Chapter 13

LeEIX2 Interactors' Analysis and EIX-Mediated Responses Measurement

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Abstract

Plant-pathogen interactions involve a large number of wide regulatory systems, necessary for plant defense responses against pathogen attack. The fungal protein ethylene-inducing xylanase (EIX) elicits defense responses in specific cultivars of tobacco and tomato. The response to EIX is controlled by a single locus encoding for LeEIX2, a leucine-rich-repeat receptor-like-protein (LRR-RLP). As an RLP, LeEIX2 does not possess an obvious cytoplasmic signaling moiety such as a kinase domain. To study LeEIX2 mode of action, it is essential to identify the potential interactors involved after EIX perception. Here, we describe the in vivo co-IP methodology used for protein interaction verification and ethylene and ROS (reactive oxygen species) measurements used for physiological effects assessment.

Key words LRR-RLP, Ethylene-inducing xylanase, MAMP, Defense responses, Co-IP, ROS

1 Introduction

Plants are constantly being exposed to a wide variety of microorganisms such as bacteria and fungi. As plants lack a network of circulating cells, their defense relies on the innate immunity of each cell and systemic signals emanating from infection sites [1]. Plant cells exploit various types of cell surface receptor molecules to perceive extracellular signals. These signals can be molecular-associated microbial patterns (MAMPs) or self-derived [2]. Often, these receptors contain extracellular leucine-rich repeats (LRRs) that mediate protein-protein interaction [3].

Two types of LRR receptors have been reported in plants: RLKs composed of an LRR domain, a single-pass transmembrane domain, and a cytoplasmic kinase domain. RLPs, which differ from RLKs by carrying a short cytoplasmic tail without a kinase domain. RLKs and RLPs were shown to play roles in innate immunity and development in several plant species [4, 5]. A total of 647 RLKs were identified in the tomato genome [6]. The number of RLP-type receptors predicted from genomic sequences varies according

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to the plant species studied. Arabidopsis has 57 [7] and the number of RLPs for the tomato genome can be expected to be much higher. Among the tomato RLPs involved in defense responses the best characterized members include the Cf family against *Cladosporium fulvum* [8, 9], Vel against *Verticillium* [10], and LeEIX2 which recognizes EIX [11].

Since RLPs lack a kinase domain it is crucial to identify the interactors responsible for downstream signaling upon elicitation. Different screening procedures such as yeast two hybrid screens and proteomics analysis will yield numerous putative interactors. Such interactions probably involve protein-protein interaction or even complex formation [2]. Co-IP methodology can be used to verify and characterize such interactions. Here, we describe an efficient procedure to detect co-IP of membrane integrated proteins and their interactors both cytoplasmatic and membranal.

Once a putative interactor is verified, its relevance to defense responses induction must be investigated. Several defense responses are known and can be monitored. Some of them occur rapidly upon elicitation such as the production of reactive oxygen species (ROS) burst. ROS production promotes cell wall strengthening and H_2O_2 triggers local PCD [12, 13]. Other responses develop at a slower rate such as ethylene production. Ethylene is a gaseous plant hormone whose level increases after elicitation [14]. We will describe the methodology to measure and quantify both these parameters.

All the methods described refer to the LeEIX2/EIX system. Other systems may require different concentrations or incubation times. The methodology can apply both to transiently expressing and stably transgenic plants.

2 Materials

2.1 Co-IP of Membrane Proteins

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at 4 °C (unless indicated otherwise).

1. Extraction buffer (EB): 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl.

   Immediately prior to use add the following: 140 mM β-mercaptoethanol, 2 mM PMSF, 1 mM protease inhibitor cocktail (EDTA free).
2. Wash buffer 1: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% Nonidet P40.
3. Wash buffer 2: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 0.1% Nonidet P40.
4. Wash buffer 3: 10 mM Tris–HCl, pH 7.5, 0.1% Nonidet P40.

5. 4× Sample buffer: 8% SDS, 40% glycerol, 200 mM Tris-Cl, pH 6.8, 388 mM dithiothreitol (DTT), and 0.1 mg/mL bromophenol blue dye.

2.2 ROS Burst from Leaf Discs

Prepare all solutions using ultrapure water and analytical grade reagents.

1. Luminol 17 mg/mL in DMSO (100 mM) prepare freshly prior to use. Keep in the dark at room temperature.
2. HRP 10 mg/mL in 0.1 M Phosphate buffer pH 6.0. Store at −20 °C in the dark (*see* **Note 1**).
3. EIX 1 mg/mL.
4. White 96-well multiplate suitable for optical reading.
5. Luminometer suitable for 96-well multiplate.

2.3 Ethylene Induction from Leaf Discs

Prepare all solutions using ultrapure water and analytical grade reagents at room temperature.

1. Ethylene assay buffer: 10 mM MES pH 6.0, 250 mM sorbitol.
2. EIX 1 mg/mL.
3. 10-mL erlenmeyer flasks tightly septa sealed with septum rubber caps sleeve type that can be needle perforated.
4. 5-mL disposable syringes with 21G needles (to pump out the gaseous sample) and rubber stoppers (to hold the sample till injecting into the gas chromatographer; GC).

3 Methods

3.1 Co-IP of Membrane Proteins

Carry out all procedures at room temperature on ice unless otherwise specified.

1. Using precooled mortar and pestle; grind approximately 200 mg (*see* **Note 2**) plant tissue expressing the tagged membranal protein with liquid nitrogen to a fine powder.
2. When powder starts to melt, add three volumes of EB and continue grinding until a homogeneous liquid solution has been achieved.
3. Transfer solution to a precooled microfuge tube.
4. Centrifuge for 16 min at 20,000 × *g*, 4 °C.
5. Discard the supernatant (*see* **Note 3**).
6. Suspend pellet in two volumes EB containing 0.5% Triton X-100 (*see* **Note 4**).
7. Homogenize thoroughly inside the microfuge tube using a small plastic or glass pestle. Leave on ice for 20 min mixing every 5 min (*see* **Note 5**).

8. Centrifuge for 16 min at 20,000 × *g*, 4 °C.
9. Vortex for 5 s and collect the supernatant (Triton soluble Membrane (TSM) fraction).
10. Remove 5–50 μL (Input) according to the expected protein expression level, add to the input sample buffer ×4 (*see* **Note 6**).
11. Add to the TSM fraction three volumes of EB so as to dilute the Triton X-100 to 0.2% to avoid unspecific binding, and 5 μL antibody bound agarose beads previously washed twice with 1000 μL EB.
12. Incubate the samples 1 h (*see* **Note 7**) on a rotating wheel at 4 °C.
13. Centrifuge at 2500 × *g* for 3 min and discard supernatant.
14. Resuspend beads with 500 μL wash buffer 1 and transfer to a dolphin microfuge tube wash previous tube with another 500 μL of wash buffer 1 and add to dolphin tube, Final wash volume will be 1 mL. Return to rotating wheel at 4 °C for 5–7 min.
15. Centrifuge at 2500 × *g* for 3 min, discard supernatant.
16. Wash twice with 1-mL wash buffer 1 and centrifuge at 2500 × *g* for 2 min. Incubate between each wash the samples on the rotating wheel for 5 min at 4 °C.
17. Wash twice with 1-mL wash buffer 2 and once with 1-mL wash buffer 3. Incubate between each wash the samples on the rotating wheel for 5 min at 4 °C.
18. Resuspend beads with 500 μL wash buffer 3 and transfer to a new dolphin microfuge tube wash previous dolphin tube with another 500 μL of wash buffer 3 and add to the new dolphin tube.
19. Centrifuge at 2500 × *g* for 2 min and discard supernatant leaving beads as dry as possible.
20. Add to the beads 20 μL 2× sample buffer.

3.2 ROS Burst from Leaf Discs

Carry out all procedures at room temperature unless otherwise specified.

1. Sample leaf discs (LD) with a cork borer (5 mm inside diameter; *see* **Note 8**).
2. Place one LD in each well of a white 96-well multiplate containing 250 μL of ddH_2O water, abaxial side facing water. Handle LD carefully to prevent wounding and avoid taking any curvy or curly LD.
3. Incubate the LDs with a transparent lid on, for 4–24 h at room temperature with gentle agitation.
4. After incubation, remove water. Take care not to damage the LDs and make sure that they lay at the bottom of the well

(*see* **Note 9**). Immediately add 50 μL ddH_2O water to each well to prevent LDs drying up (*see* **Note 10**).

5. Prepare ROS assay solution concentrated 1.5 fold: Luminol 150 μM, HRP 15 μg/mL, EIX 1 μg/mL or control.
6. Add 100 μL to each well and immediately start measuring the light emission in repeating cycles till you observe emission decay (*see* **Note 11**).

3.3 Ethylene Induction from Leaf Discs

Carry out all procedures at room temperature unless otherwise specified.

1. Dispense 1-mL ethylene assay buffer with or without EIX 1 μg/mL in each erlenmeyer flask.
2. Sample leaf discs (LD) with a cork borer (7–8 mm inside diameter).
3. Place six LDs in each erlenmeyer flask, abaxial side facing solution. Avoid taking any curvy or curly LDs. Take care to distribute equally LDs from all sampled leaves.
4. Tightly cap flasks and incubate with gentle agitation for 4 h.
5. For each sample inject the needle connected to a 5-mL syringe, pump up and down gaseous sample two to three times and finally extract a 3-mL sample and immediately stick the syringe into a rubber stopper (*see* **Note 12**).
6. Inject and read samples in GC.

4 Notes

1. HRP is sensitive to repeated defrosting cycles, therefore prepare solution and divide into small aliquots before freezing.
2. For genes with low expression level, protocol can be scaled up to 1 g tissue.
3. This is the cytosolic soluble fraction. If one of the proteins is present also in the cytosolic fraction, keep this fraction on ice and use it to dilute the Triton X-100 concentration in **step 11**.
4. It is useful to prepare 10–25% Triton X-100 stock solution and add the required amount to EB to avoid pipetting concentrated viscous Triton X-100.
5. Alternatively, the samples can be incubated on the rotating wheel at 4 °C at a slow speed.
6. Input samples can be stored at −20 °C after boiling.
7. For low protein expression and/or weak interaction, incubation may be prolonged up to overnight.
8. When using *Agrobacterium* for transient expression, sample LD 24–44 h after infiltration.

9. To thoroughly remove all water, use a blunt needle connected to a vacuum source.
10. To avoid LD drying up, remove water from one row at a time.
11. Longer integration time will yield fewer readings with higher level of emission. It is crucial to match the time elapsed between adding the solution to each well to the integration time between well readings.
12. Samples can be stored for 1–2 h before injecting them into the GC if required.

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