

Immunoprecipitation of Cullin-RING Ligases (CRLs) in *Arabidopsis thaliana* Seedlings

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Abstract

CRL (Cullin-RING ubiquitin ligase) is the major class of plant E3 ubiquitin ligases. Immunoprecipitation-based methods are useful techniques for revealing interactions among Cullin-RING Ligase (CRL) subunits or between CRLs and other proteins, as well as for detecting poly-ubiquitin modifications of the CRLs themselves. Here, we describe two immunoprecipitation (IP) procedures suitable for CRLs in *Arabidopsis*: a procedure for IP analysis of CRL subunits and their interactors and a second procedure for in vivo ubiquitination analysis of the CRLs. Both protocols can be divided into two major steps: (1) preparation of cell extracts without disruption of protein interactions and (2) affinity purification of the protein complexes and subsequent detection. We provide a thorough description of all the steps, as well as advice on how to choose proper buffers for these analyses. We also suggest a series of negative controls that can be used to verify the specificity of the procedure.

Key words Cullin-RING ubiquitin ligase, Immunoprecipitation, Coimmunoprecipitation, Ubiquitin, Immunoblot

1 Introduction

Cullin-RING Ligases (CRLs) are the largest family of E3 ubiquitin ligases and recruit specific substrates for poly-ubiquitination [1]. Since their discovery in yeast almost 20 years ago [2, 3], CRLs have also been involved in almost all developmental and physiological plant processes [4]. CRLs are modular assemblies built on a backbone cullin subunit (CUL1, CUL3, and CUL4 in *Arabidopsis*) holding at their carboxy-terminal domain a RING-box protein (RBX1), which serves as a site for the interaction with the E2 ubiquitin conjugating enzyme, and at their amino-terminal domain a variable substrate receptor subunit, often connected via a bringing adaptor [5] (Fig. 1). Depending on the type of the cullin subunit, each recruiting an interchangeable substrate receptor, distinct subclasses of CRLs can be assembled, with different substrate specificity. Detection of protein-protein interactions among CRL

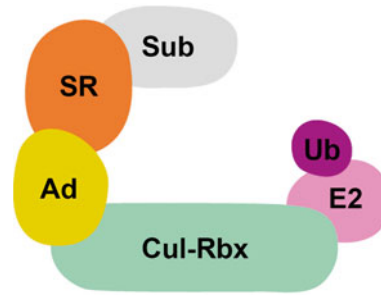


Fig. 1 Model of a CRL ubiquitin ligase. A typical CRL is composed of a cullin scaffold subunit, which interacts with RBX1 (Cul-Rbx), that in turn binds the E2 ubiquitin conjugating enzyme. Specific substrates are recruited by a variable substrate receptor (SR) anchored to the cullin through an adaptor (Ad)

subunits, as well as CRL subunit interaction with other proteins is therefore essential to provide insights on the individual cellular function of a given CRL.

CRLs activity is dynamically regulated. In absence of the substrate, CRLs can ubiquitinate their own substrate receptor, thus providing a mechanism to decrease the activity of a specific CRL when it is not necessary. CRL subunits can also be ubiquitinated by other E3 ubiquitin ligases [6]. Indeed, the turnover of several substrate receptors has been shown to be controlled by specific E3s. Therefore, determining the stability or the poly-ubiquitination status of a particular substrate receptor can offer a more complete overview on the biological role of a given CRL.

Two detailed step-by-step procedures are described here. The first one (*see* Subheading 3.1) illustrates how to immunoprecipitate a tagged CRL subunit to detect its direct or indirect interaction with another CRL subunit or other proteins of interest for which antibodies are available. Because the interaction between the protein of interest and its binding partner may be transient, a cross-linking step before protein extraction can be employed. Next, total proteins need to be extracted, and the composition of the grinding buffer may need to be adjusted (i.e., salt concentration, pH, amount of detergents), depending on the strength of the protein–protein interaction to be investigated. In addition, to enhance the overall yield of the immunoprecipitation (IP) and to increase the likelihood of immunoprecipitating interacting proteins, two classes of compounds could be added to the grinding buffer immediately before use: protease inhibitors, to avoid unwanted proteolysis during protein extraction, and phosphatase inhibitors, to preserve the phosphorylation state of immunoprecipitated proteins. Once proteins are extracted, the CRL complex is affinity purified by capturing the CRL subunit and its binding partners with a commercially available antibody immobilized on a solid support (beads). The CRL

complex attached to the beads is then precipitated and isolated (IP sample) through centrifugation, while the unbound proteins are washed out. Finally, the IP sample is analyzed by immunoblotting using both an antibody against the tagged protein, to control that the CRL subunit has been correctly immunoprecipitated, and an antibody against other proteins to investigate their suspected interaction with the CRL of interest.

The second protocol (*see* Subheading 3.2) describes an IP-based procedure to examine whether a substrate receptor subunit of a given CRL is poly-ubiquitinated *in vivo*. The critical aspect of this experiment consists in preserving the integrity of the poly-ubiquitin chain conjugated to the protein of interest. Thus, before the protein extraction and the IP steps, it might be useful to incubate Arabidopsis seedlings with a proteasome inhibitor (such as MG132) in order to stabilize the poly-ubiquitinated proteins. In addition, it might be necessary to use a denaturing protein extraction buffer supplied with *N*-ethylmaleimide (NEM). NEM blocks a cysteine residue of the active site of the deubiquitinating enzymes, thus avoiding their unwanted activity. The second part of this protocol follows the same principles described for the first protocol: the CRL subunit is subjected to affinity purification using an antibody-coupled resin, and the samples are later analyzed by immunoblotting. The presence of an ubiquitin chain on the protein of interest can be observed by using an epitope tag antibody and an anti-ubiquitin antibody. This protocol may be also used to investigate whether a protein, which is not a component of CRL complexes, is covalently conjugated to an ubiquitin chain. The procedure can be used either with epitope tag antibodies or with native antibodies/affinity matrixes. For a protocol for antibody-resin coupling, please refer to [7].

2 Materials

2.1 Co-IP of CRLs

2.1.1 Plant Material and Growth

1. MS solid medium: 4.4 g/L Murashige and Skoog medium including Gamborg B5 vitamins, 10 g/L sucrose, 0.5 g/L MES, 0.8% plant agar, pH adjusted to 5.7 with KOH.
2. DSP (dithiobis(succinimidyl propionate)) cross-linker stock solution: 10 mM dissolved in DMSO (*see* Note 1).
3. Cross-link reaction buffer: Phosphate Buffer Saline (PBS). Add 1 mM DSP before use.
4. Cross-link stop solution: 1 M Tris-HCl pH 7.5.

2.1.2 Total Protein Extraction

1. Grinding buffer A: 50 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 150 mM NaCl, 0.1% NP-40. Add 20 mM β -glycerophosphate, 20 mM NaF, 5 mM Na₃VO₄, phosphatase inhibitors, and

100 mM PMSF and 1× complete protease inhibitors (Roche; Sigma) immediately prior to use.

2. 2× Loading Buffer: 125 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 4% (w/v) SDS, 10% w/v glycerol, 0.01% Bromophenol Blue. Store at 4 °C.
3. Liquid nitrogen.
4. Mortar and pestle.
5. Refrigerated centrifuge.

2.1.3 Immuno-precipitation

1. Washing buffer A: 50 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 150 mM NaCl, 0.1% NP-40. Store at 4 °C.
2. Primary antibody against the protein to be pulled down.
3. 2× Loading Buffer: 125 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 4% (w/v) SDS, 10% w/v glycerol, 0.01% Bromophenol Blue. Store at 4 °C.
4. Refrigerated centrifuge.
5. Rotator with 1.5 mL tube holders.

2.1.4 SDS-PAGE

1. Mini-PROTEAN TGX precast gel (Biorad), stored at 4 °C (*see Note 5*). The range of acrylamide concentration should be chosen depending on the predicted molecular weight of the proteins being separated.
2. Running Buffer 10×: 250 mM Tris, 1.92 M glycine, 1% SDS. Store a room temperature.
3. Prestained molecular mass marker.
4. Mini-PROTEAN precast gel cassette (Biorad) (*see Note 5*).
5. Power supply.

2.1.5 Immunoblotting and Detection

1. Transfer Buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol.
2. Methanol.
3. PVDF membrane cut slightly larger than the dimensions of the gel.
4. Filter paper cut slightly larger than dimensions of the gel.
5. Phosphate buffer saline with Tween-20 (PBS-T): 10 mM Na phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween-20.
6. Blocking Buffer: 1% blocking reagent (Roche) dissolved in PBS-T.
7. Primary antibody against the immunoprecipitated protein.
8. Primary antibody against the coimmunoprecipitated protein.
9. HRP-conjugated secondary antibody.
10. Enhanced chemiluminescent (ECL) reagent.

11. X-ray films.
12. Mini Trans-Blot cell (Biorad) (*see Note 5*).
13. Power supply.
14. Shaker.
15. Image acquisition system (e.g., ChemiDoc, Biorad).

2.2 In Vivo Ubiquitination Analysis of CRLs

2.2.1 Plant Material and Growth

1. MS solid medium: 4.4 g/L Murashige and Skoog medium including Gamborg B5 vitamins, 10 g/L sucrose, 0.5 g/L MES, 0.8% plant agar, pH adjusted to 5.7 with KOH.
2. MS liquid medium: 4.4 g/L Murashige and Skoog medium including Gamborg B5 vitamins, 10 g/L sucrose, 0.5 g/L MES, pH adjusted to 5.7 with KOH.
3. MG132 stock solution: 50 mM MG132 dissolved in DMSO. Store at -20°C .

2.2.2 Total Protein Extraction

1. Grinding buffer B: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol. Add 50 μM MG132, 10 mM NEM, 100 mM PMSEF, and 1 \times Complete protease inhibitor cocktail (Roche) immediately prior to use.
2. 2 \times Loading Buffer: 125 mM Tris-HCl pH 6.8, 5% β -mercaptoethanol, 4% (w/v) SDS, 10% w/v glycerol, 0.01% Bromophenol Blue. Store at 4°C .
3. Liquid nitrogen.
4. Mortar and pestle.
5. Refrigerated centrifuge.

2.2.3 Immuno-precipitation

1. Washing buffer B: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol. Store at 4°C .
2. Primary antibody against the protein to be pulled down (*see Note 3*).
3. 2 \times Loading Buffer: 125 mM Tris pH 6.8, 5% β -mercaptoethanol, 4% (w/v) SDS, 10% w/v glycerol, 0.01% Bromophenol Blue. Store at 4°C .
4. Refrigerated centrifuge.
5. Rotator with 1.5 mL tube holders.

2.2.4 SDS-PAGE

1. Gradient Mini-PROTEAN TGX precast gel (Biorad), stored at 4°C (*see Note 5*).
2. Running Buffer 10 \times : 250 mM Tris, 1.92 M glycine, 1% SDS. Store at room temperature.
3. Prestained molecular marker.
4. Mini-PROTEAN precast gel cassette (Biorad) (*see Note 5*).
5. Power supply.

2.2.5 Immunoblot and Detection

1. Transfer Buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol.
2. Methanol.
3. PVDF membrane cut slightly larger than the dimensions of the gel.
4. Filter paper cut slightly larger than dimensions of the gel.
5. Phosphate buffer saline with Tween-20 (PBS-T): 10 mM Na phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween-20.
6. Blocking Buffer: 1% blocking reagent (Roche) dissolved in PBS-T.
7. Primary antibody against the immunoprecipitated protein.
8. Primary antibody against ubiquitin.
9. HRP-conjugated secondary antibody.
10. Enhanced chemiluminescent (ECL) reagent.
11. X-ray films.
12. Mini Trans-Blot cell (Biorad) (*see Note 5*).
13. Power supply.
14. Shaker.
15. Image acquisition system (e.g., ChemiDoc, Biorad).

3 Methods

3.1 IP of CRLs

3.1.1 Plant Material

1. Grow *Arabidopsis* seedlings on MS solid medium for 5–7 days at 22 °C.
2. Transfer 300–500 mg of seedlings in cross-linking reaction buffer (*see Notes 1 and 2*).
3. Incubate for 30' at room temperature with gentle shaking.
4. Add the cross-linking stop solution to a final concentration of 10 mM and incubate for 15' at room temperature.
5. Collect the seedlings in a 1.5 mL microcentrifuge tube and immediately freeze the sample in liquid nitrogen.

3.1.2 Total Protein Extraction

1. Transfer the frozen plant material in a mortar and grind them while keeping it frozen, until a fine powder is obtained. Collect the powder in a microcentrifuge tube and immediately add 300–500 μ L of Grinding Buffer A. Vortex to homogenize the sample and then place the tube on ice.
2. Centrifuge the sample at 16,000 $\times g$ for 15' at 4 °C, and transfer the supernatant in a new tube.
3. Remove a 20 μ L aliquot to serve as a total extract control. Add 20 μ L of 2 \times Loading Buffer and boil for 5'. Store at –20 °C for later analysis.

3.1.3 Immuno-precipitation

1. Equilibrate the antibody-coupled beads (*see Note 3*). Add 500 μL Grinding Buffer A to a 30 μL of beads. Centrifuge at $1500\times g$ for 4' at a room temperature and remove the supernatant.
2. Add the crude extract (from step 2 in Subheading 3.1.2) to the antibody-coupled beads.
3. Place the tube in a rotator and incubate with gentle agitation from 1 to 4 h at 4 °C (*see Note 4*).
4. Pellet the beads by centrifuging the tube at $1000\times g$ for 5' at 4 °C. Add 1 mL of Washing Buffer A and incubate for 5' with gentle agitation at 4 °C.
5. Repeat the washing (step 4) three times.
6. Pellet the beads at $1000\times g$ for 5' at 4 °C and add 30 μL of 2 \times Loading Buffer. Boil for 5'.

3.1.4 SDS-PAGE

1. Prepare the Mini-PROTEAN TGX precast gel in the apparatus as indicated in the manufacturer's instruction (*see Note 5*). Fill the cassette with Running Buffer 1 \times .
2. Load on the gel the prestained molecular marker and an equal volume of the protein samples from step 3 in Subheading 3.1.2 (total extract) and from step 6 in Subheading 3.1.3 (immuno-precipitate) (*see Note 4*).
3. Connect the electrophoresis chamber to the power supply and run the gel from 100 to 200 V until the dye reaches the bottom of the gel.

3.1.5 Immunoblot and Detection

1. Before transferring the separated proteins from the gel to the membrane, activate the PVDF membrane in methanol for 10' with gentle shaking. Transfer the PVDF membrane in Transfer Buffer to avoid its drying.
2. Prepare the gel sandwich with the filter papers, the gel, and the membrane in the Mini Trans-Blot (Biorad) cassette holder as indicated by the manufacturer's instruction. Fill the tank with Transfer Buffer, and connect the apparatus to the power supply.
3. Set the power supply at 100 V and run for 1 h.
4. After transfer, block membrane in 1% blocking reagent in PBS-T for 1 h at room temperature or at 4 °C overnight with gentle shaking.
5. Pour off the blocking solution and replace it with fresh 0.5–1% blocking solution containing the primary antibody.
6. Incubate for 3–6 h at room temperature or at 4 °C overnight with gentle shaking.

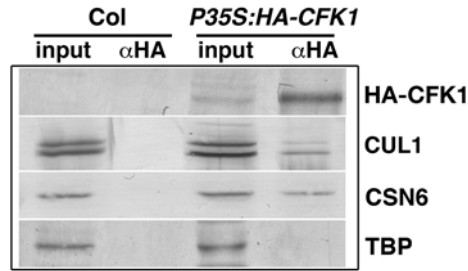


Fig. 2 Co-IP of the CRL substrate adaptor subunit CFK1 with CUL1 and CSN6. The Arabidopsis CRL substrate adaptor subunit CFK1 was fused to HA epitope to obtain plants expressing HA-CFK fusion protein [10]. Total protein extract from wild-type (Col-0) and HA-CFK1 expressing seedlings were immunoprecipitated using anti-HA affinity matrix followed by immunoblot to detect the interaction between CFK1 and other proteins. HA-CFK1 coimmunoprecipitates with CUL1, a subunit of the CRL complex, and with CSN6 protein. TBP (TATA Binding Protein) antibody was used as negative control of the interaction. “input” indicates the total extract [10]

7. Pour off the primary antibody and replace it with PBS-T. Wash with gentle shaking for 10’.
8. Repeat step 7 two more times.
9. Pour off the PBS-T and add the secondary antibody in 0.5–1% blocking reagent in PBS-T. The secondary antibody is chosen based on the primary antibody used in the step 4.
10. Incubate at 1–2 h at room temperature or at 4 °C overnight with gentle shaking.
11. Pour off the secondary antibody and replace it with PBS-T. Wash with gentle shaking for 10’.
12. Repeat step 11 four more times.
13. Pour the PBS-T off the membrane and add ECL reagent on the blotted side of the membrane. Incubation time depends on the ECL reagent used.
14. Expose the membrane to the X-ray film or use an image acquisition system. The time of exposure may vary from experiment to experiment. Figure 2 represents an example of this procedure.

3.2 In Vivo Ubiquitination Analysis of CRLs

3.2.1 Plant Material and Growth

1. Grow Arabidopsis seedlings on MS solid medium for 5–7 days at 22 °C.
2. Transfer 300–500 mg of seedlings in MS liquid medium supplied with 50 μM MG132, and 300–500 mg in MS liquid medium with DMSO as negative control (*see Note 6*).
3. Incubate from 2 to 4 h in the *Arabidopsis* growth chamber.
4. Collect the seedlings in a 1.5 mL microcentrifuge tube and immediately freeze the sample in liquid nitrogen.

3.2.2 Total Protein Extraction

1. Transfer the plant material in a mortar and pestle, under liquid nitrogen, to a fine power. Collect the power in a microcentrifuge tube and immediately add 300–500 μL of Grinding Buffer B. Vortex to homogenize the sample, and then place the tube on ice.
2. Centrifuge the sample at $16,000\times g$ for 15' at 4 °C, and transfer the supernatant in a new tube.

3.2.3 Immuno-precipitation

1. Equilibrate the antibody-coupled beads (*see* **Note 3**). Add 500 μL Grinding Buffer B to a 30 μL of beads. Centrifuge at $1500\times g$ for 4' a room temperature and remove the supernatant.
2. Add the crude extract (from step 2 in Subheading 3.2.2) to the beads.
3. Put the tube in the tube rotator and incubate with gentle agitation from 1 to 4 h at 4 °C (*see* **Note 4**).
4. Pellet the beads in a centrifuge at $1000\times g$ for 5' at 4 °C. Add 1 mL of Washing Buffer B and incubate for 5' with gentle agitation at 4 °C.
5. Repeat the washing (step 4) three times.
6. Pellet the beads at $1000\times g$ for 5' at 4 °C and add 30 μL of 2 \times Loading Buffer. Boil for 5'.

3.2.4 SDS-PAGE

1. Load on the gel the prestained molecular marker and samples from the step 6 in Subheading 3.2.3. Follow the procedure as described in Subheading 3.1.4.

3.2.5 Immunoblot and Detection

1. Follow the procedure as described in Subheading 3.1.5. For the *in vivo* ubiquitination analysis an antibody against the immunoprecipitated protein and an antibody against ubiquitin are used. A representative result of this procedure is shown in Fig. 3.

4 Notes

1. We use the chemical cross-linker DSP to covalently preserve the interactions among the CRL subunits. This step is not always required to detect protein–protein interaction and depends on the strength of the interaction. If this step is omitted, proceed directly to step 5 in Subheading 3.1.1.
2. The total protein extract (indicated as “input” in Fig. 2) serves as a positive control of the extraction. Extracts and immunoprecipitates from wild-type seedlings, not expressing the tagged protein, can be used as a negative control of the experiment. In addition, antibodies against proteins not supposed to

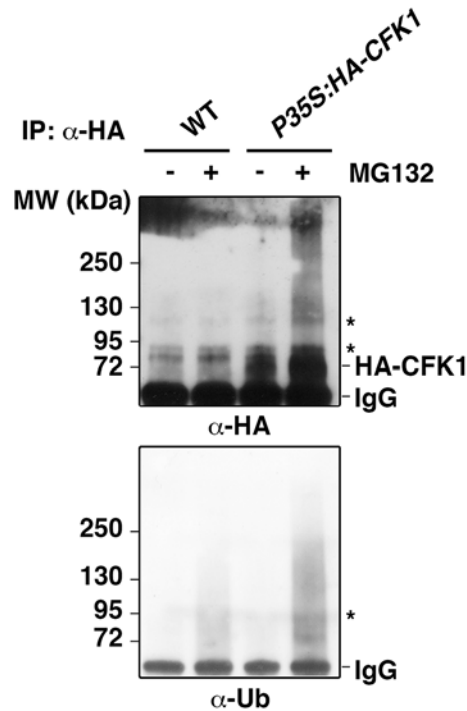


Fig. 3 In vivo ubiquitination analysis of the CRL substrate adaptor subunit CFK1. Wild-type and HA-CFK1 expressing seedlings were incubated with the proteasome inhibitor MG132. The crude extracts were prepared and immunoprecipitated with an anti-HA resin. The immunoprecipitated proteins were detected with anti-HA (top panel) and anti-ubiquitin (bottom panel) antibody. The increase in the higher molecular mass species in presence of MG132 recognized by the antibody against HA and against ubiquitin indicates that CFK1 is ubiquitinated in vivo [10]

interact can be employed. We suggest to use them in step 5 in Subheading 3.1.5 as a further negative control of the IP.

3. Both native antibodies or epitope antibodies can be used. Table 1 shows a list of epitope tags and their corresponding antibodies and matrices. We have successfully employed anti-HA agarose affinity gel from Sigma-Aldrich (Figs. 2 and 3), but other commercially available antibodies and resins (e.g., Covance, Roche) can be used. If a direct antibody against the protein to be immunoprecipitate is available, it can be coupled directly to protein A or protein G matrix and used for the IP. For this procedure, refer to other general IP protocols [7].
4. The incubation time might depends also on the antibody–resin that will be employed. Refer to the manufacturers’ instruction to set up the IP time.
5. Here we provide the instructions for the SDS-PAGE based on the Mini-PROTEAN precast gels from Biorad, but other commercially systems or handcast gel can be employed [8, 9].

Table 1
List of epitope tags, commercially antibodies, and antibodies-conjugated matrices (and their corresponding catalog numbers) successfully used in Arabidopsis for IP protocols

TAG	Antibody	Matrix
HA	Sigma-Aldrich (H3663) Covance (MMS-101P) Santa Cruz (sc-805)	Sigma-Aldrich (E6779) Roche (11815016001)
c-myc	Sigma-Aldrich (M4439) Covance (MMS-164P) Santa Cruz (sc-40)	Covance (AFC-150P) Sigma-Aldrich (E6654)
FLAG	Sigma-Aldrich (F3165)	Sigma-Aldrich (A2220)
GFP	Sigma-Aldrich (G1544) Abcam (ab290)	ChromoTek (gta-20) Miltenyi Biotec (130-091-288)

6. Wild-type Arabidopsis seedlings, not expressing the tagged protein, can be used as negative control of the experiment. Because the MG132 proteasome inhibitor is dissolved in DMSO, immunoprecipitates from seedlings treated only with DMSO can be used as an additional negative control for the *in vivo* ubiquitination assay.

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