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Apoplastic Expression of CARD1-ecto Domain in *Nicotiana benthamiana* and Purification from the Apoplastic Fluids

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Abstract

The protein expression and purification process is an essential initial step for biochemical analysis of a protein of interest. Traditionally, heterologous protein expression systems (such as *E. coli*, yeast, insect cells, and cell-free) are employed for plant protein expression, although a plant expression system is often desirable for plant proteins, to ensure proper post-translational modifications. Here, we describe a method to express and purify the ectodomain of one of the leucine-rich repeat receptor-like kinase called CARD1/HPCA1, from *Nicotiana benthamiana* apoplastic fluid. First, we express His-tagged CARD1 ectodomain in the apoplastic space of *N. benthamiana* by the Agroinfiltration method. Then, we collect apoplastic fluids from the leaves and purify the His-tagged protein by Ni^{2+}-affinity chromatography. In addition to plant-specific post-translational modifications, protein accumulated in the plant apoplastic space, rather than in the cytosolic space, should be kept under an oxidizing environment. Such an environment will help to maintain the property of intrinsic disulfide bonds in the protein of interest. Further, purification from the apoplastic fluids, rather than the total protein extract, will significantly reduce contaminants (for instance RuBisCO) during protein extraction, and simplify downstream processes. We envisage that our system will be useful for expressing various plant proteins, particularly the apoplastic or extracellular regions of membrane proteins.

Keywords: Protein expression and purification, *Nicotiana benthamiana,* Leucine-rich repeat receptor-like kinase, Apoplastic fluids, Signal peptide

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Background

The use of *Nicotiana benthamiana* as a host organism for protein expression has increasingly become an attractive system, in addition to well-established expression systems like *E. coli*, yeast, insect cells, and cell-free. However, it is challenging to obtain near homogeneity protein by one-step affinity purification in the *N. benthamiana* system, possibly due to the complexity of the total protein extract (Souza, 2015). An alternative option is to ensure that the proteins of interest are expressed and accumulated in specialized compartments, which are spatially separated from contaminants derived from other compartments. Thus, by collecting proteins of interest accumulated in a specific compartment, we can simplify the heterogeneity of the starting material for column chromatography.

Many plant proteins passing through the secretory pathway need to get oxidized and form disulfide bonds, to mature into a stable form (Meyer *et al.*, 2019). The appropriate disulfide bonding is required in many apoplastic proteins or extracellular regions of membrane proteins, including membrane-bound leucine-rich repeat (LRR) receptor-like kinase, and secreted peptide hormones (Meyer *et al.*, 2019). However, the cytoplasm is normally maintained in a reducing environment. It is desirable to accumulate the expressed proteins in the apoplast, and retrieve them with appropriate disulfide bond modifications.

In this protocol, we describe a method to express and purify the ectodomain of an LRR receptor-like kinase CARD1/HPCA1 (CARD1ecto) from *N. benthamiana* apoplastic fluid. We will primarily focus on its expression and accumulation in the apoplast (Procedure A), its extraction from the apoplastic space (Procedure B), and its purification by Ni^{2+}-affinity chromatography (Procedure C). This system should prove useful not only for CARD1ecto, but also for other plant proteins of interest, particularly the apoplastic or extracellular regions of membrane proteins, which may require redox-related modifications to function correctly.

Materials and Reagents

1. 1 mL needleless plastic syringe (TERUMO, catalog number: SS-01T)
2. 20 mL needleless plastic syringe (TERUMO, catalog number: SS-20ESz)
3. 50 mL centrifuge tube (FALCON, catalog number: 352070)
4. GD/X syringe filter (PES 0.45 μm) (GE healthcare, catalog number: 6876-2504)
5. 500 mL Pyrex beaker
6. 300 mL Pyrex beaker
7. Vacuum desiccator
8. 15 mL centrifuge tube (FALCON, catalog number: 352097)
9. Soil-grown *Nicotiana benthamiana*
10. pEAQ-HT plasmid (Sainsbury *et al.*, 2009)
11. *Agrobacterium tumefaciens* C58C1 carrying pCH32 (Hamilton *et al.*, 1996; Hellens *et al.*, 2000)
12. LB Broth (Lennox) (Sigma-Aldrich, catalog number: L7275-500TAB)
13. Kanamycin (FUJIFILM Wako Chemicals, catalog number: 113-00343)
14. Rifampicin (FUJIFILM Wako Chemicals, catalog number: 185-01003)
15. 4'-Hydroxy-3',5'-dimethoxyacetophenone (acetosyringone) (Sigma-Aldrich, catalog number: D134406-25G)
16. Bis-Tris (Dojindo, catalog number: 6976-37-0)
17. Tween 20 (polyoxyethylene sorbitan monolaurate) (Nacalai Tesque, catalog number: 35624-15)
18. cOmplete™ ULTRA Tablets, EDTA-free, Protease Inhibitor Cocktail (Merck, catalog number: 5892953001)
19. Sodium Chloride (FUJIFILM Wako Chemicals, catalog number: 195-01663)
20. Magnesium Chloride (FUJIFILM Wako Chemicals, catalog number: 136-03995)
21. Immobilized Ni^{2+}-affinity column, HisTrap excel (Cytiva, catalog number: 17371205)

> *Note: Whilst any standard Ni^{2+}-affinity column should be acceptable, we recommend using the HisTrap excel column by Cytiva. In this method, the His-tagged protein will be purified in buffer at pH 6.0, given that apoplast space is usually weakly acidic. According to the manufacturer's instructions, HisTrap excel can capture His-tagged proteins even at pH 6.0.*

22. Superloop, 1/16" fittings (ÄKTAdesign), 50 mL (Cytiva, catalog number: 18111382)

23. Imidazole (FUJIFILM Wako Chemicals, catalog number: 095-00015)
24. Vivaspin Turbo 15 (10 kDa molecular weight cut off) (Sartorius, catalog number: VST15T01)
25. Coomassie protein stain, such as InstantBlue (Expedeon, catalog number: ISB01L)
26. Agroinfiltration buffer (see Recipes)
27. Vacuum infiltration buffer (see Recipes)
28. Equilibration buffer (see Recipes)
29. Wash buffer (see Recipes)
30. Elution buffer (see Recipes)

Equipment

1. Vacuum pump (*e.g.*, ULVAC DTC-41, or equivalent model)
2. Electroporation system (*e.g.*, Bio-Rad Gene Pulser XCell™, or equivalent)
3. Centrifuge with a swing rotor for 50 mL centrifuge tubes (*e.g.*, Hitachi CF16RXII with swing rotor T4SS31, or equivalent)
4. Centrifuge with a fixed angle rotor for 15 mL centrifuge tubes (*e.g.*, Hitachi CR20GIII with fixed angle rotor R15A, or equivalent)
5. Fast Protein Liquid Chromatography machine (we used the equivalent of GE healthcare AKTA pure 25 M1 (Cytiva, catalog number 29018227), with optional accessories installed).
   Note: Any fast protein liquid chromatography machine from companies such as Cytiva (https://www.cytivalifesciences.com/) or from Bio-Rad (https://www.bio-rad.com/) are fine.
6. Spectrophotometer (*e.g.*, Thermo Fisher Scientific NanodDrop One^{C}, or equivalent)
7. Standard SDS-PAGE equipment (such as Mini-PROTEAN® Tetra Cell from Bio-Rad). For the SDS-PAGE protocol, please refer to Green and Sambrook (2014).

Procedure

A. Expression of His-tagged CARD1 ectodomain in apoplastic space of *N. benthamiana*

Note: We expressed non-tagged CARD1ecto, and purified it by conventional column chromatographies in our previous study (Laohavisit et al., 2020). Although it is possible to purify non-tagged proteins, the purification procedure is simpler in His-tagged proteins.

1. Clone the nucleotide sequence encoding CARD1 from *Arabidopsis* genomic DNA (amino acids 1–546) into the pEAQ-HT vector, in frame with a His tag at C-terminus (CARD1ecto-His), using standard molecular biology techniques (Figure 1).

   Notes:
   a. The native signal peptide of CARD1 should be intact, so that CARD1ecto-His is secreted into the apoplastic space.
   b. In the pEAQ-HT vector system, a gene of interest is inserted between a modified 5'-untranslated region (UTR) and the 3'-UTR from Cowpea mosaic virus RNA-2, and co-expressed together with silencing suppressor p19 (Sainsbury et al., 2009; Sainsbury and Lomonossoff, 2008). This permits an extremely high-level and rapid production of proteins of interest (see Figure 1).
   c. We used genomic DNA to clone the construct, but cDNA should also work.
   d. For His-tag at C-terminus, pEAQ-HT should be digested with AgeI and SmaI restriction enzymes (Figure 1, see Sainsbury et al., 2009 for details).

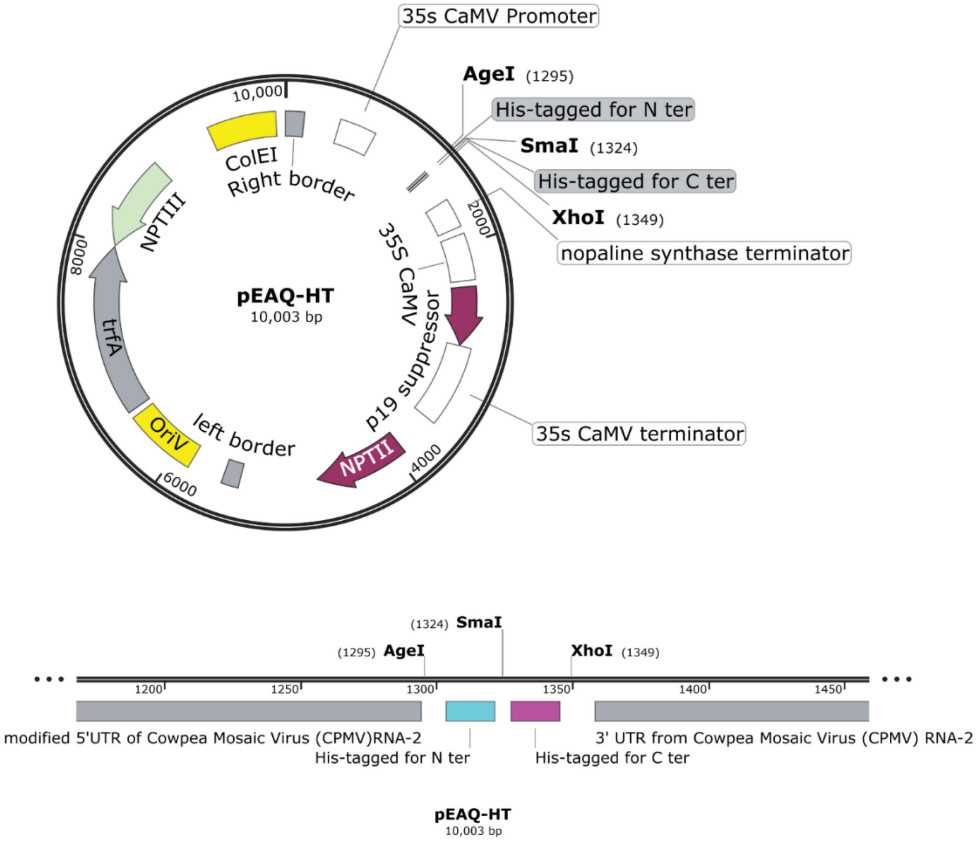

Figure 1. The pEAQ-HT vector map.
Top, a simplified pEAQ-HT vector map. Bottom, a close-up of the restriction enzyme map.

2. After sequence confirmation, transform electrocompetent cells of *Agrobacterium tumefaciens* C58C1 with pEAQ-CARD1ecto-His, using an electroporation system (machine default settings for *Agrobacterium tumefaciens* are capacitor = 25 μF, pulse controller = 200 Ω, and Voltage = 2.4 kV). Plate out the transformed cells onto LB agar plates supplemented with 100 μg/mL rifampicin and 50 μg/mL kanamycin.
3. Confirm the *Agrobacterium* colonies with correct transformation using PCR, and make glycerol stocks for long term storage.
4. Pick a fresh colony or take a small portion of the glycerol stocks of *Agrobacterium* into 5 mL of LB liquid media supplemented with 100 μg/mL rifampicin and 50 μg/mL kanamycin and incubate it with shaking at 120 rpm at 28°C overnight.
5. Dilute 2 mL of overnight culture with 18 mL of fresh LB liquid media supplemented with 100 μg/mL rifampicin and 50 μg/mL kanamycin, and incubate with shaking at 120 rpm and 28°C for 4 h.
6. Centrifuge the *Agrobacterium* at 3,000 × *g* for 5 min, and discard the supernatant.
7. Add 10 mL of Agroinfiltration buffer, and resuspend the pellet.
8. Repeat steps 6–7.
9. Measure OD_{600} of the suspension and adjust it to 0.3.
10. Infiltrate the *Agrobacterium* suspension into whole leaves of 5-weeks-old *N. benthamiana* from the abaxial side, using 1 mL needleless syringes. The infiltrated area should turn darker in color compared to the unfiltrated area (Yin *et al*., 2017).

Note: In our experiment, we infiltrated approximately 30 leaves to obtain approximately 1 mg of highly purified CARD1ecto-His protein. For other proteins of interest, it is recommended that the experimenter perform small-scale pilot experiments (for instance, 8–10 leaves per construct) to ascertain how much protein can be obtained, since different proteins will show different expression levels.

B. Extraction of apoplastic fluid from *N. benthamiana* leaves

1. After 4–7 days post inoculation, harvest the infiltrated *N. benthamiana* leaves and pile them together, such that the abaxial side of the leaves points upward.
2. Carefully place the pile of leaves into a 500-mL Pyrex beaker containing 150 mL of vacuum-infiltration buffer. Afterward, place a 300-mL Pyrex beaker on top of the pile, such that leave samples are submerged in buffer (Figure 2).

Figure 2. Set-up for vacuum infiltration.

3. Place the assembled beaker (with leave samples) into a vacuum desiccator, and apply a pressure of 60 hPa for 10 min using a pump (or other appropriate equipment). Bubbles should be released from the leaves.
4. Slowly release the pressure inside the desiccator. We usually allow at least 10 min for the pressure to return to normal.

Note: In this step, expanded air bubbles in the apoplastic space shrink significantly and vacuum-infiltration buffer will replace this space. The color of the leaves should turn darker as the apoplastic space has been filled with buffer. If you do not notice any differences, this suggests that the vacuum infiltration process has not been successful, and step 3 should be carefully repeated.

5. Carefully take out individual leaves from the beaker and gently remove excess buffer using paper towels. Set these aside.
6. Meanwhile, assemble the apoplastic fluid collection unit. This consists of the following materials:
   - 20-mL single-use needleless plastic syringe with the plunger removed (*i.e.*, only the barrel part is needed)
   - 50-mL centrifuge tubes, non-skirted

   To assemble the apoplastic fluid collection unit, simply place the barrel part of a 20-mL sized syringe into a 50-mL centrifuge tube (Figure 3A)

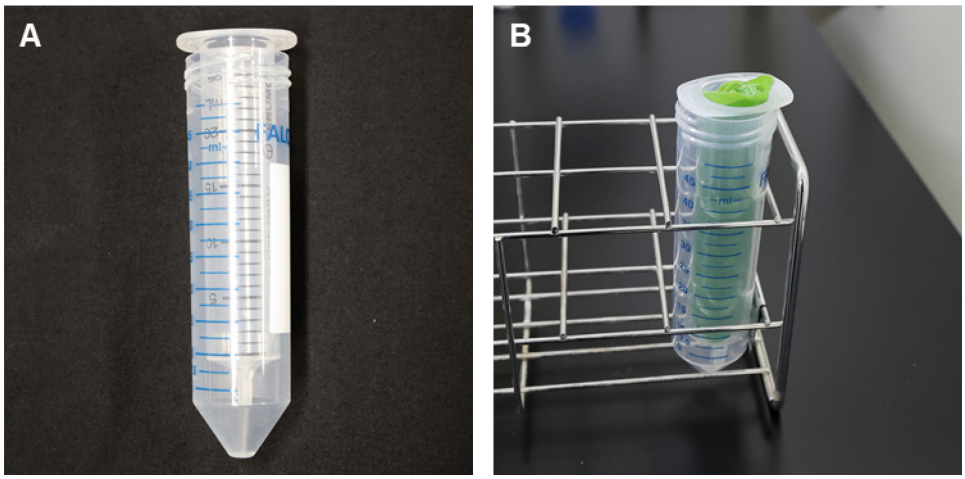

Figure 3. Set-up of the apoplastic fluid collection unit.

7. Per apoplastic fluid collection unit, take 6–8 leaves from step 5 and separate them into two piles (3–4 leaves per pile). Next, carefully roll the leaves from one pile (from the base to the apex of the leaf) and place this into the barrel part of the apoplastic fluid collection unit. The abaxial or adaxial sides can be faced in any direction. Repeat for the second pile (Figure 3B).

   Note: For our experiment, we used approximately 30 leaves to purify CARD1ecto-His protein, which corresponded to 6 apoplastic fluid collection units.

8. Place the apoplastic fluid collection units (with leaves) in a swing rotor, and centrifuge at 1,500 × *g* and 4°C for 10 min.

   Note: Centrifugation should be performed using a swing rotor, to maximize recovery of the apoplast fluid.

9. Apoplastic fluid should appear at the bottom of the 50-mL centrifuged tubes. Transfer the fluid to a separate tube and centrifuge again at 1,500 × *g* and 4°C for 3 min, to ensure all fluid has been extracted. Finally, pool all the apoplastic fluid into a single tube.
10. Centrifuge the collected apoplastic fluid from step 9 at 10,000 × *g* and 4°C for 10 min. Collect the supernatant, and centrifuge it again at 10,000 × *g* and 4°C for 10 min.
11. Filter the supernatant with a GD/X syringe filter. The resulting filtrate is now ready for the next step of purification.

    Note: The filter unit should have a 0.45 µm pore size.

C. CARD1ecto-His purification by Ni^{2+}-affinity chromatography

Note: Monitor the absorbance at 280 nm to detect proteins throughout the purification process.

1. Connect a HisTrap excel column (capacity of 5 mL) to an FPLC system (such as the AKTA pure), and wash this column with 25 mL of ultrapure water (5 column volumes, CV) at 5 mL/min (1 CV/min).

Note: Wash volume and the flow rate should be adjusted according to the column size used.

2. Equilibrate the column with 25 mL (5 CV) of equilibration buffer at 5 mL/min (1 CV/min).
3. Load the apoplastic fluid sample onto the column with a superloop at 2.5 mL/min (0.5 CV/min).

Note: Depending on your FPLC machine configuration and your sample volume, you can also load your sample manually.

4. Wash the column with 25 mL (5 CV) of wash buffer at 5 mL/min (1 CV/min).
5. Elute the His-tagged protein with 25 mL (5 CV) of elution buffer at 5 mL/min (1 CV/min).
6. Pool the peak fractions in one sampling tube and check the purity of CARD1ecto-His by SDS-PAGE, followed by Coomassie brilliant blue staining (InstantBlue; Expedeon) (Figure 4).

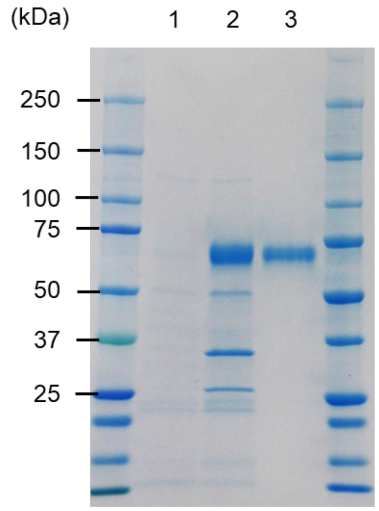

Figure 4. SDS-PAGE analysis of purified CARD1ecto-His.
Lane 1: Apoplastic fluid from intact *N. benthamiana* leaves. Lane 2: Apoplastic fluids from the CARD1ecto-His expressing *N. benthamiana* leaves. Lane 3: Purified CARD1ecto-His by Ni^{2+}-affinity chromatography. The gel was stained with Coomassie brilliant blue (InstantBlue).

7. Measure protein concentration by absorbance at 280 nm, using a spectrophotometer (such as NanoDrop One[C]).
8. (Optional) Apply the pooled fractions in an ultrafiltration unit with a 10-kDa cut-off membrane (Vivaspin turbo 15) for buffer exchange and/or concentration.

Note: When buffer exchanging or sample concentration are necessary for downstream processes.

Recipes

1. Agroinfiltration buffer

10 mM MES/NaOH (pH 5.6)
10 mM $MgCl_2$
150 µM acetosyringone

2. **Vacuum infiltration buffer**
   20 mM Bis-Tris/HCl (pH 6.0)
   0.01% Tween20
   1× cOmplete™ Protease Inhibitor Cocktail

3. **Equilibration buffer**
   20 mM Bis-Tris/HCl (pH 6.0)
   300 mM NaCl

4. **Wash buffer**
   20 mM Bis-Tris/HCl (pH 6.0)
   300 mM NaCl
   5 mM Imidazole

5. **Elution buffer**
   20 mM Bis-Tris/HCl (pH 6.0)
   300 mM NaCl
   300 mM Imidazole

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Competing interests

The authors declare no competing interests.

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