Published in final edited form as:

Methods Mol Biol. 2017 ; 1564: 169–180. doi:10.1007/978-1-4939-6813-8_14.

Probing activation/deactivation of the BRASSINOSTEROID INSENSITIVE1 receptor kinase by immunoprecipitation

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Summary

Brassinosteroids are plant sterol-derived hormones that control plant growth and development. The BR receptor complex is encoded by the BRASSINOSTEROID INSENSITIVE1 (BRI1) and members of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE family. BR receptor complex activation and deactivation uses different post-translational modifications and recruitment of partner proteins. In this chapter, we describe optimized immunoprecipitation protocols and variants for biochemical analyses of BRI1 post-translational modification and protein-protein interaction.

Keywords

Brassinosteroids; BRI1; Immunoprecipitation; Ubiquitination; Phosphorylation; Protein-protein interaction

1 Introduction

Brassinosteroids (BRs) are plant steroid hormones that play important roles in plant growth and development (1–3). Mutants with defects in BR biosynthesis or signaling display severe developmental defects such as reduced seed germination, extreme dwarfism, photomorphogenesis in the dark, altered distribution of stomata, delayed flowering and male sterility (1,3). BRs are perceived at the plasma membrane by a pair of receptor kinases including BRASSINOSTEROID INSENSITIVE 1 (BRI1) and members of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (SERK1 to SERK5, SERK3 being also called BRI1-ASSOCIATED KINASE 1 (BAK1)) (4–12). The perception of BRs by the BRI1/BAK1 receptor complex triggers transphosphorylation between BRI1 and BAK1 kinase domains in the cytoplasm (13,14). Downstream BR signaling involves a succession of phosphorylation and dephosphorylation events with kinases and phosphatases that culminates in the dephosphorylation of BES1 (bri1-EMS-SUPPRESSOR1) and BZR1

(BRASSINAZOLE RESISTANT1) transcription factors and BR-responsive gene transcription (1,15–17). In the absence of ligand or upon deactivation of the BR receptor complex, BRI1 kinase domain is bound by the BRI1 KINASE INHIBITOR 1 that prevents interaction with BAK1 and spurious activation of the pathway (18,19).

Activation or deactivation of the receptor complex relies on post-translational modifications and interaction with positive and negative regulators. Over the past decade, extensive knowledge was gained on the phosphorylation of BRI1 (1,13,14,20–24). Several phosphorylation sites have been mapped or more recently identified by mass spectrometry analyses after ligand binding. The timing of phosphorylation after addition of BRs together with detailed analyses of mutant variants of BRI1 impaired for specific phosphorylation sites unraveled sites playing a positive and negative role on BRI1 activity (1,13,14,21–23). In addition, BRI1 was demonstrated to be post-translationally modified by ubiquitination, and more specifically K63 polyubiquitin chains (25). Although mostly independent of ligand binding to the receptor complex, BRI1 ubiquitination appears to be an important mechanism to downregulate BRI1 activity. Identification of an ubiquitination site by mass spectrometry coupled to model-based prediction allowed the generation of a non-ubiquitinatable BRI1 variant (25). Such ubiquitination-defective BRI1 show slower internalization from the cell surface and vacuolar degradation, leading to increased plasma membrane levels and BR hypersensitivity phenotypes.

The analysis of BRI1 activation and deactivation mechanisms is mostly based on protein-protein interaction detection (e.g. interaction with BAK1 and BKI1) and post-translational modifications (e.g. phosphorylation, ubiquitination) (18,26,23,13,19,25,27). Both approaches rely on prior purification of BRI1 protein by immunoprecipitation (18,25,26). Although antibodies recognizing endogenous BRI1 protein may be used, most studies aiming at immunoprecipitating BRI1 use anti-tag antibodies targeting functional tagged fusions of BRI1, such as for example functional BRI1-mCitrine (25,26,18), BRI1-Flag (13,14) or BRI1-6xHA (18). Immunoblot using anti-tag antibodies after immunoprecipitation is used to confirm the enrichment in BRI1 compared to input total protein extracts. The subsequent detection of partner proteins or post-translational modifications uses specific antibodies (anti-phosphoserine/threonine, anti-phosphotyrosine, anti-ubiquitin or antibodies targeting a given protein (e.g. native or tagged versions of BAK1, BRI1 or BKI1) (13,18,23,25,26). Subtle differences in the immunoprecipitation protocols must be considered according to the goal of the experiment. While detection of reversible post-translational modifications requires the use of specific inhibitors preventing removal of the covalently attached modification, it also requires more stringent solubilization and wash conditions to avoid co-purification of other proteins also modified and that may migrate at a molecular weight close to BRI1. Detection of protein-protein interaction however uses milder conditions to allow detection of partner proteins. Below we provide detailed protocols to investigate the activation and deactivation mechanisms of BRI1.

2 Materials

Prepare all the solutions with ultrapure water (resistivity of 18.2 MΩ·cm at 25°C). Keep the reagents at room temperature (unless indicated otherwise). Follow the regulation for waste disposals.

2.1 Proteins extraction from plants and immunoprecipitation

Keep the solutions and material at 4°C unless indicated otherwise.

1. Mortar and pestle at room temperature to grind tissues.
2. 1 M Tris-HCl (pH 8.0). In a glass beaker mix about 50 mL of water and 12.11 g of Tris-base. Once the mixture is homogenized, adjust the pH to 8.0 by adding Hydrochloric acid (HCl). Make up the volume to 100 mL and store at 4°C.
3. 0.5 M Ethylenediaminetetraacetic acid disodium salt (EDTA) (pH 8.0). In a glass beaker with 400 mL of water, mix 93.06 g of EDTA and stir with a magnetic stirrer. Adjust the pH to 8.0 with sodium hydroxide (NaOH). Make up the volume to 500 mL and autoclave the solution at 120°C.
4. Sucrose buffer: 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.33 M sucrose. In about 50 mL of water, add 2 mL of 1M Tris-HCl (pH 8.0), 200 µL of 0.5M EDTA (pH 8.0), and 11.3 g of sucrose. Dissolve the sucrose with the help of a magnetic stirrer and make up the volume to 100 mL with water. Autoclave the solution at 120°C or filter sterilize. Keep at 4°C.
5. Protease inhibitors cocktail inhibitors.
6. Phosphatase inhibitors cocktail.
7. 20 mM N-Ethylmaleimide (N-EM) solution in sucrose buffer: dissolve 25 mg of N-EM in 100 µL of ethanol 100% (*see Note 1*) and dilute this solution in 10 mL of sucrose buffer.
8. Sucrose buffer for ubiquitination studies: add 100 µL of protease inhibitors cocktail, and the 100 µL of 20 mM N-EM solution into 10 mL of freshly prepared sucrose buffer (*see Note 2*).
9. Sucrose buffer for phosphorylation studies: add 100 µL of protease inhibitors cocktail, and one tablet of phosphatase inhibitors cocktail into 10 mL of freshly prepared sucrose buffer (*see Note 2*).
10. Miracloth.
11. Sorval Legend Micro 21R Centrifuge (Thermo Fisher Scientific company, USA).
12. 1M Tris-HCl (pH 6.8). Proceed as for the preparation of 1M Tris-HCl (pH 8.0) described in step 1, but adjust the pH to 6.8 with HCl.
13. 4x Laemmli buffer: 2.8 mL of water, 2.4 mL of 1M Tris-HCl (pH 6.8), 0.8 g of SDS, 4 mL of 100% glycerol, 0.01% of bromophenol blue and 1 mL of β-mercaptoethanol (β-ME). Keep it at -20°C.

14. 2 mL tissue grinder potter.
15. Lysis buffer: 150 mM NaCl, 1% Triton[®] X-100, 50 mM Tris HCl (pH 8.0). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
16. 20% SDS: Prepare 100 mL of 20% SDS by weighing 20 g SDS in a beaker. Add 70 mL of ultrapure water and mix it gently. Adjust final volume to 100 mL with ultrapure water.
17. Rotating wheel.
18. Heated block: Eppendorf ThermoMixer[®] (Eppendorf AG, Hamburg, Germany).
19. Anti-GFP MicroBeads (*see* Note 3). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
20. Anti-HA MicroBeads (*see* Note 3). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
21. μ Columns: MACS[®] Separation Columns (MACS Milteny Biotec GmbH company, Germany).
22. Place the μ MACS[™] Separator on the MACS MultiStand (MACS Milteny Biotec GmbH company, Germany).
23. Wash buffer 1: 150 mM NaCl, 1% Igepal CA-630 (formerly NP-40), 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl (pH 8.0). This buffer is supplied with the μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
24. Wash buffer 2: 20 mM Tris HCl (pH 7.5). This buffer is provided with the μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
25. Elution buffer: 50 mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol. This buffer is provided with the μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).

2.2 Immunoblots

1. Nitrocellulose membrane.
2. Orbital Shaker.
3. Petri dishes or a small container/dish where the nitrocellulose membrane can fit with some liquid.
4. Blotting tweezer to hold the nitrocellulose membrane.
5. 10X TBS: 20 mM Tris-HCl, 500 mM NaCl. In about 800 mL of water, dissolve 24 g of Tris-base and 88 g of NaCl. Once the mixture is homogenized, adjust the

pH to 7.4 by adding Hydrochloric acid (HCl). Make up the volume to 1 L and store at room temperature.

6. Tween® 20.
7. Washing solution (TBS-T): 1X TBS, 0.1 % Tween® 20 (*see* Note 4). Dilute 100 mL of 10X TBS in 800 mL of water. Add 1 mL of Tween® 20 and make up the volume up to 1 L. Store at room temperature.
8. Bovine Serum Albumin (BSA).
9. Blocking solution: TBS-T, 1% BSA. Weight 1 g of BSA and mix gently with 90 ml of TBS-T in a beaker with the help of a stirrer (*see* Note 5). Make up the volume to 100 mL with TBS-T and store at 4°C.
10. Primary antibodies: Anti-GFP-HRP conjugated monoclonal antibody (MACS Milteny Biotec GmbH Company, Germany), Anti-HA-HRP conjugated monoclonal antibody (MACS Milteny Biotec GmbH Company, Germany), Ubiquitin P4D1 Mouse monoclonal antibody (Cell Signaling Technology®, USA), Lys63-Specific Anti-Ubiquitin rabbit monoclonal (Apu3 clone, Merck Millipore, France), Anti-phosphothreonine antibody (Cell Signaling Technology, Beverly, MA, USA), Anti-phosphotyrosine HRP-coupled monoclonal antibody (4G10, Millipore), primary antibody recognizing a downstream BRI1 partner or a tagged downstream partner (e.g. BAK1-mCherry (18,26) or BKI1-mCitrine (18)).
11. Secondary antibodies: Goat Anti-Mouse IgG (H + L)-HRP Conjugate, Goat Anti-Rabbit IgG (H + L)-HRP Conjugate.
12. Super Signal® West Dura Extended Duration Substrate (Thermo Scientific Company, USA).
13. Transparent office file sheet protector.
14. Chemiluminescence Imaging System.

3 Methods

3.1 Protein extraction from plants and Immunoprecipitation

Pre-chill down the material to be used and keep everything on ice or in the cold room (*see* Note 6).

1. Grind tissues in liquid nitrogen (*see* Note 7) and transfer with a pre-chilled spatula to 2 mL of ice-cold sucrose buffer completed with the desired inhibitors according to the post-translational modification analyzed. It is also possible to use fresh tissues and grind it directly in ice-cold sucrose buffer (*see* Note 7).
2. Centrifuge for 10 minutes at 5,000 g at 4 °C to remove cellular debris and transfer the supernatant into 2 mL Eppendorf tubes.
3. Repeat step 2 until the supernatant is clean (*see* Note 8).

4. Pre-heat a heat block to 95°C.
5. Pipet 50 µL of the total protein fraction to use as input of the immunoprecipitation, and add 15 µL of 4X Laemmli buffer.
6. Boil the input for 3 minutes at 95°C. Keep the input on ice if you are planning to proceed with western blot the same day or freeze in liquid nitrogen and keep at -80°C for later use.
7. In order to obtain the membrane protein fraction, centrifuge for 45 minutes at 20,000 g at 4°C and keep the pellet (microsomal fraction) (*see* Note 9).
8. Add 1 mL of lysis buffer to the pellet (*see* Note 10) and resuspend carefully by pipetting up and down (*see* Note 11).
9. Transfer the lysate to ice-cold potters and homogenize (*see* Note 12). Transfer the samples to new 1.5 mL tubes.
10. Solubilize the microsomal fraction on a rotating wheel at 4°C for 30 minutes (Figure 1).
11. Centrifuge the samples for 10 minutes at 20,000 g at 4°C to pellet non-solubilized microsomes. Transfer the supernatant into a new 1.5 mL tube. This fraction corresponds to solubilized microsomal proteins and will be used for BRI1 immunoprecipitation.
12. Quantify the samples and use 1 mg of proteins of the supernatant to perform the immunoprecipitation of BRI1.
13. Add 50 µL of Anti-Tag MicroBeads to the supernatant and mix well (Figure 1).
14. Incubate on ice for 30 minutes to allow the formation of the BRI1-tag-antibody complex.
15. Place the µColumn on the µMACS™ Separator and apply 200 µL of the Lysis buffer to equilibrate the column (*see* Note 10).
16. Pipette 75 µL of Elution buffer for each sample into a new 1.5 mL tube and boil it on the pre-heated 95°C block.
17. Apply the samples lysate onto the center of the column and let it run through by gravity flow.
18. Wash the column with 200 µL of the Wash buffer 1. Repeat 4 times.
19. Wash the column with 100 µL of the Wash buffer 2.
20. Apply 20 µL of Elution buffer kept at 95°C onto the center of the column and incubate at room temperature for 5 minutes (*see* Note 13).
21. Place a new 1.5 mL collecting tube under the column.
22. Apply 50 µL of Elution buffer kept at 95°C onto the center of the column and collect the eluates (*see* Note 14). Freeze samples in liquid nitrogen and keep them at -80°C, or proceed directly with immunoblotting.

3.2 Immunoblot

Run the input and immunoprecipitated proteins in a standard SDS-PAGE, using a 7.5% separating gel (*see* Note 15). Transfer the proteins from the gel to a nitrocellulose membrane (*see* Note 16). It is important to handle the membrane with blotting tweezers and to keep it wet at all time. All procedures can be done at room temperature unless specified otherwise.

3.1.1 Detection of BRI1-mCitrine or BRI1-6xHA by western-blot

1. Place the nitrocellulose membrane in a petri dish and add the blocking solution until it covers the membrane. Shake gently for 1 hour at room temperature on the orbital shaker.
2. Prepare the solution containing primary antibodies by adding Anti-GFP-HRP or Anti-HA-HRP antibodies in new blocking solution. Use 1 μ L of antibody to 5 mL of blocking solution.
3. Replace the blocking solution with antibody solution. Incubate for 1 hour shaking gently on the orbital shaker (*see* Note 17).
4. Discard the antibody solution.
5. Wash the membrane 3 times for 10 minutes in washing solution under gentle agitation.
6. Discard the washing solution.
7. Apply the Super Signal[®] West Dura Extended Duration Substrate as indicated by the manufacturer and incubate for 5 minutes.
8. Place the membrane between a transparent office file sheet protector.
9. Proceed to signal detection using a chemiluminescence imaging system.

3.1.2 Detection of BRI1 ubiquitination, phosphorylation or co-immunoprecipitated proteins

1. Place the nitrocellulose membrane in a petri dish and add the blocking solution until it covers the membrane and shake gently during 1 hour at room temperature on the orbital shaker (*see* Note 17).
2. Prepare the solution with the primary antibody in new blocking solution. Use the following dilutions: anti-P4D1 1:2,500; anti-Apu3 1:2,000; anti-pThr 1:1,000, anti-Anti-pTyr 1:5,000. *Ee* Figure 2 for an example of western blot with anti-GFP and anti-Ubiquitin antibodies.
3. Discard the blocking solution and add the antibody solution. Incubate for 1 hour and shake gently on the orbital shaker.
4. Discard the antibody solution.
5. Wash the membrane 3 times for 10 minutes in washing solution under gentle agitation.
6. Discard the washing solution.

7. Prepare the HRP-conjugated secondary antibody in blocking solution (dilution 1:20,000).
8. Wash the membrane 3 times for 10 minutes in the washing solution with gentle shaking.
9. Discard the washing solution.
10. Apply the Super Signal® West Dura Extended Duration Substrate according to the manufacturer instruction and incubate for 5 minutes.
11. Place the membrane between a transparent office file sheet protector.
12. Proceed with signal detection using a chemiluminescence imaging system.

4 Notes

1. The N-EM does not dissolve in the sucrose buffer; therefore it is necessary to first dissolve it in 100% ethanol.
2. N-EM is an efficient deubiquitinating enzymes (DUBs) inhibitor, which prevents the potential deubiquitination of ubiquitinated proteins, including BRI1. Phosphatase inhibitors will prevent dephosphorylation of phosphorylated proteins. It is strongly advised to add the protease inhibitors cocktail, phosphatase inhibitor cocktail and N-EM extra-temporaneously to the sucrose buffer just before protein extraction and immunoprecipitation. The protease inhibitors cocktail is important to avoid protein degradation.
3. To perform the immunoprecipitation, we routinely use tagged form of BRI1, BRI1-6xHA (18) or BRI1-mCitrine (25,18,26), which can be recognized by the Anti-HA or Anti-GFP MicroBeads used in this protocol, respectively.
4. The TBS is recommended for proteins that are phosphorylated since it avoids a higher background on the nitrocellulose membrane during the Western-blot washes. The Tween® 20 is a detergent that will help to avoid non-specific binding to the membrane.
5. The BSA is a bovine protein that can be degraded at high temperatures mechanically, therefore it should be stored at 4°C. The BSA is important for blocking the membrane prior and during the incubation with the antibodies specific to the protein of interest in order to avoid non-specific binding of the antibody to the membrane.
6. Work and/or use a cold room (4°C). It's important that everything is cold at all time.
7. Keep the mortar and pestle at room temperature to avoid water condensation and the formation of ice crystals when in contact with the liquid nitrogen. Cool down the mortar and pestle with liquid nitrogen. Leave a small quantity of liquid nitrogen inside the mortar to help keep the tissue frozen while it is grinded. The best is to have at least 1g of tissue. Tissues must be kept in liquid nitrogen or at -80°C before being grinded. It is also possible to grind fresh tissue directly in the

ice-cold sucrose buffer. This latter option is preferable when the plant material (e.g. seedlings) has been submitted to liquid treatments prior to extraction in order to avoid the formation of ice following incubation in liquid nitrogen.

8. These steps are critical since it is important to have a clean supernatant to avoid carrying over cellular debris to the membrane fraction. Usually, it takes 5 extra centrifugations at 4°C at 5,000 g for 10 minutes to obtain a clean supernatant. It is advisable to stay well clear of the pellet when pipetting the supernatant. Before the initial first two centrifugations, it is possible to quickly filter out the bulk of the cellular debris by passing the solution through Miracloth. It is important to always use ice-cold buffer and work at 4°C or on ice.
9. The supernatant resulting from the 20,000 g centrifugation corresponds to the fraction enriched in soluble proteins and the pellet contains the microsomal fraction, in which BRI1 is enriched.
10. The analysis of BRI1 post-translational modifications requires more stringent conditions, to strip off interacting proteins, by adding 0.1% SDS to the lysis buffer. In that case, the μ MACS column must be equilibrated with the SDS containing lysis buffer.
11. If the same sample is separated in two 2 mL tubes, pipette 500 μ L of lysis buffer into each tube. Resuspend the pellet carefully by pipetting up and down, and avoid pipetting the foam.
12. To avoid foaming while using the potter, we recommend to keep the pestle submerged in the sample.
13. The μ Column has a capacity of 20 μ L. When applied the first 20 μ L of Elution buffer is important to make sure that the Wash buffer 2 runs completely through the column to avoid residual salts and detergents from this Wash buffer on the eluate sample.
14. It is possible to improve the collection of the eluate sample, by placing parafilm on the top of the column and push it in order to create a little pressure and allow the eluate that is retained in the column to come out.
15. BRI1 is a 120 kDa protein, which reaches 160 kDa when coupled with GFP. For this reason we use a 7.5% polyacrylamide gels, but if low molecular weight interacting proteins are expected, it is also possible to use gels with higher polyacrylamide concentration.
16. In the case of BRI1, we recommend to use 20% methanol during the transfer.
17. It is important that the membrane is always in contact with the solution during the antibody incubation time. It is possible to adjust the speed of the agitation of the orbital shaker in order to optimize that.

Acknowledgment

We thank Youssef Belkhadir for providing tips during the establishment of this protocol. Y.J. has received funding from the European Research Council - ERC Grant Agreement n. (3363360-APPL) and from the Marie Curie Action

– CIG Grant Agreement n. (PCIG-GA-2011-303601) under the European Union's Seventh Framework Programme (FP/2007-2013). G.V. has received funding from Agence Nationale de la Recherche (ANR-13-JSV2-0004-01), and from the Marie Curie Action (PCIG12-GA-2012-334021 under the European Union's Seventh Framework Programme (FP/2007-2013). S.M. is sponsored by a PhD fellowship from the Saclay Plant Sciences LabEx initiative (ANR-10-LABX- 0040-SPS) funded by the French government.

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Figure 1. Protein extraction and purification scheme described in this protocol

Figure 2. Example of western blots obtained following BRI1-mCitrine immunoprecipitation. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from wild-type and transgenic plants expressing BRI1-mCitrine and subjected to immunoblotting with anti-GFP and anti-ubiquitin antibodies. IP, immunoprecipitation; IB, immunoblotting