bio-protocol

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Isolation of Intact Vacuoles from *Arabidopsis* Root Protoplasts and Elemental Analysis

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Abstract

The vacuole is one of the most conspicuous organelles in plant cells, participating in a series of physiological processes, such as storage of ions and compartmentalization of heavy metals. Isolation of intact vacuoles and elemental analysis provides a powerful method to investigate the functions and regulatory mechanisms of tonoplast transporters. Here, we present a protocol to isolate intact vacuoles from *Arabidopsis* root protoplasts and analyze their elemental content by inductively coupled plasma mass spectrometry (ICP-MS). In this protocol, we summarize how to prepare the protoplast, extract the vacuole, and analyze element concentration. This protocol has been applied to explore the function and regulatory mechanisms of tonoplast manganese (Mn) transporter MTP8, which is antagonistically regulated by CPK4/5/6/11 and CBL2/3-CIPK3/9/26. This protocol is not only suitable for exploring the functions and regulatory mechanisms of tonoplast transporters, but also for researching other tonoplast proteins.

Keywords: Isolation of intact vacuoles, *Arabidopsis* protoplasts, Elemental analysis, Mn transporter, MTP8

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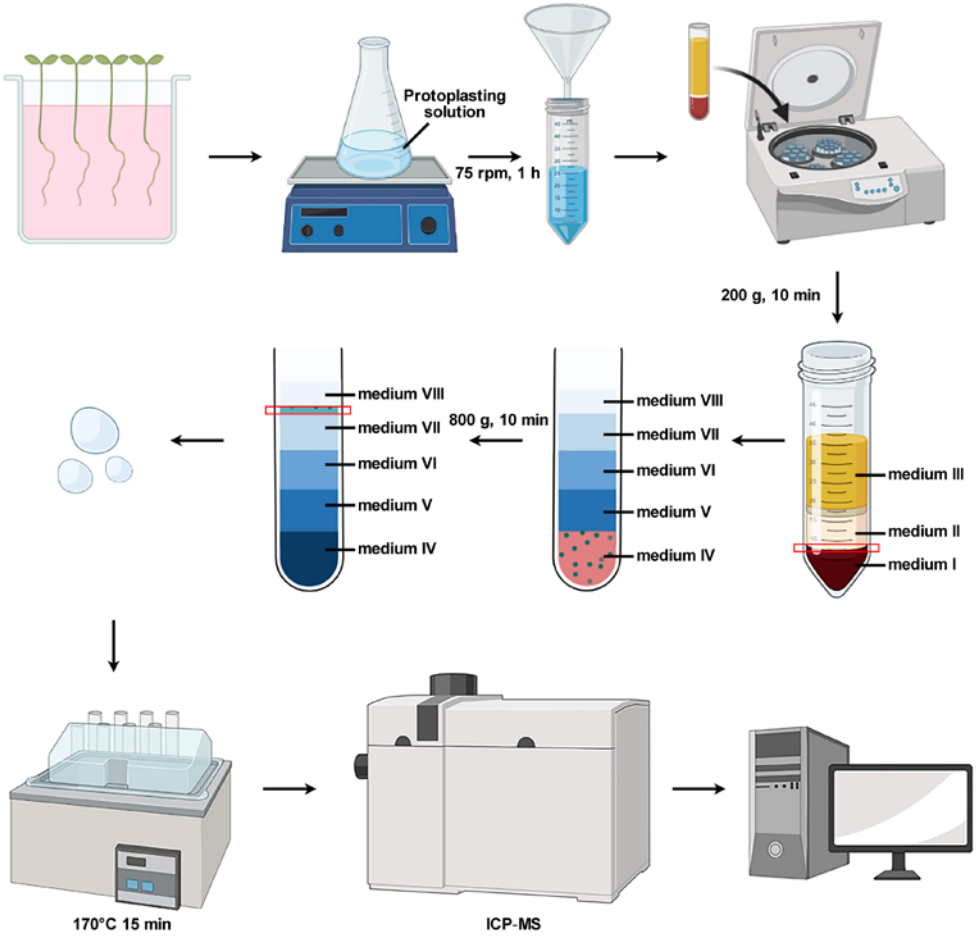
Graphical abstract



Background

The central vacuole is the largest compartment of a mature plant cell and participates in plenty of physiological processes. The determination of its elemental concentrations is essential for researching the function and regulation of plant tonoplast transporters. The transport rates of different anions and the factors affecting the uptake of chloride ions across the tonoplast were detected in isolated barley vacuoles (Martinoia et al., 1986). Transport of phosphate across the tonoplast was also detected in intact vacuoles, which were isolated from suspension-cultured cells of *Catharanthus roseus* (L.) G. Don (Massonneau et al., 2000). Shimaoka et al. (2004) modified the method for extracting intact vacuoles, which is summarized in this article, and detected the tonoplast proteins combined with proteomic analysis. The function and regulatory mechanisms of the tonoplast manganese (Mn) transporter MTP8 were resolved via this method (Eroglu et al., 2016; Zhang et al., 2021; Ju et al., 2022). In conclusion, this protocol is feasible and also applied for the research to investigate tonoplast transporters in the future.

Materials and Reagents

1. 40 μm cell strainer (Corning, catalog number: 431750)
2. 50 mL tubes (Sangon Biotech, catalog number: F602788)
3. Cellulase R10 (Yakult, catalog number: L0012)
4. Macerozyme R10 (Yakult, catalog number: L0021)
5. D-mannitol (Sigma-Aldrich, catalog number: M9647)
6. MES hydrate (Sigma-Aldrich, catalog number: M2933)
7. Tris-base (Thermo Fisher Scientific, catalog number: BP152-5)
8. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C5670)
9. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
10. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
11. Percoll (GE Healthcare, catalog number: 17-0891-09)
12. HEPES (Sigma-Aldrich, catalog number: H3375)
13. EGTA (Sigma-Aldrich, catalog number: H3889)
14. Sucrose (Sigma-VETEC, catalog number: V900116)
15. K-gluconate (Sigma-Aldrich, catalog number: G4500)
16. D-sorbitol (Sigma-Aldrich, catalog number: S1876)
17. $MnSO_4$ (Sigma-Aldrich, catalog number: M7634)
18. HNO_3 (GHTECH, catalog number: 1.14003.018)
19. H_2O_2 (Sinopharm Chemical Reagent, catalog number: 10011208)
20. Murashige and Skoog (MS) base salts with vitamins (Phytotech Labs, catalog number: M519)
21. Agar (Sigma-Aldrich, catalog number: A1296)
22. KNO_3 (Sinopharm Chemical Reagent, catalog number: 10017218)
23. $Ca(NO_3)_2{\cdot}4H_2O$ (Sigma-Aldrich, catalog number: SC278601)
24. $NH_4H_2PO_4$ (Sinopharm Chemical Reagent, catalog number: 10002808)
25. $MgSO_4{\cdot}7H_2O$ (Sinopharm Chemical Reagent, catalog number: 10013018)
26. H_3BO_3 (Sinopharm Chemical Reagent, catalog number: 10004808)
27. $MnCl_2{\cdot}4H_2O$ (Sigma-Aldrich, catalog number: SM500501)
28. $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (Sinopharm Chemical Reagent, catalog number: 10002318)
29. $ZnSO_4{\cdot}7H_2O$ (Sinopharm Chemical Reagent, catalog number: 10024018)
30. $CuSO_4{\cdot}5H_2O$ (Sinopharm Chemical Reagent, catalog number: 10008218)
31. EDTA-Fe(III)Na (Biotopped, catalog number: Q0028-100g)
32. BSA (Sigma-Aldrich, catalog number: V900933)
33. β-mercaptoethanol (14.3 M) (Millipore, catalog number: 444203)
34. 1/2 MS medium (see Recipes)
35. 1/5 Hoagland solution (see Recipes)
36. Protoplasting solution (see Recipes)
37. Medium B (see Recipes)
38. Medium I (see Recipes)
39. Medium II (see Recipes)
40. Medium III (see Recipes)
41. Medium IV (see Recipes)
42. Medium V (see Recipes)
43. Medium VI (see Recipes)
44. Medium VII (see Recipes)
45. Medium VIII (see Recipes)

Equipment

1. Artificial illumination incubator (PERCIVAL, model: LT-36VL)
2. Horizontal centrifuge (Eppendorf, model: Centrifuge 5810R)
3. Dissolver (LabTech, model: DigiBlock ED54)
4. ICP-MS (Thermo Fisher Scientific, model: ICAP Qc)

5. Perfluoroalkoxy alkane (PFA) vessels (LabTech, model: GC-36-L)
6. Fuchs-Rosenthal hemacytometer (Marienfeld, model: Dark-Line 0650010)

Procedure

A. Protoplast preparation

1. Sterilize approximately 50 *Arabidopsis* seeds, plant on 1/2 MS medium, and then grown in an artificial illumination incubator under normal light conditions (100 μmol m^{-2} s^{-1}) with a long-day cycle (16:8 h light/dark) at 22 °C. After seven days, clamp the seedlings with a small sponge and place them on a reticulated floating board; immerse the roots in 1/5 Hoagland solution and then grow in an artificial illumination incubator under normal light conditions (100 μmol m^{-2} s^{-1}) with a short-day cycle (8:16 h light/dark) at 22 °C. Change the solution every three days. After seven weeks, transfer the seedlings to the same solution containing 240 μM $MnSO_4$ for another seven days.
2. Take 10–15 seedling roots and cut them into small pieces with a blade, depositing them into a 100 mL flask containing the protoplasting solution (see Recipes). Generally, 20 mL of protoplasting solution is used per 15 seedling roots.
3. Shake the flasks gently (75 rpm) at room temperature for 1 h. A longer incubation time may increase the protoplast yield.
4. Filter the protoplast solution with a 40 μm cell strainer and carefully pour into a 50 mL tube.

B. Vacuole extraction

1. In order to reduce the disruption of protoplasts, first add 5 mL of medium I to the bottom of the tube and then gently mix the filtered protoplasts. After centrifugation (200 × *g*, 10 min), discard the supernatant with a pipette to obtain sedimented protoplasts.
2. Slowly add 5 mL of medium I into the tube and gently mix with the sedimented protoplasts.
3. Using a pipette, add 5 mL of medium II to the tube against the wall, and then 5 mL of medium III. Each layer of liquid should be added slowly to ensure that the layers do not break apart. After adding medium III, the gradient is formed and can be clearly seen in the tube. After centrifugation (800 × *g*, 10 min), purified colorless protoplasts are obtained in the interfaces between medium I and II or between medium II and III. Discard the latter protoplasts (between medium II and III) with the pipette to avoid contamination of the vacuolar fractions with protoplasts.
4. Gently mix approximately 2 mL of the remaining purified protoplasts with 2 mL of medium B and incubate on ice for 5 min.
5. Gently mix the mixture of protoplasts, vacuoles, and lysate (from step B4) with 5 mL of medium IV first. Then, a gradient is formed by consecutive overlaying with 5 mL each of medium V, medium VI, medium VII, and medium VIII.
6. After centrifugation (800 × *g*, 10 min), discard medium VIII with a pipette. Carefully aspirate approximately 1 mL of liquid between medium VII and medium VIII as the purified vacuoles.

C. Elemental analysis

1. Determine the number of vacuoles using a Fuchs-Rosenthal hemacytometer. (Approximately 10 vacuoles per square millimeter of the Fuchs-Rosenthal hemacytometer, whose height is 0.1 mm through microscope observation; so, there are 100 vacuoles in each cubic millimeter of solution.) The total number of vacuoles in the solution is obtained by multiplying the number of vacuoles per cubic millimeter by the volume of the solution.
2. Digest the vacuoles in PFA vessels with 1.5 mL of HNO_3 (65%) and 0.6 mL of H_2O_2 (30%) for 15 min in the dissolver at 170 °C.
3. After cooling down to room temperature, transfer the digestion solution to a plastic volumetric flask and dilute to 5 mL with ultrapure water (18.25 MΩ cm^{-1}).
4. Determine Mn in the digestion solution using an inductively coupled plasma mass spectrometer (ICP-MS).

Data analysis

The vacuolar elemental concentration of each plant material requires three biological replicates to be measured. The mean value of three biological replicates was calculated and statistically analyzed (Student's *t*-test). Detailed data and statistical methods could be found in the previously published articles (Figure 5B in Zhang et al., 2021 and Figure 4B in Ju et al., 2022).

Recipes

1. 1/2 MS medium (adjust the pH to 5.7 using Tris)

Reagent	Final concentration	Amount
MS base salts with vitamins	0.222%	2.22 g
Sucrose	1%	10 g
Agar	1%	10 g
ddH_2O	n/a	Up to 1 L
Total	n/a	1 L

2. 1/5 Hoagland Solution

Reagent	Final concentration	Amount
KNO_3	1 μM	0.51 g
$Ca(NO_3)_2·4H_2O$	1 μM	1.18 g
$NH_4H_2PO_4$	0.2 μM	0.12 g
$MgSO_4·7H_2O$	0.4 μM	0.49 g
H_3BO_3	3 nM	1.85 μg
$MnCl_2·4H_2O$	0.5 nM	0.99 μg
$(NH_4)_6Mo_7O_{24}·4H_2O$	1 nM	12.36 μg
$ZnSO_4·7H_2O$	0.4 nM	1.15 μg
$CuSO_4·5H_2O$	0.2 nM	0.05 μg
EDTA-Fe(III)Na	20 nM	8.42 μg
ddH_2O	n/a	Up to 1 L
Total	n/a	1 L

3. MES-Tris buffer

Reagent	Final concentration	Amount
MES hydrate	0.5 M	9.76 g
Tris-base		Adjust pH to 5.7
ddH_2O	n/a	Up to 100 mL
Total	n/a	100 mL

4. Protoplasting solution

Reagent	Final concentration	Amount
Cellulase R10	1.25%	1.25 g
Macerozyme R10	0.3%	0.3 g
D-mannitol (0.8 M)	0.4 M	50 mL
MES-Tris buffer (0.5 M, pH 5.7)	20 mM	4 mL
KCl (1 M)	20 mM	2 mL
Heat the solution to 55 °C for 10 minutes and let it cool down to room temperature		
BSA	0.1%	0.1 g
$CaCl_2$ (1 M)	10 mM	1 mL
β-mercaptoethanol (14.3 M)	5 mM	35 µL
ddH_2O	n/a	Up to 100 mL
Total	n/a	100 mL

5. Medium B

Reagent	Final concentration	Amount
HEPES-Tris (1 M, pH 7.2)	30 mM	3 mL
K-gluconate	30 mM	0.7 g
$MgCI_2$ (1 M)	2 mM	0.2 mL
EGTA (0.5 M)	2 mM	0.4 mL
ddH_2O	n/a	Up to 100 mL
Total	n/a	100 mL

6. Medium I

Reagent	Final concentration	Amount
Sucrose	400 mM	13.69 g
Percoll	50%	50 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

7. Medium II

Reagent	Final concentration	Amount
Sucrose	400 mM	13.69 g
Percoll	7.5%	7.5 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

8. Medium III

Reagent	Final concentration	Amount
D-sorbitol	400 mM	7.27 g
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

9. **Medium IV**

Reagent	Final concentration	Amount
D-sorbitol	200 mM	3.64 g
Percoll	25%	25 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

10. **Medium V**

Reagent	Final concentration	Amount
Sucrose	200 mM	6.85 g
Percoll	7.5%	7.5 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

11. **Medium VI**

Reagent	Final concentration	Amount
Sucrose	200 mM	6.85 g
Percoll	5%	5 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

12. **Medium VII**

Reagent	Final concentration	Amount
Sucrose	200 mM	6.85 g
Percoll	2.5%	2.5 mL
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

13. **Medium VIII**

Reagent	Final concentration	Amount
Sucrose	200 mM	6.85 g
Medium B	n/a	Up to 100 mL
Total	n/a	100 mL

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Competing interests

Non-financial competing interests on behalf of all authors.

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