins to the antibody.

Reduce the incubation time samples/Dynabeads.

To avoid degradation of the antibody during the Co-IP, add fresh protease inhibitors and MG132.

Use the Clean-Blot™ IP detection reagent (HRP) during developing the western blot.

Problem 6

High amount of antibody eluting with the target protein (step 45).

Potential solutions

Crosslink the antibody with the Dynabeads before running the Co-IP experiment (step 45).

Problem 7

No eluted target protein detected (step 45).

Potential solutions

Increase the amount of antibody.

Ensure to elute the target proteins from the Dynabeads.

Do not add non-ionic detergents such as NP-40 or Triton X-100 to the lysis buffer.

Use less stringent washing buffer conditions and reduce the number of washing steps.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wolfgang Schmidt (wosh@gate.sinica.edu.tw).

Materials availability

Unique reagents (i.e., plasmids or purify antibodies) generated in this study can be requested from Wolfgang Schmidt (wosh@gate.sinica.edu.tw).

Data and code availability

The published article includes the figures generated with this protocol.

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AUTHOR CONTRIBUTIONS

I.C.V.-B. and J.E.S.-H. performed the experiments and optimized the protocols that are described in this method article. I.C.V.-B., J.E.S.-H., M.R., D.C.-R., and W.S. performed data analysis and wrote the protocol. W.S. provided funding and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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