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Measurement of ^{33}P-PO_4 Absorption Kinetic Constants in *Arabidopsis*

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[Abstract] Based on the Michaelis-Menten kinetics model (Hofstee, 1952), this method allows calculation of the kinetic parameters (V_{max}, K_m) of phosphate uptake by *Arabidopsis* roots. This method is based on the quantification of phosphate uptake by *Arabidopsis* roots as described in Thibaud and Marin (2016), except that a range of phosphate concentration is applied in the incubation medium.

Plants are grown in high or low Pi giving access to kinetic parameters corresponding to low and high affinity respectively. In high Pi, the high-affinity transporters are not induced giving access to the low-affinity transport only. When plants are grown in low Pi, high affinity transporters are active, and the corresponding kinetic parameters can be measured. The calculation of K_m and V_{max} values is based on the Michaelis-Menten kinetics model.

Materials and Reagents

1. Plastic 12-well plates (Denmark, Nunc)
   Note: One for the absorption step, one for the desorption step for each treatment (plant type or culture condition).
2. Plastic 20 ml vials for radioactivity measurement (Ratiolab GmbH, Dreieich)
   Note: You will need one vial per plant, or 2 vials per plant if you want to quantify ^{33}P in both roots and leaves. The vials should be numbered from 1 to N before you start the experiment. They also should be placed in order in appropriate racks (PerkinElmer) adapted to the beta counter.
3. Tips
4. Young in vitro plantlets
5. MES hydrate (Sigma-Aldrich, catalog number: M8250)
6. $CaCl_2$ (Sigma-Aldrich)
7. ^{33}P-PO_4 5 mCi/ml (185 MBq/ml, 1.48-5.84 TBq/mg, >99% isotopically pure, less than 0.5 µM Pi) (PerkinElmer)
8. Scintillation cocktail (PerkinElmer, Ultima Gold™)
9. $MgSO_4$
10. NH_4NO_3
11. KNO_3
12. NaH_2PO_4
13. KI
14. $FeCl_2$

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15. $MnSO_4$
16. $ZnSO_4$
17. $CuSO_4$
18. $CoCl_2$
19. Na_2MoO_4
20. Thiamine
21. Pyridoxine
22. Nicotinic acid
23. Inositol
24. Sucrose
25. Agar
26. MS/10 medium (see Recipes)
27. Stock solution (see Recipes)
28. 1 M KH_2PO_4 (Sigma-Aldrich) (see Recipes)
29. Incubation medium (see Recipes)
30. Desorption medium (see Recipes)

Equipment

1. Liquid scintillation counter (PerkinElmer, Packard Instrument Company, model: TRI-CARB)
2. Ice-containing large boxes for the desorption step (all 12-well plates will be placed horizontally on ice for 2 h)
3. Tweezers for handling plantlets
4. Micropipets
5. Shield for protection against radiation (Plexiglas)
6. Scanner or camera (Epson America, model: Perfection V850Pro or Canon, model: Powershot SX130), respectively but other devices from other manufacturers could suit perfectly

Software

1. ImageJ version 1.46r with NeuronJ plugin (http://imagej.nih.gov/ij)
2. PRISM 6.0 software (GraphPad)

Procedure

A. Preparation of plant materials

9 to 11 day-old plantlets grown in high and low Pi are convenient for the experiment.

1. When plating, space the seeds in order to maintain root systems independent for every single plantlet; this will help when measuring the length of the root system (see below). 6 or 10 seeds

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are sown per plate depending on the growth medium (respectively with high or low Pi, see below). 9 or 8 plates will be necessary per plant type (respectively in high and low Pi).

2. Plants are grown vertically in 12 x 12 cm Petri plates in modified MS/10 medium.
3. Number the plants on each Petri plate, in order to identify each root system individually.
4. 12 plants per treatment (Pi concentration in the incubation medium) will be necessary plus 10 plants for blanks.
5. Scan or photograph the plates (in black and white and jpg format). Use graph paper as a scale bar for measurement of the root length. The scan should be at 300 dpi and photos should be about 1,000 x 800 pixels (this is recommended by ImageJ).
6. Root length measurement and calculation: We use ImageJ version 1.46r with NeuronJ plugin (http://imagej.nih.gov/ij). Figure 1 shows a photo of a plate, and how root length is measured with ImageJ. The scale (1 cm using the graph paper) is measured (Figure 1A); then with the NeuronJ plugin the primary and lateral roots are traced (pink trace, Figure 1B). Values (in cm) are transferred in an Excel file. Total root length is calculated by adding primary and lateral root lengths (see protocol in Figure 1 legend).

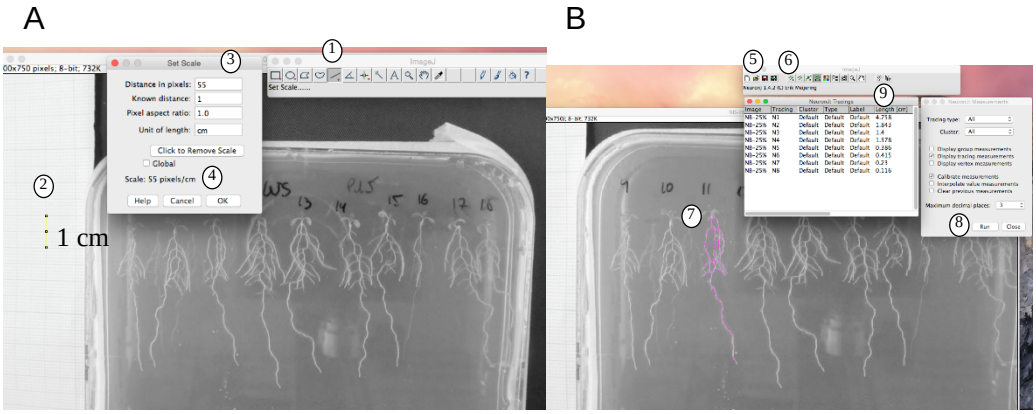

Figure 1. Root length measurement. A. Set of the scale: With ImageJ, trace a 1 cm scale on the graph paper (1, 2) then 'set scale' (3). This gives pixels/cm (4). B. Measurement of the root length: with NeuronJ plugin, load the image file (5) then set scale (as defined in A). Add tracings (6) by drawing the along the roots (pink trace, 7) then measure tracings (RUN, 8). Results of root length in cm (9) can be copied to an excel file. A and B are screenshots of the process.

B. Incubation

1. This step should be performed behind a shield for protection against radiation.
2. Before starting a series of experiments, you must check that Pi absorption is linear in your conditions (plant specificity, temperature, light). To do that, a time course is performed between 30 min and 2 h following the protocol as described in Thibaud and Marin (2016).
3. Prepare ten 12-well plates, one for each point of Pi concentration: 2, 5, 10, 20, 50, 100 µM for plants grown in low Pi and 200, 500, 1,000 and 2,000 µM for plants grown in high Pi. Number the wells: 1 to 12 (for 12 replicates) for each Pi concentration.

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4. Add 4 ml of incubation medium per well. Place 1 plant per well with tweezers (Figure 2), the roots should be immersed and the leaves outside the well. To avoid excessive dehydration of the young plantlets during the incubation, carefully put a cover on the plate avoiding the immersion of the leaves in the solution (Figure 2B). Incubate during 2 h at room temperature (22 - 24 °C) under white light (150-180 $\mu E \cdot m^{-2} s^{-1}$). Discard plants that are fully immersed in the liquid, if any.

A B

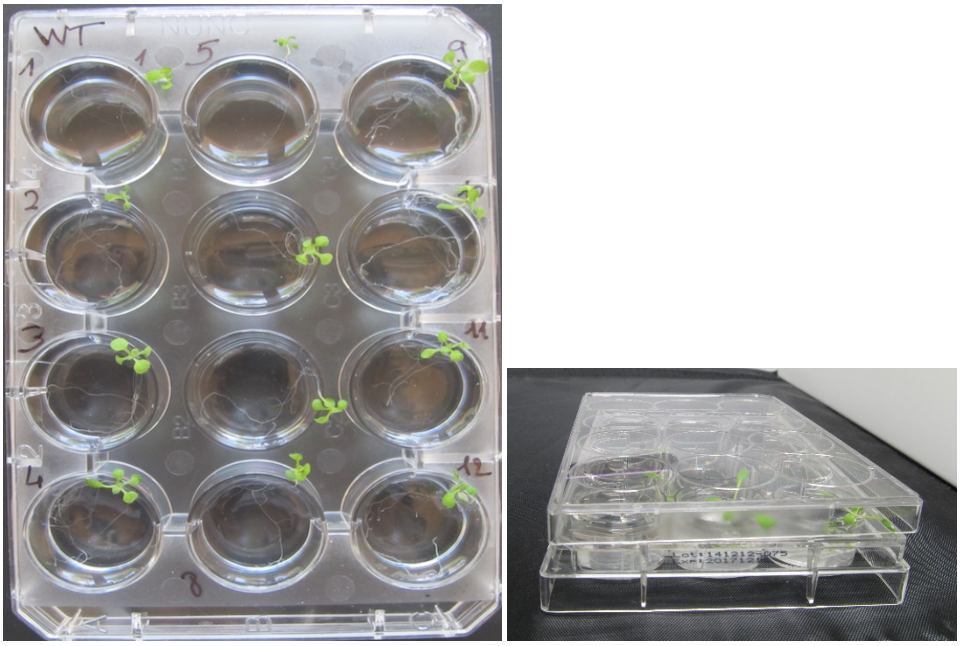

Figure 2. Incubation experiment. A. Photo of a 12-well plate with plants numbered 1 to 12 in the incubation medium. The cover plate has been removed for clarity. Please note that rosettes are out of the medium and roots are fully immersed. B. Photo of the plate and the cover (side view) showing how the cover is placed onto the 12-well plate.

5. In order to evaluate Pi adsorption on the root (mechanical or chemical adsorption can occur on cells, but in that case, Pi does not enter inside the root cells), blank samples are treated as follows: The plant root is dipped in the incubation medium for 2 sec (use tweezers) and directly transferred in the desorption medium for 2 h (see below for the desorption procedure).

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C. Desorption (see Video 1)

Video 1. Desorption

1. Prepare new 12-well plates, with 3 ml of cold desorption medium per well. Place the plates on ice.
2. Rinse the plants in water (1-2 sec).
3. With the help of tweezers, transfer the plants (in the same order you put them in the incubation solution, 1 plant/well) with both leaves and roots immersed into the desorption medium for 2 h on ice.
4. Transfer each plant into a counting vial (use tweezers). It is convenient to put the vials in the counting racks as soon as you harvest the plants (in order to avoid mismatches).

D. Dry the plants in an oven at 50 °C overnight

E. Radioactivity measurement

1. Under a chemical hood, add 2 ml of scintillation cocktail in each counting vial and tighten a top on it. Then place the vials in racks adapted to the counter.
2. With a beta counter, count ^{33}P in each sample (Cs) and in blanks (Cb is a mean of the blank replicates); and also in the incubation medium (C_{10}, 10 µl of incubation medium are placed in a counting vial and 2 ml scintillation cocktail are added). In the scintillation cocktail, beta radiations are transformed in photons that are detected by the beta counter. The measure is in cpm (count per min).
3. Data analysis:

   The amount of PO_4 absorbed per hour per root cm (V in nmol/h/cm) is calculated as follows:

   $V = (Cs-Cb) * (S *10^{-3}) * B / (T * L_{root} * C_{10})$

   Cs: Radioactivity in the sample (cpm)

   Cb: Mean value of radioactivity in the blanks (cpm)

   S: Pi concentration in the incubation medium (S=2 to 2,000 µM).

   $S*10^{-3}$: Pi content (nmol/µl) in the incubation medium (S=2 to 2,000 µM).

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C_{10}: Radioactivity in 10 µl of the incubation solution (cpm). $C_{10}/(S*10^{-2})$ is the specific activity of ^{33}P in the incubation medium

B=10: Volume of the incubation solution for measurement of radioactivity before incubation of the plants (10 µl is convenient).

T=2: Incubation is 2 h

L_{root}: Root length (cm)

4. Kinetics parameters are calculated by drawing Eadie-Hofstee plots where V (mean value of Pi uptake for each Pi concentration in the medium) is plotted against V/S (mean value, S=Pi concentration in the incubation medium). This representation reveals two uptake systems (high or low affinity, Figure 3). Kinetics parameters (V_{max} and K_m) are calculated by linear regression for each uptake system: K_m is the slope of the equation and V_{max} is the extrapolated value for V/S=0 (Table 1).

   Mean and standard deviation (SD) can be calculated from several experiments (not shown here).

5. Kinetics parameters can also be calculated by nonlinear regression (based on Michaelis-Menten kinetics model on V values *vs* S) using PRISM 6.0 software (GraphPad) for high and low affinity systems separately (Table 1). With this procedure, mean and standard deviation (SD) of V_{max} and K_m can be calculated.

Representative data

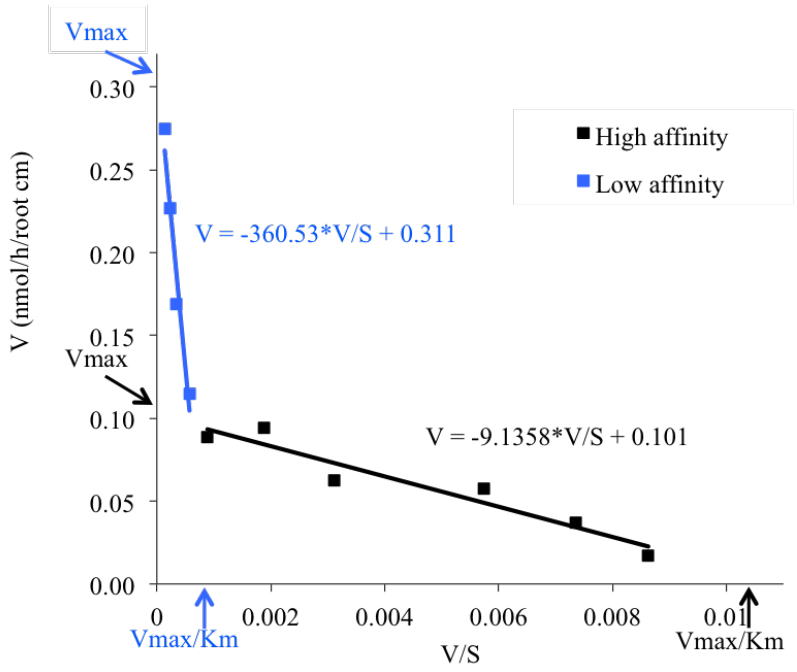

Figure 3. Eadie-Hofstee plot showing the mean values for each Pi concentration uptake rate, the linear regression and the equations giving the Km and Vmax values for both low (in blue) and high (in black) Pi transport activity

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Table 1. V_{max} and K_m values obtained with Eadie-Hofstee plot (from Figure 1) or with Prism software

		High affinity	Low affinity
Eadie-Hofstee plot	V_{max} (nmol·h^{-1}·cm^{-1})	0.101	0.311
	K_m (µM)	9.1	360
Prism (GraphPad) software (mean ± SD)	V_{max} (nmol·h^{-1}·cm^{-1})	0.100 ± 0.006	0.327 ± 0.054
	K_m (µM)	8.85 ± 1.8	423 ± 210

Recipes

1. MS/10 medium
   10x diluted Murashige and Skoog medium containing:
   0.15 mM $MgSO_4$
   2.1 mM NH_4NO_3
   1.9 mM KNO_3
   0.5 (high Pi) or 0.005 (low Pi) mM NaH_2PO_4
   0.34 mM $CaCl_2$
   0.5 µM KI
   10 µM $FeCl_2$
   10 µM H_3BO_3
   10 µM $MnSO_4$
   3 µM $ZnSO_4$
   0.1 µM $CuSO_4$
   0.1 µM $CoCl_2$
   1 µM Na_2MoO_4
   5.9 µM thiamine
   4.9 µM pyridoxine
   8.1 µM nicotinic acid
   55 µM inositol
   3.4 mM MES
   0.5% sucrose and 0.8% agar at pH 5.7
2. Stock solution
   0.1 mM $CaCl_2$ in 5 mM MES, adjusted at pH 5.5
   For 1 L: 0.976 g MES + 0.0147 g $CaCl_2$, Adjust pH to 5.5 with 10 N NaOH
3. 1 M KH_2PO_4
   For 1 L: 136 g in water
4. Incubation medium (4 ml /plant are necessary)
   5,550 Bq ^{33}P/ml (0.15 µCi ^{33}P/ml) in stock solution
   Then prepare aliquots with appropriate volume (see table below) of 1 M or 100 mM KH_2PO_4 for 2 to 2,000 µM final concentration in 40 ml stock solution supplied in ^{33}P as follows:

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Final Pi concentration (µM)	2	5	10	20	50	100	200	500	1,000	2,000
1 M KH_2PO_4 (µl)							10	20	40	80
100 mM KH_2PO_4 (µl)	0.8	2	4	8	20	40				

5. Desorption medium (3 ml/plant are necessary)
   1 mM KH_2PO_4 in stock solution: 1 ml 1 M KH_2PO_4 in 1 L stock solution

Acknowledgments

This protocol was adapted from the previously published studies, Narang *et al.* (2000) modified by Misson *et al.* (2004) and Aung *et al.* (2006) based on the study of Hosftee (1952). We acknowledge all these authors for their previous work. The present protocol was published by Ayadi *et al.* (2015). This work was supported by the Commissariat à l'Energie Atomique et aux Energies Alternatives.

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