A combined approach to evaluate total phosphorus/inorganic phosphate levels in plants

Inorganic phosphate (Pi) and phosphorus (P) homeostasis are essential for plant growth and yield, and reliable detection of dynamic Pi/P in different tissues is important for studying their biological functions. Here, we report a combined protocol for rapid determination of Pi/P levels. We first perform $^{31}$P NMR assay to reveal the intracellular Pi distribution and then dissect the level of Pi/P by the chromogenic reaction and ICP-MS analysis. Finally, we take μXRF element fluorescence assay to achieve the visual P distribution.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol
A combined approach to evaluate total phosphorus/inorganic phosphate levels in plants

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https://doi.org/10.1016/j.xpro.2022.101456

SUMMARY
Inorganic phosphate (Pi) and phosphorus (P) homeostasis are essential for plant growth and yield, and reliable detection of dynamic Pi/P in different tissues is important for studying their biological functions. Here, we report a combined protocol for rapid determination of Pi/P levels. We first perform 31P NMR assay to reveal the intracellular Pi distribution and then dissect the level of Pi/P by the chromogenic reaction and ICP-MS analysis. Finally, we take μXRF element fluorescence assay to achieve the visual P distribution. For complete details on the use and execution of this protocol, please refer to Ma et al. (2021).

BEFORE YOU BEGIN
Monitoring Pi/P levels both at the intracellular and tissue levels can effectively reflect the P homeostasis important for plant growth. In this protocol, we integrate four different methods to facilitate Pi/P evaluation both at the intracellular and tissue levels. Before the experiment begins, we should first prepare various plant samples, such as plant seedlings and mature plants.

Preparation of plant seedling samples (rice is taken as an example below)

© Timing: 2 weeks

1. Preparation of rice hydroponic nutrient solution.
   a. The hydroponic nutrient solution with 12 reagents provides all the necessary elements for the healthy growth and development of rice plants. For convenience, first prepare the stock solution of each reagent, and then prepare the 1 L working solution easily by adding 1 mL of each stock solution in 988 mL ddH2O.
   b. Adjust the pH of working solution to 5.5 with HCl for a reduced pH and KOH for an increased pH as soon as it is prepared.

   Note: 1 L working solution can be used for 96 seedlings grown in a 96-well plant hydroponic box for one week.
Note: The stock solution of each reagent can be stored at 4°C after auto-claving for 1 year, and the working solution can be stored at or below 25°C for 1 month.

2. Hydroponic cultivation of rice seedlings:
   a. Pre-germinate the rice seeds (no need for sterilization) in water for 2 days in a petri dish and then germinate them in a 0.5 mM CaCl₂ solution for 4 days.

   Note: The CaCl₂ solution can be prepared from the 1/2 dilution of the 1 mM stock solution as described above.

   b. Transfer the germinated seeds with about 2 mm young buds into a 96-well plant hydroponic box full of hydroponic nutrient solution, and replace the nutrient solution every 3 days to make fresh for 10 days.

Preparation of adult rice tissue samples

⊕ Timing: up to 17 weeks

3. Sow the germinated seeds directly in the rice nursery for seedling growth, and transfer the 30-days old seedlings into the paddy field or pots in growth chamber.

   Note: The growth chamber was set with a 16-h-light (30°C)/8-h-dark (22°C) photoperiod, and the relative humidity was controlled at approximately 70%. The rice plants were supplied with normal N, P and K fertilizer for normal growth, and interested tissues can be sampled at different developmental stages.

△ CRITICAL: All experiments need to be repeated at least three times with reasonable replicates for each trial.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Ammonium nitrate (NH₄NO₃) | MilliporeSigma | CAT#A9642 |
| Sodium phosphate monobasic dihydrate (NaH₂PO₄·2H₂O) | MilliporeSigma | CAT#V900328 |

(Continued on next page)
### Protocol

#### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Potassium sulfate (K$_2$SO$_4$) | MilliporeSigma | CAT#P0772 |
| Calcium chloride dihydrate (CaCl$_2$•2H$_2$O) | MilliporeSigma | CAT#449709 |
| Magnesium chloride hexahydrate (MgCl$_2$•6H$_2$O) | MilliporeSigma | CAT#M0250 |
| Sodium metasilicate nonahydrate (Na$_2$SiO$_3$•9H$_2$O) | MilliporeSigma | CAT#E6760 |
| Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA) | MilliporeSigma | CAT#B6768 |
| Boric acid (H$_3$BO$_3$) | MilliporeSigma | CAT#M5005 |
| Copper(II) sulfate pentahydrate (CuSO$_4$•5H$_2$O) | MilliporeSigma | CAT#C7631 |
| Zinc sulfate heptahydrate (ZnSO$_4$•7H$_2$O) | MilliporeSigma | CAT#Z4750 |
| Sodium molybdate dihydrate (Na$_2$MoO$_4$•2H$_2$O) | MilliporeSigma | CAT#M1003 |
| Trichloroacetic acid (TCA) | MilliporeSigma | CAT#T4885 |
| Nitric acid (HNO$_3$) | MilliporeSigma | CAT#438073 |
| Potassium phosphate monobasic (KH$_2$PO$_4$) | Merck | CAT#104873 |
| Ammonium molybdate tetrahydrate (NH$_4$H$_2$Mo$_7$O$_24$•4H$_2$O) | MilliporeSigma | CAT#09878 |
| Ammonium metavanadate (NH$_4$VO$_3$) | MilliporeSigma | CAT#205559 |
| Ascorbic acid | MilliporeSigma | CAT#A92902 |
| Sulfuric acid (H$_2$SO$_4$) | MilliporeSigma | CAT#258105 |
| Agarose LE | Promega | CAT#V3121 |
| Deuterium oxide (D$_2$O) | MilliporeSigma | CAT#1.13366 |
| Methyleneidiphosphonic acid (MDP) | MilliporeSigma | CAT#64255 |
| Glucose | MilliporeSigma | CAT#G7528 |
| Potassium nitrate (KNO$_3$) | MilliporeSigma | CAT#P6291 |
| Calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$•4H$_2$O) | MilliporeSigma | CAT#C955 |
| Potassium chloride (KCl) | MilliporeSigma | CAT#P9333 |
| Magnesium sulfate (MgSO$_4$) | MilliporeSigma | CAT#M7506 |
| 4-Morpholineethanesulfonic acid monohydrate (MES) | MilliporeSigma | CAT#69892 |
| P standards | PerkinElmer | CAT#N9303788 |
| Phosphoric acid (H$_3$PO$_4$) | MilliporeSigma | CAT#345245 |
| Double distilled water (ddH$_2$O) | Self-prepared | n/a |
| Deionized water (diH$_2$O) | Self-prepared | n/a |

#### Software and algorithms

| Software and algorithms | | |
|-------------------------|------------------|-----------------|
| Gen5 | BioTek | Instrument Software |
| NexION 350D | PerkinElmer | Instrument Software |
| M4 TORNADO | Bruker | Instrument Software |
| Bruker TopSpin 3.2 | Bruker | Instrument Software |
| MestReNova 8.1 | Bruker | Instrument Software |
| GraphPad Prism 8 | GraphPad Software Inc. | http://www.graphpad.com/ |

#### Other

| Other | | |
|-------|------------------|-----------------|
| Multipurpose Shaker | Kylin-Bell | QB-210 |
| Microplate Reader | BioTek | Gen5 Take3 |
| Multiwave3000 Microwave Reaction System | Anton PAAR | Microwave3000 |
| Black heater | SCP Science | DigiPREP MS |
| ICP mass spectroscopy | PerkinElmer | NexION 350D |
| X-ray fluorescence spectrometer | Bruker | M4 Tornado |
| 600 MHz Digital NMR Spectrometer | Bruker | AVANCE III NMR Spectrometer |
| Tissue lyser | Jing Xin | TissueLyser-192 |
| Oven | Jing Hong | DHG-9203A |
| Centrifuge | Eppendorf | Centrifuge 5424 R |
| Mill-Q Advantage | Merck Millipore | Mill-Q Advantage |
| 96-well plant hydroponic box | Maisinuo (China) | HZQ-12 |
| NMR tube (5 mm) | Norell | ST500-7 |
| Capillary (0.5 mm and 0.3 mm) | This paper | n/A |
| Double channel peristaltic pump | Ismatec | ISM5%E |
| Air pump | RESUN (China) | AC-9904 |

(Continued on next page)
**Materials and Equipment**

**Perfusion solution in nuclear magnetic resonance spectroscopy (NMR) analysis (prepare freshly)**

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Glucose                  | 5 mM                | 0.90 g |
| KNO₃                     | 10 mM               | 1.01 g |
| KH₂PO₄                   | 0.2 mM              | 0.03 g |
| Ca(NO₃)₂·4H₂O            | 0.5 mM              | 0.12 g |
| KCl                      | 1 mM                | 0.07 g |
| MgSO₄                    | 0.5 mM              | 0.06 g |
| MES                      | 2 mM                | 0.43 g |
| ddH₂O                    | n/a                 | Up to 1 L |
| Total                    | n/a                 | 1 L    |

**Note:** Prepare the perfusion solution immediately before use and perform high-pressure steam sterilization by autoclave to make the solution amicrobic.

**Optional:** Glucose is not required when sampling early-filling seeds since the tissue contains high content of starch and sugar.

**10 mM MDP solution**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| MDP     | 10 mM               | 0.0176 g |
| ddH₂O   | n/a                 | Up to 10 mL |
| Total   | n/a                 | 10 mL  |

**Note:** The prepared solution can be stored at 4°C for 1 month.

**Caution:** Methylene phosphonic acid (MDP) is a corrosive solid material and can cause severe eye and skin burns. This material should only be handled by appropriately trained staff, and while using appropriate gloves and goggles. In case of contact with skin or eyes, wash thoroughly with plenty of water and seek medical advice.

**Inorganic phosphate (Pi) extraction buffer**

| Reagent       | Final concentration | Amount |
|---------------|---------------------|--------|
| TCA (100%v/v) | 12.5%v/v            | 12.5 mL |
| MgCl₂ (1 M)   | 25 mM               | 2.5 mL |
| ddH₂O         | n/a                 | 85 mL  |
| Total         | n/a                 | 100 mL |

**Note:** This paper, N/A
**Note:** The Pi extraction buffer should be stored well-capped and at 4°C for no more than 1 month.

**CAUTION:** The reagent TCA is harmful to human skin and eyes and toxic to aquatic organisms. Appropriate gloves, googles, and an acid apron should be used when handling this material. In case of contact with skin or eyes, rinse immediately with plenty of water and seek medical advice.

| Reagent                          | Final concentration | Amount |
|----------------------------------|----------------------|--------|
| (NH₄)₆Mo₇O₂₄·4H₂O               | 100 g/L              | 10 g   |
| NH₄VO₃                          | 2.35 g/L             | 2.35 g |
| HNO₃ (70%)                      | 165 mL/L             | 16.5 mL|
| ddH₂O                           | n/a                  | Up to 100 mL |
| **Total**                       | n/a                  | 100 mL |

**Note:** The reagents (NH₄)₆Mo₇O₂₄·4H₂O and NH₄VO₃ are hard to dissolve at room temperature, (NH₄)₆Mo₇O₂₄·4H₂O and NH₄VO₃ should be dissolved in hot ddH₂O (80°C–90°C), respectively. After cooling, add HNO₃ to the NH₄VO₃ solution and cool again. Then add (NH₄)₆Mo₇O₂₄ solution and make up the volume with ddH₂O to the final volume. The developer solution should be prepared immediately before use and protected from light.

**CAUTION:** The reagents (NH₄)₆Mo₇O₂₄·4H₂O and NH₄VO₃ are irritating to eyes, respiratory system, and skin, and harmful if swallowed. Hence the experimenters should wear gloves, googles, and masks and operate in a chemical fume hood.

**Alternatives:** Ammonium molybdate ascorbate developer solution (prepare freshly).

| Reagent                          | Stock solution | Amount |
|----------------------------------|----------------|--------|
| (NH₄)₆Mo₇O₂₄·4H₂O               | 2.5% (w/v)     | 20 mL  |
| Ascorbate acid                   | 10% (w/v)      | 20 mL  |
| H₂SO₄                            | 3 M            | 20 mL  |
| ddH₂O                            | n/a            | 40 mL  |
| **Total**                       | n/a            | 100 mL |

**CAUTION:** The reagents concentrated nitric acid (HNO₃) and concentrated sulfuric acid (H₂SO₄) are extremely dangerous substance to handle. HNO₃ and H₂SO₄ are considered an oxidizing liquid that can cause fires, is severely corrosive to metal and tissues (i.e., eyes, skin, mouth and throat, and respiratory tract), is considered acutely toxic by inhalation. Concentrated HNO₃ and H₂SO₄ should only be handled by appropriately trained staff, and while using appropriate gloves, goggles, acid apron, and a chemical fume hood.

**STEP-BY-STEP METHOD DETAILS**

**Measurement of intracellular inorganic phosphate (Pi)**

© Timing: 2 days

NMR technology is one of the main techniques to study living cells under physiological conditions without causing damage to the organism and is suitable for studying life phenomena closer to physiological states. The 31P-NMR technique proved to be feasible to detect Pi concentrations in vivo in the plant (Roby et al., 1987; Bligny et al., 1989; Wang et al., 2015; Xu et al., 2019). In this step, rice seedling samples are well-adapted and packed into 5 mm NMR tubes and cytoplasmic and vacuolar specific Pi peaks are dissected by the perfusion system on a Bruker Ascend 600 NMR spectrometer (Figure 1).
1. Transfer the 2-week-old seedling growing in the nutrition solution into perfusion solution, and soak the root of testing samples (about 5 seedlings) for 1 h to adapt to the perfusion solution.

2. Preparation of perfusion system:
   a. Add D$_2$O into two 0.5 mm capillaries, and pack MDP solution into a 0.3 mm capillary, respectively (see below).
   b. Seal both ends of the three capillaries (place one end of the capillary tube at a 45° angle on the edge of the fire generated by the alcohol lamp, keep rotating while heating the tube, and complete the seal when both ends are closed) and then place them into a 5 mm NMR tube (Figure 1).

3. Pack roots or leaves (about 50 mg fresh weight) from 2-week-old seedlings into the 5 mm NMR tube and connected it to a peristaltic pump by two polytetrafluoroethylene tubes, followed by adding perfusion solution (Aubert et al., 1998; Stefanovic et al., 2011).

△ CRITICAL: The samples should not exceed one-third of the height of the NMR tube (Figure 1).

CAUTION: The strong magnetic field of NMR can suddenly pull nearby unrestrained magnetic objects into the magnet with considerable force. The analyzer should keep all tools, equipments and personal items containing ferromagnetic material (e.g., steel, iron) at least 2 metres away from the magnet. In particular, keep the peristaltic pump outside of 5-gauss lines (Figure 1), and do not use metal seats in the operating room.

4. Setting the parameters in NMR Spectrometer:

| Reagents/items                  | Parameter                                      |
|---------------------------------|------------------------------------------------|
| Pulse program                   | zgpg30                                         |
| MestReNova software version     | 8.1                                            |
| Data collection method          | 242.9-MHz lock with H$_2$O and D$_2$O          |
| Scans                           | 3000–4500                                      |
| Spectral window                 | 24 kHz                                         |
| Line broadening factor          | 30 Hz                                          |
| Sampling time                   | 0.33 s                                         |
| Sampling temperature            | 25°C                                           |
5. Test the samples according to the above parameter setting, with a run time of about 2 h. Record the in vivo $^{31}$P-NMR spectra on the Bruker Ascend 600 NMR spectrometer and measure the chemical shifts ($\delta$) relative to the signal from a glass capillary containing 10 mM MDP solution.

**Note:** Set the phosphate peak at 0 ppm by 85% H$_3$PO$_4$ determination, and the standard MDP peak will occur at 18.9 ppm using same parameters. In this way, different Pi components in the testing sample can be discriminated by their relative positions to MDP peak based on the previous report (Quiquampoix et al., 1993).

6. Assign specific peaks to cytoplasmic and vacuolar Pi pools following the methods given previously (Roby et al., 1987; Bligny et al., 1989).

⚠️ **CRITICAL:** The $^{31}$P-NMR method is used to detect not only the phosphate concentration in plants but also distinguish the metabolic pools of phosphate in different organelles of cells (such as Vac-Pi, Cyt-Pi) in vivo.

**Determination of inorganic phosphate (Pi) in different tissues**

�� **Timing:** 3 days

The ammonium vanadate molybdate method has been widely used to detect Pi content, which is suitable for a variety of plant tissues, including both dry and fresh samples. In this step, we sample different rice tissues and extract the Pi crudes for chromogenic reaction.

7. Preparation of testing samples.
   a. Sample different tissues directly from the mature plants in the field. For dry samples preparation, dry the tissues (root, leaf, stem, seed, etc.) at 65°C in an oven for 48 h and then grind them into powders directly by Tissue Lyser at or below 25°C. For fresh samples, snap-freeze the tissues in liquid nitrogen, grind them into powders rapidly by Tissue Lyser and proceed with subsequent testing immediately.
   b. Place 50 mg of each powdered samples into separate 1.5 mL centrifuge tubes.

   **Optional:** In the case of a high-throughput assay, the samples can be transferred to a 96-well plate for rapid determination.

8. Extraction of Pi crudes.
   a. Resuspend each 50-mg sample in 1 mL phosphate extraction buffer, followed by shaking the extraction mixture on a Multipurpose Shaker (20 g) at 4°C for 12–16 h.
   b. Centrifuge the extraction mixture at 4°C (10,000 g, 15 min), then collect the supernatant (500 μL) and load it into new 1.5 mL centrifuge tubes to produce the Pi extraction crudes.

   **Pause point:** Pi extraction crudes can be stored at 4°C for several days.

9. Preparation of Pi standard solution and standard curve.
   a. Take the appropriate amount (2 g) of KH$_2$PO$_4$ into a 100 mL Erlenmeyer flask, and dry it in 105°C temperature in an oven for 1 h (Keep the mouth of the flask open).
   b. After cooling, use an analytical balance to accurately weigh 0.2195 g powder and dissolve it in diH$_2$O in a 1 L volumetric flask. Then, add 3 mL of 70% HNO$_3$, and make up the volume with diH$_2$O to 1 L to generate the Pi standard solution (50 μg/mL).
   c. Transfer exactly 0.0 mL, 1.0 mL, 2.0 mL, 4.0 mL, 8.0 mL and 16.0 mL Pi standard solution to the 50 mL centrifuge tubes (polypropylene with scale) to make 0 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL gradients, respectively.
d. Add 10 mL ammonium vanadate molybdate developer solution into each above tube, and dilute the solution up to 50 mL with diH₂O. Fasten the cap securely and mix by inversion and let stand for more than 10 min.

e. Set the 0 µg/mL Pi standard solution as the blanking control, and measure the OD400 values of various Pi standard solutions by spectrophotometer or microplate reader. Draw the standard curve with Pi concentration as abscissa and OD400 absorbance as ordinate (Figure 4A).

10. Chromogenic reaction and data collection.
a. Dilute the Pi extraction crudes in step 8 five times with diH₂O, and mix the diluted crudes and the ammonium vanadate molybdate developer solution in a 5:1 ratio to 300 µL volume.
b. Record the OD400 values in the microplate reader after 10 min rest.
c. Calculate the Pi concentration of each sample based on OD400 value and Pi standard curve and further process it into Pi content per unit weight based on the total buffer volume and sample weight.

Optional: In case of low-Pi concentration samples, the ammonium vanadate molybdate developer could be substituted as the ammonium molybdate-ascorbic developer (see materials and equipment) which contains H₂SO₄, ammonium molybdate and ascorbic acid.

△ CRITICAL: The recording of OD400 for all samples should be finished within 1 h since the ammonium vanadate molybdate developer undergoes photo-decomposition, and the mixture must be protected from light damage by packing the containers with tinfoil.

Determination of total phosphorus (P) of different tissues

Timing: 3 days

The total P levels are measured with an inductively coupled plasma mass spectrometer (ICP-MS, NexION 350D, PerkinElmer). This method is widely used in detecting P content and other elements, and even for genetic screens in plants (Gong et al., 2004; Zhao et al., 2016). In this step, we sample different rice tissues and digest the sample for ICP-MS analysis.

11. Preparation of plant samples.
a. Rinse the plant samples from the field with diH₂O 3–4 times to remove impurities.
b. Prepare powdered samples following step 7.
c. Dry the powdered samples in an oven at 65°C for 24 h, and weigh 50 mg powder each into Pyrex test tubes (16 × 100 mm) or Round Bottom High Clarity PP Test tube (14 mL).

12. Samples digestion.
a. Add 1 mL 70% HNO₃ to each sample tube.
b. Digest the samples completely in a block heater (DigiPREP MS, SCP Science; Essex, UK) or Microwave 3000 (Anton PAAR, Graz, Austria) at 115°C for 5 h.

Note: Sample tube caps should be loosened or removed during digestion.

Alternatives: If there is no block heater or Microwave 3000 available, the sample digestion can be performed by placing the samples tubes in boiling water for 4–5 h.

13. Dilute the digested samples with diH₂O to a final volume of 14 mL.
14. Detect the total P signal by ICP-MS (NexION 350D; PerkinElmer, USA) coupled with an Apex desolvation system and an SC-4 DX autosampler (Elemental Scientific Inc., USA).
15. Finally, calculate the total P concentration based on the signal value of the P standards.

Optional: Other elements, such as Li, B, Na, Mg, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, and Cd can be detected as well in the presence of standards. In the case of a high-
throughput assay, it is not necessary to weigh each sample, and part of the samples are accurately weighed to calculate the weights of the rest samples (Weight$_{unknown}$ = (Signal$_{unknown}$/Concentration$_{known}$) x dilution factor), and all samples are normalized with a heuristic algorithm using the best-measured elements as reported (Lahner et al., 2003).

μXRF element fluorescence imaging

Timing: 2 days

μXRF element fluorescence spectrometer (M4 TORNADO) is the preferred device for element characterization using small spot micro area X-ray fluorescence. The measurement can provide information about the composition and element distribution of the sample, especially for the element distribution under the surface of the sample. It is widely used to detect P distribution in plant samples as reported (Dong et al., 2018; Ma et al., 2021). In this step, we prepare rice tissues with flatten surface and generate the visualization of P fluorescence intensity using μXRF spectrometer.

16. Preparation of image samples.
   a. For mature samples, cut the plant tissues (seed, kernel, husk, etc.) smoothly with a single side razor, and then dry the samples at 37°C in oven for 24 h.
   b. For fresh samples, embed the plant tissues in 5% agarose rapidly to prevent water loss and proceed with testing immediately.

17. Put the samples carefully on the Sample Chamber of the X-ray fluorescence spectrometer (M4 Tornado, Bruker. Figures 2A–2E).
CRITICAL: The surface of all samples must be kept on the same plane during these measurements.

18. Operate the X-ray generator at 40 kV and 300 μA, and set X-Ray Beam Spot Size smaller than 20 μm for the Molybdenum K-shell X-ray Emission Spectrum (Mo-K). Then observe the P fluorescence under Rh X-ray tube illumination.

19. Collect the element distribution images for further analysis (Figure 5).

Optional: Other elements, such as Na, Mg, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, etc. can be analyzed at the same time with the X-ray generator operated at 50 kV and 600 μA.

EXPECTED OUTCOMES

For $^{31}$P-NMR determination, examples of the expected outcomes of intracellular inorganic phosphate (mainly Vac-Pi and Cyt-Pi) were provided in Figures 3A and 3B, and the specific peak of Vac-Pi and Cyt-Pi can be distinguished from other components including MDP, Glc-Pi, and Fru-Pi (Figure 3A), and the concentrations are calculated based on the normalization of peak area of Cyt-Pi and Vac-Pi for final statistical analysis (Figures 3B and 3C).

For Pi detection, a standard curve was drawn and Pi concentration in various plant tissues are calculated and compared accordingly (Figures 4A and 4B). The total P concentration of samples from the
same tissues was measured by ICP-MS measurement simultaneously and the variations among tissues are shown (Figure 4C). This protocol may apply to the high-throughput measurement of plant samples to evaluate both Pi and total P levels.

Figure 4. Expected outcomes for Pi and total P content determination of various tissues in rice
(A) Pi standard curve. Y-Axis: Pi concentration (µg/mL).
(B) Pi concentration (mg/gram tissue) detection in various rice tissues, including brown rice, husk, rachis, flag leaf, node and stem at mature stage. Values are mean ± S.D. (n = 6 plants).
(C) Total P concentration of various rice tissues, including brown rice, husk, rachis, flag leaf, node and stem at mature stage. For box-and-whisker plots, the central line, box and whiskers indicate the median, interquartile range (IQR) and 1.5 times the IQR, respectively (n = 6 plants).
(D and E) Raw data of Pi/P concentration corresponding to B and C, respectively.
For μXRF analysis, total P distribution can be observed visually by μXRF element fluorescence, and the image of μXRF is given in Figure 5. In this way, the researchers can get a direct view of the P content and distribution in the detected tissues.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses of the data were performed using GraphPad Prism 8 (GraphPad software, www.graphpad.com) or Excel 2019 for Windows. Significant difference between two groups were analyzed by Student’s t-tests, and significant difference between multiple groups were performed by one-way ANOVA followed by Tukey’s HSD test. The values are presented as mean ± s.d. or box-plot with median, interquartile range (IQR), and 1.5 times the IQR. The numbers (n) of analyzed samples or replicates should be more than three.

LIMITATIONS

This protocol describes a method for the detection of Pi and P concentrations in plant tissues/cells, which prove to be efficient for evaluating P/Pi accumulation and homeostasis in the plant, and even for the genetic screening of low P cereals and phosphate/phosphorus use efficiency (PUE). However, there are still several limitations to this protocol. For the $^{31}$P-NMR assay, the seedlings can be easily loaded into NMR tubes, however, in the case of large samples, such as mature leaf and leaf sheath, which are hard to pack into NMR tubes due to the size limitations. Besides, the application of $^{31}$P-NMR assay is partly limited due to the long time and high costs. For Pi detection, although ammonium vanadate molybdate can be used to rapidly detect Pi concentrations, the detected Pi concentration may deviate from the true values, as it is calculated by using absorbance spectroscopy (OD400) on the color evolved by the production of the reaction product, ammonium phosphomolybdate efficiency. The μXRF analysis is excellent for rapid detection of a large number of samples due to the easiness in sample preparation. However, the μXRF element fluorescence assay provides only qualitative results but cannot present the exact values of elemental concentrations. Nevertheless, this protocol provides a viable method to evaluate plant phosphate/phosphorus levels from different tissues and even organelles at different development stages.

TROUBLESHOOTING

Problem 1

$^{31}$P-NMR does not work (step 5).

Potential solution

For $^{31}$P-NMR assay, successful $^{31}$P-NMR spectra are collected from plant samples in a standard NMR tube, therefore the status of plant samples directly influence NMR system. The system failing to work...
may arise from multiple reasons. Firstly, be sure to transfer only alive seedlings to the experimental NMR tubes. Weak samples should be discarded and not transferred. The operator should be careful to avoid injuring seedlings during transfer, and discard any seedlings with potential injury and replace them by unbroken ones. Secondly, perfusion solutions need to be kept fresh without contamination, and discard the solution if it is contaminated by bacteria which would be turbid and look cloudy. Besides, the preparation of MDP solution and D$_2$O capillaries are also important, and carefully check whether capillaries were sealed intactly.

**Problem 2**
Peak noise in $^3$P-NMR assay (step 6).

**Potential solution**
Peak noise often occurs during $^3$P-NMR assay, and the specific peaks of Vac-Pi and Cyt-Pi are difficult to define in this case (example as Figure 6). In order to get a better signal-to-noise ratio, $^3$P-NMR spectra are recorded from compressed cells within the detection coil at 25°C in a circulating system (perfusion solution can be circulated using double channel peristaltic pump, namely, one polytetrafluoroethylene tube pumping in and another pumping out in an NMR tube, see Figure 1) based on a standard NMR tube. In fact, the intensities of Pi peaks depend on the relative volumes of cytoplasm and vacuoles in the plant cells, and peak noise always appears in case of samples exposed to stress. To avoid this, samples must be kept alive and unbroken, and replicate samples should be analyzed. When plant seeds samples (early-filling rice seeds) are assayed, the glucose in perfusion solution should be removed to eliminate the noise peak.

**Problem 3**
Weak OD400 absorbance in ammonium vanadomolybdate assay (step 10).

**Potential solution**
The reaction color of ammonium vanadomolybdate is weak when Pi concentration is very low in some samples, and the standard error may be large. In this case, the Pi supernatant can be directly used for color reaction, with no need for dilution. In addition, the ammonium vanadomolybdate developer solution can be substituted as ammonium molybdate-ascorbic acid to enhance the color.
reaction, by which the ammonium phosphomolybdate can be reduced by reducing agents (such as ascorbic acid, etc.) to a blue compound with strong chromogenic ability. The preparation and procedure are similar to the ammonium vanadomolybdate method as mentioned above in steps 9–10.

Problem 4
How samples with uneven surface planes are addressed (steps 16 and 17)?

Potential solution
Samples with uneven surface planes may cause background noise in μXRF fluorescence assay, and reducing the thickness of samples can decrease background noise effectively. Thinner samples cause smaller irradiation shadow as usual, for example, the image quality of leaf-derived samples is typically much better than those produced for seed-derived samples. Besides, spacing and orientation of samples should also be taken into account as well. For seed samples, seeds should be cut with a single-sided blade to obtain similar thickness; In case of samples with largely different thickness, the surface planes can be adjusted to the same level by the double faced adhesive tape.

Problem 5
Background noise in μXRF element fluorescence assay (step 19).

Potential solution
For better visualization, a set of filters (Bruker) are placed between the X-ray tube and the lens entrance, which compose of three foils of Al/Ti/Cu with thicknesses of 100/50/25 μm for P element detection. This creates a region of low Bremsstrahlung radiation immediately above the filter’s absorption edge energy. When the filter metals fluoresce, some portion of this energy makes it to the sample surface where most of it (> 90%) is probably scattered inelastically (with energy loss) without causing any K or L-line excitations of sample elements. The elemental mappings are performed with a focused X-ray beam (spot size < 20 μm), and a pixel spacing of 30 μm to completely cover the kernels or rice seeds samples, with a measurement time of 2 ms per pixel. In addition, the researchers could mark the border of each scanned sample with dashed lines during data presentation to facilitate comparison (Figure 7).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zuhua He (zhhe@cemps.ac.cn).

Materials availability
All reagents generated in this study are available from the lead contact upon completing a Materials Transfer Agreement.
Data and code availability
No unique datasets or codes were generated in this study.

ACKNOWLEDGMENTS
We thank Engineer Liqing Sun and Boyue Instruments (Shanghai) Co., Ltd for technical help and useful suggestions. This work was supported by the National Natural Science Foundation of China (32100206 and 31871217) and Yangzhou University Interdisciplinary Research Foundation for Crop Science Discipline of Targeted Support (yzuxk202006). The work was supported in part by China National Postdoctoral Program for Innovative Talents (BX2021314) and China Postdoctoral Science Foundation (2021M693173). This work was also supported by the National Key Research and Development Program of China (2016YFD0100600) and the National Key Laboratory of Plant Molecular Genetics.

AUTHOR CONTRIBUTIONS
B.M., L.Z., and Z.H.H. conceived and designed the project. B.M., L.Z., X.Y.L., Z.J.F., and Y.L. performed all the experiments. B.M., Y.L., and L.Z. analyzed the data. B.M., L.Z., and Z.H.H. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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