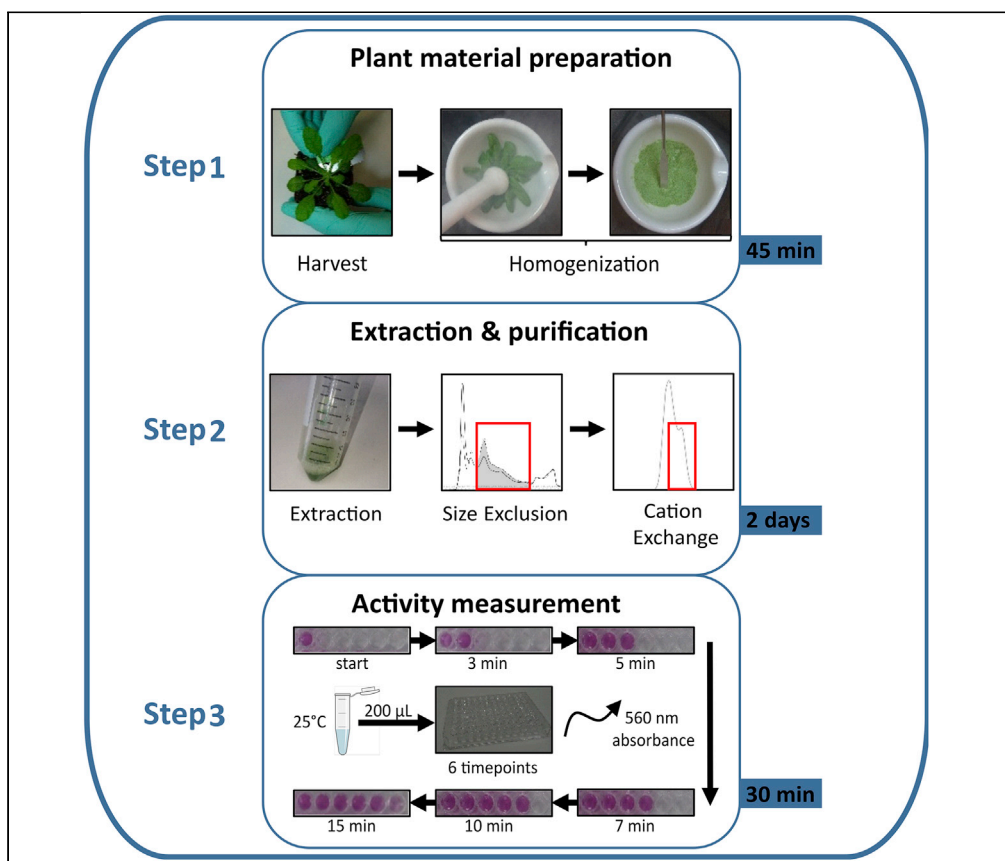


## Protocol

# Native protein purification of ferroxidase LPR1 from leaf extracts of a transgenic *Arabidopsis thaliana* line



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### Highlights

Protocol to produce and purify plant ferroxidase AtLPR1 from leaf extracts

Protocol for tag-free purification, yielding highly pure protein for *in vitro* assays

Specific ferroxidase activity measurements in mild acetic pH for protein biochemistry

LPR1 (LOW PHOSPHATE ROOT 1), a bacterial-type plant ferroxidase, is crucial for local root phosphate (Pi) sensing. Here, we present a detailed protocol for native (tag-free) protein purification of LPR1 from leaf extracts by differential ammonium sulfate precipitation, size exclusion, and cation exchange chromatography of a transgenic *Arabidopsis thaliana* line overexpressing LPR1. We outline steps for LPR1 purification tracking via immune blot analysis and ferroxidase activity assay. The protocol yields highly pure and active LPR1 protein for biochemical analysis.

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

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## Protocol

Native protein purification of ferroxidase LPR1 from leaf extracts of a transgenic *Arabidopsis thaliana* lineNancy Tang<sup>1,2,\*</sup> and Christin Naumann<sup>1,2,3,\*</sup><sup>1</sup>Department of Molecular Signal Processing, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [nancy.tang@ipb-halle.de](mailto:nancy.tang@ipb-halle.de) (N.T.), [christin.naumann@ipb-halle.de](mailto:christin.naumann@ipb-halle.de) (C.N.)  
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## SUMMARY

LPR1 (LOW PHOSPHATE ROOT 1), a bacterial-type plant ferroxidase, is crucial for local root phosphate (Pi) sensing. Here, we present a detailed protocol for native (tag-free) protein purification of LPR1 from leaf extracts by differential ammonium sulfate precipitation, size exclusion, and cation exchange chromatography of a transgenic *Arabidopsis thaliana* line overexpressing LPR1. We outline steps for LPR1 purification tracking via immune blot analysis and ferroxidase activity assay. The protocol yields highly pure and active LPR1 protein for biochemical analysis.

For complete details on the use and execution of this protocol, please refer to Naumann et al. (2022).

## BEFORE YOU BEGIN

We generated stable transgenic (CaMV 35Spro:LPR1) *Arabidopsis thaliana* lines, which overexpress native LPR1 in roots and leaves (deposited at NASC, Stock ID: N2111056). Plants were grown on soil for 6–8 weeks in short-day conditions (8 h/16 h, light/dark) at constant 21°C.

## Plant seeding and growth

⌚ Timing: 6–8 weeks

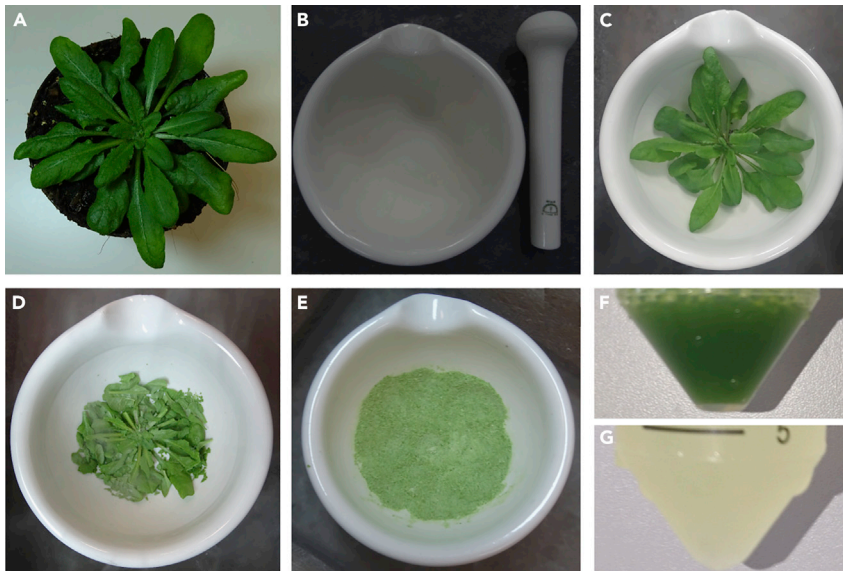
1. Seeds of CaMV 35Spro:LPR1 were sown on steamed soil mixture “Einheitserde Classic Kokos”, consisting of 45% white peat, 20% clay, 15% block peat, 20% coco fibers, 25% vermiculite (grain size 2–3 mm), and 300–400 g Exemptor per m<sup>3</sup> soil substrate (ca. 100 g thiacloprid per kg soil). Seeds were stratified for 2 d in the dark at 4°C prior to germination in short day conditions (8 h/16 h, light/dark) at constant 21°C.
  - a. After germination for 2–3 weeks, plants were pricked into single pots and grown for additional 4–5 weeks until 18–22 rosette leaves were fully developed (Figure 1A).

## Harvesting rosette leave material

⌚ Timing: 10–30 min

2. Place mortar and pestle (Figure 1B) into a container (e.g., polystyrene box) filled with liquid nitrogen for pre-cooling.
3. Harvest stepwise (4–8 rosettes per step) all above-ground tissues of short-day grown CaMV 35Spro:LPR1 plants into the mortar (Figure 1C) by cutting off the rosette with a sharp razor blade.





**Figure 1. Plant material processing to extract AtLPR1**

(A) 8-week-old plant grown in short-days conditions.

(B–G) (B–E) Homogenization of plant material with mortar and pestle (F) solubilized 40% ammonium sulfate precipitation pellet (G) solubilized 80% ammonium sulfate precipitation pellet.

4. Homogenize leaf material with liquid nitrogen to a fine powder by first shortly (Figure 1D) crushing the leaf surface with the pestle, prior to fine homogenization of the whole rosette to a fine green powder by continuous grinding. [Troubleshooting 1](#).

**△ CRITICAL:** Please note that harvesting prior to bolting is crucial. Plants should contain 18–22 fully developed leaves without flower bud induction. Protein yield will increase with homogenization degree of the plant material.

**▣ Pause point:** Homogenized material can be stored, without addition of any buffer at  $-80^{\circ}\text{C}$  for at least one year. Before storage make sure that all residual liquid nitrogen is evaporated.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rabbit polyclonal anti-LPR1 (1:1000)	<a href="#">Naumann et al., (2022)</a>	N/A
Goat monoclonal anti-rabbit IgG-HRP (1:5000)	Thermo Scientific	31460
<b>Chemicals, peptides, and recombinant proteins</b>		
Ammonium sulfate	Carl Roth	9218.1
Tris	Carl Roth	0188.3
HCl 37%	Carl Roth	6331.1
NaCl	Carl Roth	HN00.2
EDTA	Sigma-Aldrich	Cat# E6635
Glycerol	Carl Roth	6962.1
Glycine	Carl Roth	HN07.3
PMSF	Carl Roth	6367.3
Na <sub>2</sub> HPO <sub>4</sub>	Carl Roth	T877.1
NaH <sub>2</sub> PO <sub>4</sub>	Carl Roth	T879.2
SDS pellets	Carl Roth	8029.4

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DTT	Sigma-Aldrich	1114740005
Tween-20	Carl Roth	9127.1
Na-acetate	Carl Roth	6773.2
Acetic acid	Carl Roth	T179.1
cOmplete, EDTA-free protease inhibitor cocktail	Merck	11873580001
Thiourea	Carl Roth	9531.1
3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine (ferrozine)	Sigma-Aldrich	82940
Chloroform	Merck	1024451000
PageBlue™ protein-stain	Thermo Scientific	24620

### Critical commercial assays

2D-Quant	GE Healthcare	80-6483-56
Power Blotter 1-Step™ Transfer Buffer (5×)	Thermo Scientific	PB7100
ECL Select Western Blotting Detection Reagent	Amersham	RPN2235

### Experimental models: Organisms/strains

<i>Arabidopsis thaliana</i> 35S <sub>pro</sub> :LPR1	Müller et al., 2015	35S <sub>pro</sub> :LPR1
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### Other

Amersham Hybond PVDF (0.45 P) membrane	Amersham	10600038
Mortar	IDL GmbH	ID2100185090
Pestle	IDL GmbH	ID2100195100
Nylon mesh 100 µm mesh size	Carl Roth	XA96.1
Centrifuge	Eppendorf	5804R
AKTA Purifier 10	GE Healthcare	N/A
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad	# 1658004
Power Blotter XL System	Thermo Scientific	PB0013
HighLoad Superdex 16/60 200 pg	GE Healthcare	GE28-9893-35
Superloop, 1/16" fittings (ÄKTAdesign), 50 mL	Cytiva	18111382
HiTrap CM Fast Flow, 1-mL	GE Healthcare	GE17-5056-01
Fuji Super RX-N 13 × 18 100 BI.	Röntgen Express	N/A
96-well-plate, polystyrol	Sigma-Aldrich	M2936-100EA

## MATERIALS AND EQUIPMENT

### Extraction buffer

Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 6.8)	50 mM	2.5 mL
NaCl (5 M)	100 mM	1 mL
EDTA (0.5 M, pH 8)	0.5 mM	0.5 mL
Glycerol	10%	5 mL
PMSF (0.1 M in DMSO)	1 mM	0.5 mL
EDTA-free Protease inhibitor	1×	1 tablet
ddH <sub>2</sub> O		up to 50 mL
<b>Total</b>		<b>50 mL</b>

Fully supplemented with protease inhibitor the buffer can be stored for 1 day at 4°C.

△ **CRITICAL:** Extraction buffer without PMSF and protease inhibitor can be stored at 4°C for 2 month.

### Buffer A (size exclusion chromatography)

Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 6.8)	50 mM	50 mL
NaCl (5 M)	100 mM	20 mL

(Continued on next page)

**Continued**

Reagent	Final concentration	Amount
EDTA (0.5 M, pH 8)	0.5 mM	1 mL
Glycerol	10%	100 mL
ddH <sub>2</sub> O		829 mL
<b>Total</b>		<b>1,000 mL</b>

Store Buffer A at 4°C for maximum 2 month.

⚠ **CRITICAL:** Buffer A must be degassed and filtered before usage.

**Buffer B (cation exchange chromatography)**

Reagent	Final concentration	Amount
Na <sub>2</sub> HPO <sub>4</sub> -NaH <sub>2</sub> PO <sub>4</sub> (0.1 M, pH 7)	20 mM	200 mL
ddH <sub>2</sub> O		800 mL
<b>Total</b>		<b>1,000 mL</b>

Store Buffer B at 4°C for maximum 2 month.

⚠ **CRITICAL:** Buffer B must be degassed and filtered before usage.

**Buffer C (elution buffer)**

Reagent	Final concentration	Amount
Na <sub>2</sub> HPO <sub>4</sub> -NaH <sub>2</sub> PO <sub>4</sub> (0.1 M, pH 7)	20 mM	200 mL
NaCl (5 M)	1 M	200 mL
ddH <sub>2</sub> O		600 mL
<b>Total</b>		<b>1,000 mL</b>

Store Buffer C at 4°C for maximum 2 month.

⚠ **CRITICAL:** Buffer C must be degassed and filtered before usage.

**5 × SDS-PAGE loading buffer**

Reagent	Final concentration	Amount
Tris-HCl (1 M pH 6.8)	0.35 M	3.5 mL
Glycerol	30% (v/v)	3 mL
SDS	10%	1 g
DTT	0.6 M	0.93 mg
Bromphenol blue (1% w/v)	0.012%	0.12 mL
ddH <sub>2</sub> O		up to 10 mL
<b>Total</b>		<b>10 mL</b>

1 mL aliquots can be stored at –20°C for a maximum of 1 year.

**SDS running buffer**

Reagent	Final concentration	Amount
Tris base	25 mM	3 g
SDS	0.1%	1 g
Glycine	192 mM	14.4 g
ddH <sub>2</sub> O		up to 1,000 mL
<b>Total</b>		<b>10 mL</b>

Store at 22°C–25°C (room temperature).

△ CRITICAL: Never adjust pH.

### Tris-buffered saline with Tween 20 (TBST)

Reagent	Final concentration	Amount
Tris-HCl (1 M pH7.6)	20 mM	10 mL
NaCl (5 M)	150 mM	15 mL
Tween-20	0.2% (v/v)	1 mL
ddH <sub>2</sub> O		up to 500 mL
<b>Total</b>		<b>500 mL</b>

Store at 22°C–25°C (room temperature) for a maximum of 3 months.

### Sodium acetate solution

Reagent	Final concentration	Amount
Na-acetate	0.45 M	36.91 g
ddH <sub>2</sub> O		1,000 mL
<b>Total</b>		<b>1,000 mL</b>

Sodium acetate solution can be stored for a year at 22°C–25°C (room temperature).

### Acetic acid solution

Reagent	Final concentration	Amount
Acetic acid	0.45 M	25.87 mL
ddH <sub>2</sub> O		974.13 mL
<b>Total</b>		<b>1,000 mL</b>

Acetic acid solution can be stored for one year at 22°C–25°C (room temperature).

△ CRITICAL: When preparing acetic acid solution, first the water, then add the acid.

### Acetate buffer

Reagent	Final concentration	Amount
Sodium acetate solution	0.45 M	940 mL
Acetic acid solution	0.45 M	60 mL
<b>Total</b>		<b>1,000 mL</b>

Acetate buffer can be stored at 22°C–25°C (room temperature) for one year.

### Ferrozine substrate solution

Reagent	Final concentration	Amount
Thiourea	0.13 M	2.475 g
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> × 6 H <sub>2</sub> O	367 μM	36 mg
Chloroform	0.1%	250 μL
ddH <sub>2</sub> O		add 250 mL
<b>Total</b>		<b>250 mL</b>

Ferrozine substrate solution is stable for 3 weeks at 4°C.

△ CRITICAL: It is crucial to first dissolve the thiourea in water and add the chloroform before solubilizing the substrate (Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> × 6 H<sub>2</sub>O), otherwise it is directly oxidized and not suitable for the assay.

Ferrozine solution		
Reagent	Final concentration	Amount
Ferrozine (3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine)	18 mM	88.64 mg
ddH <sub>2</sub> O		10 mL
<b>Total</b>		<b>10 mL</b>

Ferrozine solution can be stored at 4°C for 1 year.

## STEP-BY-STEP METHOD DETAILS

This protocol aims to purify overexpressed AtLPR1 in a native form from frozen leaf material. The purification process includes protein extraction, size exclusion and cation exchange chromatography to obtain pure and active LPR1 protein for further biochemical analysis.

### Protein extraction

⌚ Timing: 1 h

Protein extraction from plant material is dependent on homogenization degree of the frozen plant material with the extraction buffer.

1. To extract whole leaf proteins, vortex 15 g frozen plant material in a 50 mL Falcon tube with 40 mL pre-cooled extraction buffer for a minimum of 1 min, followed by incubation on ice for 5 min.
2. Vortex suspension again for 1 min and incubate at 4°C for a minimum of 30 min on a rotating wheel (20 rpm) or shaker (800 rpm). [Troubleshooting 2](#).
3. Centrifuge at 500 × g for 30 min at 4°C.
4. Discard the pellet (cell fragments) and filter supernatant through a nylon mesh (100 μm mesh size) to remove residual plant fragments. Take 40 μL of the supernatant as sample for Western blot analysis, this sample refers to "crude extract (E)".

⚠ **CRITICAL:** The extraction process is crucial, therefore effective vortexing increases protein yield.

### Protein concentration by differential ammonium sulfate precipitation

⌚ Timing: 3–5 h

Ammonium sulfate precipitation is one of the most commonly used first steps for classical protein purification. Pre-fractionation is used to enrich LPR1 from whole plant extract based on its chemical properties. Hereafter, work at 4°C on ice and store protein preparations on ice when transported.

5. 40 mL of supernatant are subjected to a beaker glass with magnetic stirrer.
6. Slowly add 10.56 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to the supernatant while moderately stirring (aliquots of ca. 2 g will be helpful). Make sure ammonium sulfate is dissolved before adding the next aliquot. [Troubleshooting 3](#).
7. After dissolving the ammonium sulfate, stir the solution for one hour at 4°C on ice.
8. Centrifuge (4,500 × g) for 45 min at 4°C (precooled).
9. Transfer supernatant to a fresh beaker glass with magnetic stirrer and solubilize greenish (40% pellet, [Figure 1F](#)) protein pellet in extraction buffer to take 40 μL sample for Western blot analysis, this sample refers to "pellet 1 (P1)". Discard the rest of the pellet and continue working with the supernatant from the precipitation reaction before.

10. Slowly add another 10.56 g  $(\text{NH}_4)_2\text{SO}_4$  to the supernatant while moderately stirring (aliquots of ca. 2 g will be helpful). Make sure ammonium sulfate is dissolved before adding the next aliquot. [Troubleshooting 3](#).
11. After dissolving the ammonium sulfate, stir the solution for one hour at 4°C on ice.
12. Centrifuge (4,500 × g) for 45 min at 4°C (precooled).
13. Discard supernatant (take 40 μL for Western blot analysis, this sample refers to “80% supernatant”), solubilize pellet in 2 mL extraction buffer ([Figure 1G](#)) and take 5 μL for Western blot analysis, this sample refers to “pellet 2 (P2)”.

▣▣ **Pause point:** The solubilized protein pellet obtained can be stored at –80°C for 1 week.

### Size exclusion chromatography and cation exchange chromatography

⌚ **Timing:** 7 h

To desalt, enrich and concentrate LPR1, size exclusion chromatography followed by cation exchange chromatography is performed, which yields pure LPR1 protein with high ferroxidase activity. Before you start, make sure that all buffers and solution used are filtered and degassed.

14. Thaw protein pellet on ice and centrifuge (>15,000 × g) for 20 min at 4°C to clear precipitates.
15. Attach a HighLoad Superdex 200 gel filtration column (HL 16/60, GE Healthcare) to an FPLC-system.
16. Use a 5 mL loop for online sample application. Before loading, wash the loop with 5 mL water followed by 5 mL NaOH (1 M) followed by 5 mL water and 10 mL Buffer A.
17. Equilibrate the column with 2 column volumes of Buffer A at 1 mL/min flow rate or according to the column manufacturer’s instructions before sample application at 0.5 mL/min flow rate.
18. Elute proteins at a flow rate of 0.5 mL/min with 1.5 column volumes Buffer A.
19. Start sampling 1 mL fractions after 20% of the column volume.
20. After completion, combine fractions corresponding to the “F3” in [Figure 2A](#) (in our system fraction E4-F4) and dilute with 10–20 mL  $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$  (0.02 M, pH 7) to a final volume of 25–30 mL. Take 40 μL sample for Western blot analysis, this sample refers to “size exclusion (SE)”. [Troubleshooting 4](#).
21. Attach a carboxymethyl-sepharose column (HiTrap CM FF, 1-mL, GE Healthcare) to the FPLC-system and equilibrate it with 10 mL Buffer B.
22. Load the sample at 0.5 mL/min and continue to run Buffer B at 1 mL/min through the system until the baseline returns to zero.
23. Wash the column with 10 mL of Buffer B at 1 mL/min flow rate.
24. Elute the bound proteins with Buffer B and increasing concentrations of Buffer C up to 100% Buffer C at 1 mL/min flow rate and collect 1 mL fractions. [Troubleshooting 5](#).
25. Analyze the fractions by SDS-PAGE and use the desired fractions.

⚠ **CRITICAL:** Size exclusion chromatography and cation exchange chromatography must be performed on the same day. Longer storage of the combined fractions after size exclusion chromatography results in complete loss of LPR1 protein.

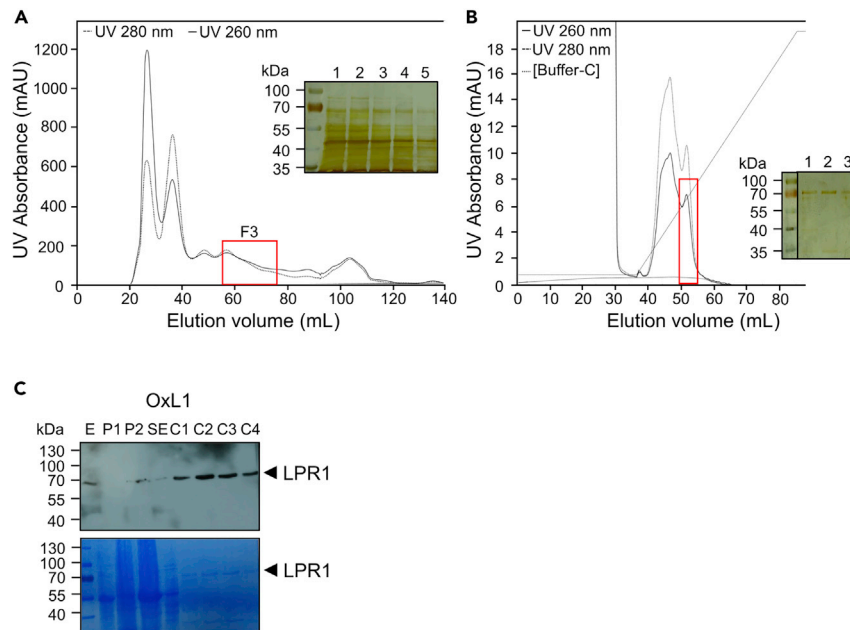
*Optional:* To identify protein samples that contain LPR1, you should use either Western blot analysis followed by Coomassie-staining or silver-staining to visualize protein abundance.

▣▣ **Pause point:** The protein fractions can be stored at –20°C for 2 weeks.

### Tracking LPR1 purification by immune blot analysis

⌚ **Timing:** 6–7 h





**Figure 2. Purification of AtLPR1**

(A) A representative size-exclusion chromatogram for 50%–80% ammonium sulfate pellet from a Superdex 200 HL 16/60 column using a flow rate of 1 mL/min. UV absorbance at 260 nm and 280 nm are shown as solid and dotted line respectively. The insert shows a representative silver stained SDS-PAGE run of the F3 elution peak fractions (marked with red box).

(B) A representative cation-exchange chromatogram for LPR1. Increasing buffer-C concentration (0%–100%) are indicated as linear straight line. Note, LPR1 elutes in the shoulder (marked with red box) of the main peak at 350 mM NaCl.

(C) A representative Western blot and Coomassie stained membrane. 40  $\mu$ L sample loaded per lane. E: Extraction; P1: 40% ammonium sulfate pellet; P2: 40% ammonium sulfate pellet; SE: combined size exclusion fractions; C1–C4: cation exchange elution fractions 1–4.

Purification success of LPR1 is validated by SDS-PAGE and Western blot analysis.

26. Thaw the frozen samples collected at the different purification steps.
27. Add 10  $\mu$ L 5 $\times$  SDS loading buffer to 40  $\mu$ L sample and incubate for 5 min at 95°C.
28. Run the samples on an SDS-PAGE gel. We use self-casted 10% polyacrylamide gels in the BioRAD-system.
  - a. Assemble the chamber with the gel according to the manufacturer's instructions and fill up the chamber with SDS Running Buffer.
  - b. Load 45  $\mu$ L of each sample in each well of the gel including the protein ladder (5  $\mu$ L pre-stained 1 kb protein ladder; Thermo Fisher).
  - c. Run the gel at 85 volts (constant) for 15 min.
  - d. Run the gel at 130 volts (constant) for another 45 min.
29. Transfer the protein bands from the gel to a PVDF (Amersham Hybond 0.45 PVDF) blotting membrane. We use a PowerBlotter system (Thermo Scientific).
30. Block the membrane with 5% (w/v) dry milk in TBST for 1 h at 22°C–25°C or 12 h at 4°C.
31. Incubate with primary epitope-specific anti-LPR1 polyclonal antibody (1:1,000 in TBST with 3% dry milk) for 1 h at 22°C–25°C or 12 h at 4°C.
32. Wash the membrane 3  $\times$  10 min with TBST.
33. Incubate the membrane with a suitable horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 in TBST with 3% dry milk) for 1 h at 22°C–25°C or 12 h at 4°C.
34. Wash the membrane 5  $\times$  10 min with TBST.

35. For detection, add ECL Select Western Blotting Detection Reagent (Life Technologies) to the membrane following the manufacturer's protocol (<https://www.thermofisher.com/order/catalog/product/32109>).
36. Analyze chemiluminescence with X-ray films (incubation time 2–10 min).
37. Stain PVDF membrane with Coomassie for 5 min (we use PageBlue protein-stain from Thermo Fisher Scientific).

### Ferroxidase activity assay

⌚ Timing: 15–30 min

The assay used to determine protein activity is an end-point determination as the substrate and 3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine (ferrozine) as a specific  $\text{Fe}^{2+}$ -chelator to scavenge the remaining substrate after the reactions. The rate of  $\text{Fe}^{2+}$  oxidation can be calculated from the decreased absorbance at 560 nm using a molar extinction coefficient of  $\epsilon_{560}=25,400 \text{ M}^{-1} \text{ cm}^{-1}$  for the  $\text{Fe}^{2+}$ -ferrozine complex.

38. Use a 96 flat-bottom multi-well plate and add 14  $\mu\text{L}$  ferrozine solution to 5 wells per reaction tube.
39. Prepare 1.5 mL tubes with 1,191  $\mu\text{L}$  acetate-buffer, prepare at least 3 technical replicates per sample.
40. Add 1  $\mu\text{g}$  total protein to the tube. LPR1 protein purification normally yields 2–3 mL protein solution with a LPR1 concentration from 0,02–0,08  $\mu\text{g}/\mu\text{L}$ . The final assay volume will be between 1287.5  $\mu\text{L}$  and 1,325  $\mu\text{L}$  in total. [Troubleshooting 6](#).
41. Reaction starts with addition of 84  $\mu\text{L}$  substrate-solution to the tube, invert 2 times, take 200  $\mu\text{L}$  from the reaction and add it to the first well with ferrozine solution (time point "0").
42. Place the tube in an incubator at 25°C until time point 1 (e.g., 2 min).
43. Reaction amount serves for six different time point elucidations. We normally take samples at the start of the reaction (e.g., 0 min) and further 2, 5, 7, 10 and 15 min after substrate addition ([Figure 3](#)).
44. Measure absorbance of each well at 560 nm in a microplate reader.
45. Display decreasing absorbance at 560 nm in a linear regression curve and calculate  $\Delta E = \frac{\Delta A}{t}$  based on the following formular:  $y = \frac{\Delta A}{t}x + n$ .
46. To calculate the specific enzyme activity based on the Lambert-Beer law, make sure to know the diameter of the well plate or cuvette used to measure absorbance ("d").

$$E = c * d * \epsilon \Delta C = \frac{|\Delta E|}{d * \epsilon} \quad d = 0,52 \text{ cm} \quad \epsilon = 0,0254 \frac{\text{L}}{\mu\text{mol} * \text{cm}}$$

$$\Delta c = \frac{|\Delta E|}{0,52 \text{ cm} * 0,0254 \frac{\text{L}}{\mu\text{mol} * \text{cm}}} * \frac{1}{\text{min}} = \frac{|\Delta E| \frac{1}{\text{min}}}{0,52 \text{ cm} * 0,0254 \frac{\text{L}}{\mu\text{mol} * \text{cm}}} = \frac{|\Delta E|}{0,013208} \frac{\mu\text{mol}}{\text{L} * \text{min}}$$

$$\Delta n = \Delta c * V = \frac{|\Delta E|}{0,013208} \frac{\mu\text{mol}}{\text{L} * \text{min}} * (1191 + 84 + V(\text{protein})) * 10^{-6}$$

$$A_{\text{spec}} = \frac{\Delta n}{m_{\text{protein}}}$$

E: Extinction.

c: concentration ( $\text{Fe}^{2+}$ ).

d: diameter of the cuvette/well used.

e: extinction coefficient.

n: amount of substance.

V: (reaction) volume.

$A_{\text{spec}}$ : specific enzyme activity.

## EXPECTED OUTCOMES

Using this protocol, we typically obtain 60–90  $\mu\text{g}$  LPR1 protein per 30 g leave material.

## LIMITATIONS

This protocol is optimized for Arabidopsis LPR1 purification. It has to be modified according to the target protein.

## TROUBLESHOOTING

### Problem 1

Homogenization of plant material to a fine powder may be difficult due to the rigid leaf stems ([before you begin](#), steps 2–4).

#### Potential solution

If leaf stems are hard and fail to homogenize it may help to add sea sand (0,5 g) into the mortar. Otherwise it is also possible to remove them from the plant material with a tweezer.

### Problem 2

Extraction from plant material results in low protein concentration (steps 1–4).

#### Potential solution

Protein extraction efficiency highly depends on homogeneity of the frozen plant material, vortex-time and repetition and incubation (in rotation or shaking). Increasing the vortex time or incubation time at 4°C might improve protein yield.

### Problem 3

Ammonium sulfate precipitation is insufficient (steps 6–13).

#### Potential solution

To improve ammonium sulfate precipitation, one could increase the incubation time at 4°C. Always make sure to not add all salt directly to the supernatant solution, instead portion the salt and solubilize it stepwise.

### Problem 4

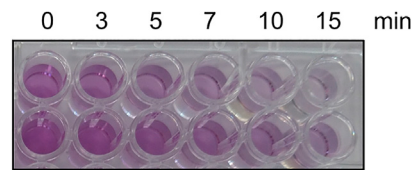
LPR1 protein is not present in the corresponding elution fractions (step 20).

#### Potential solution

Size exclusion chromatography might differ in sample identity dependent on the column and system used. To identify LPR1 containing elution fractions, perform a Western blot with all fractions to identify the correct fractions according to the system used.

### Problem 5

LPR1 protein is not eluting from the CM-sepharose at the indicated NaCl concentration (steps 22–25).



**Figure 3. Ferrozine assays in 96-well plate format**

Displayed 12 of 96 wells of 96-well plate.

### Potential solution

Ion exchange chromatography is highly sensitive to altered pH values. Make sure that Buffer B and Buffer C have the correct pH value. Also, test sample after size-exclusion chromatography for a neutral pH-value.

### Problem 6

LPR1 protein concentration is below the suggested value (step 40).

### Potential solution

LPR1 displays high ferroxidase activity. If the amount of pure protein is below  $0,02 \mu\text{g}/\mu\text{L}$  it is also possible to reduce the amount of protein (e.g.,  $0,5 \mu\text{g}$  total LPR1). Assay duration might be adjusted (e.g., expanded to 30 min instead of 15 min).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christin Naumann ([cnaumann@ipb-halle.de](mailto:cnaumann@ipb-halle.de)).

### Materials availability

All plant lines generated in this study have been deposited to the National Arabidopsis Stock Center (NASc), CaMV 35Spro:LPR1, Stock ID: N2111056.

All other unique/stable reagents generated in this study are available from the [lead contact](#) with a completed Materials Transfer Agreement.

### Data and code availability

This study did not generate/analyze [datasets/code].

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## AUTHOR CONTRIBUTIONS

N.T. and C.N. conceived and designed the project. N.T. conducted the major experiments. C.N. analyzed data and wrote the article.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

Müller, J., Toev, T., Heisters, M., Teller, J., Moore, K.L., Hause, G., Dinesh, D.C., Bürstenbinder, K., and Abel, S. (2015). Iron-dependent callose deposition adjusts root

meristem maintenance to phosphate availability. *Dev. Cell* 33, 216–230.

Naumann, C., Heisters, M., Brandt, W., Janitza, P., Alfs, C., Tang, N., Toto Nianguesso, A., Ziegler, J.,

Imre, R., Mechtler, K., et al. (2022). Bacterial-type ferroxidase tunes iron-dependent phosphate sensing during Arabidopsis root development. *Curr. Biol.* 32, 2189–2205.e6.