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Combining multiple high-resolution in situ techniques to understand phosphorous availability around rice roots

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ABSTRACT

Differentiating chemical-versus-biotic drivers for P-behaviour in wetland rice paddies is key to improving the sustainability of our most important global crop. As these reactions occur within intensely localized environmental clines of sub-mm distances, there is a lack of suitable methods for deciphering these intertwined soil-solute-plant processes. In this study, we integrated three advanced soil imaging systems (DGT-LA-ICPMS, O_2 planar optode, and soil zymography) to deliver a low disturbance and high-resolution (HR) measurement of root influenced P availability. 2D visualization of solute-P pools within the rhizosphere were obtained by DGT and combined with associated O_2, Fe, and phosphatase activity mapping for process/mechanism identification. HR visualization revealed a uniform P depletion, but high heterogeneities in Fe/P interactions associated with rice roots. The linking of HR-DGT and soil zymography, for the first time, revealed that these P-depletion zones are sufficiently developed to stimulate a biological response, which was demonstrated by the overlapping of P depletion and phosphatase activities hotspots in spatial distributions and their negative association in solute contents.

With the intensification of agricultural food production, excessive input with subsequent accumulation of phosphorus (P) in agricultural ecosystems has become a major contributor to the eutrophication of surface waters[1,2]. Compared with other arable soils, the threat to water quality from P-fertilization is more serious for paddy fields since P is more mobile and highly soluble under flooded soil conditions[3]. Changes in the chemical bonding of P_i to soil substrates along with phosphatase mediated mineralization of P_{org} are the dominant soil-to-porewater transitions releasing P in paddy soils. Conversely, removal of labile-P is mainly from root uptake, rhizosphere iron-plaque sequestration, and leaching losses, thus, completing this cycle. It is this interplay of processes that determines the spatial-temporal characteristics of P-bioavailability in rice soils.

More specifically, P_i release is dominated by the chemical behavior of Fe (hydr)oxides and to a lesser extent Al and Mn (hydr)oxides which are important reactive surfaces for P adsorption[4]. Though orthophosphate itself is generally not reduced in submerged/rice paddy soils, P_i tends to be mobilized under anaerobic conditions when precipitated Fe(III) species that bind with P_i are transformed to soluble Fe(II)[5]. Although the ratio/proportion P_i/ P_{org} varies in soils, up to 80% of the total P pool within a soil can be in organic form[6]. Here, microbial and plant release of extracellular enzymes (phosphatase) catalyze the hydrolysis and mineralization of unavailable P_{org} to soluble P_i [7,8]. With the microbiota capable of producing both acid and alkaline phosphatases, while plants can excrete only acid phosphatase[9].

~~The rice root is a crucial linkage in the whole P cycling processes in paddy field.~~

Rice roots not only directly participate in the P cycling of soils as sinks or sources of P, but also influence the processes of P_i release and P_{org} mineralization[10, 11]. A living/active rhizosphere differs considerably in O_2 concentration, pH, and microbial abundances/activities[12, 13, 14], compared to its surrounding bulk soils. However, even after the rice has been harvested, the residue of dead root in soils transforms the rhizosphere to a detritusphere hotspot[15]. In the paddy field, soils are commonly drained for several months between the two flooded cropping periods[5]. The root detritus will remain in soils after the drainage and it can provide abundant available organic C[16], which stimulates microbial mineralization of P_{org} and enhances phosphatase activities[17]. Therefore, P availability is influenced strongly by roots no matter whether they are active or not. Measuring and therefore understanding of P-fluxes within the rhizosphere/detritusphere's is challenging. The common/traditional methods which have drawbacks of i) not able to make measurements at suitable spatial sensitivities/scales[18], ii) not suitable for two dimensional (2D) applications, iii) disturbing the local environment in which they are sampling, and iv) cannot distinguish enough relevant parameters to enable mechanistic interpretation[19].

A solution to this technology limitation, and one that has recently be used to study plant-soil interactions[20, 21, 22], in in situ sub-mm High Resolution (HR) DGT chemical imaging. A recent study using HR-DGT, revealed, for the first time, a decoupling behavior between Fe and P in the oxic-anoxic transition zone within the rhizosphere, where a flux maxima in Fe around root tips corresponded to a significant minima in P release[21]. This is in contrast to previous observations of a strong positive relationship

between Fe and P availability in both rhizosphere and bulk soils based on traditional rhizobox and root-mat approaches[23, 24, 25]. However, all these studies were focused on the chemical processes and P-fluxes without simultaneous in situ biological information at appropriate scale.

In this study, HR-DGT was combined with another two 2D mapping methods of O_2 planar optode and soil zymography to investigate the P bioavailability regulation within rice rhizosphere and detritusphere zones. The coupling of techniques can achieve the simultaneous visualization of P/Fe availability distributions and other relevant biogeochemical parameters including O_2 dynamics and phosphatase activities which are needed for interpreting the underlying processes of P at appropriate spatial resolutions. This is the first study which amalgamates HR-DGT with soil zymography techniques, providing integrated measurements of P_{org} mineralization by phosphatase, which is an important source for P_i in the rhizosphere and detritusphere.

Results

Depletion of P flux within the rhizosphere and detritusphere zone. As revealed by PZ-SPR-IDA DGT, a decrease in labile-P developed within all root zones, including living or dead roots (Figure 1d and Figure 2c). Within the rhizosphere, even though there was a well-developed spatial heterogeneity in chemical behavior, for example in ROL (R1-R3, Figure 1b), with this then influencing the oxidation-adsorption reactions controlling P-availability, the root associated P minima was consistently observed/detected (Figure 1d). The average P flux in the rhizosphere zone (R2) was 9.37±0.38 pg cm^{-2} s^{-1}, significantly lower than in bulk soil (B2) (29.5±2.4 pg cm^{-2} s^{-1},

Table 1, t-test, $p<0.01$). To have a better identification of the depletion zone, we quantitatively defined the P flux depletion as the zone with intensities lower than the average flux of the sampling area. Based on the histogram distribution of the P flux (Figure S3) around living roots, it was calculated that the zone of P depletion accounted for ~56.6% of the total sampling area, indicating substantial/extensive influence of living root activities on P availability.

Regarding the drained soils with dead roots, 67.8% of the total sampling zone was under the condition of P depletion. The depletion zone was higher in the drained soils (Figure 2c) compared with submerged soils (Figure 1d) the since the P flux was quite low, approximately 16.6 pg cm^{-2} s^{-1}, in the whole upper soils (0–2.5 cm) due to the oxic condition. In the anoxic deeper soils, mobilization of P was significant in the bulk soils, but P depletion occurred in the detritusphere zone, with the P flux in detritusphere soils (D1) being 52% lower than bulk soil (B3).

Fe and P flux relationship. Since Fe (hydr)oxide is the dominant reactive surface for P, assessing the relationship between Fe and P lability can help to understand the processes of Fe reductive desorption or oxidative adsorption underlying the P spatial distribution. High resolution visualization revealed that Fe and P show a general commonality in spatial distribution in bulk soils and some parts of rhizosphere soil without significant ROL. As shown in Figure 1, there were prominent mobilizations of both Fe and P in the anaerobic bulk soils (B1, B2), due to the reductive dissolution of P from Fe (hydr)oxides. However, their fluxes in bulk soils B2 were about 62% for P and 40% for Fe lower than those in bulk soils B1. Diffusion of O_2 from the atmosphere

can lead to the slight oxygenation in the upper soil zones which are close to soil water interface (SWI), favoring the immobilization of P by Fe oxidation-adsorption process[26].

In the rhizosphere zone without significant ROL, such as R2 and R3, both Fe and P are significantly immobilized. The minima of Fe and P fluxes simultaneously occurred in the basal parts of root (R2), with the Fe and P fluxes of 7.72±1.23 pg cm^{-2} s^{-1} and 9.37±0.38 pg cm^{-2} s^{-1}, respectively. Similarly, Fe and P fluxes in the zone of root axial (R3) were also significantly lower than the surrounding bulk soils (B1) (Table 1). Fe and P fluxes transects presented gradual decrease approaching the root surface and P flux was significantly positively related to Fe flux based on Pearson analysis (r=0.875, $p<0.01$), implying the strong affinity between Fe and P in this zone.

The decoupling between Fe and P availability can be found at the periphery of the aerobic root zone (R1) where there was Fe flux maxima, but the labile P flux kept decreasing from anaerobic bulk soils to aerobic zone (Figure 1 & Figure S4). At the periphery of the aerobic root zone, the Fe flux reached up at ~127 pg cm^{-2} s^{-1}, the maxima value in the sampling area, while the average flux of P in the same zone was only 10.5±0.9 pg cm^{-2} s^{-1}. A chemical decoupling between Fe/P was also observed at the periphery of root apices with strong O_2 release in another study using HR-DGT technique[21]. In this study, we ascertained that the decoupling between Fe/P occurred only in the root tips zone with O_2 maxima.

Different from the variation in Fe-P relationship around living roots, spatial pattern of P flux was similar to Fe flux in both detritusphere and bulk soils. The distribution characteristics of both Fe and P availability were strongly related to soil depth and the

existence of dead roots (Figure 2). Fe flux in the upper soils (0–2.5 cm) was about 94% lower than the corresponding value in the lower soils (B3). Similarly, the Fe flux in deteritusphere zone (D1) was approximately half of the flux in the surrounding bulk soils.

P depletion and phosphatase maxima hotspots in rhizosphere and detritusphere. As the organic P mineralization by phosphatase is one of the important source for inorganic P, the spatial patterns of P fluxes and phosphatase activities were compared to resolve the biological control on the chemical processes of P. 2D mapping of phosphatase activities showed that high activities occurred around both living and dead roots (Figure S5 and Figure S6). Activities of both alkaline and acid phosphatase in the rhizosphere zone were up to 8–10 times higher than activities in the bulk soils. This is mainly due to the activities of phosphatase production by plant roots and abundant rhizosphere microorganism. In this study, when we coupled soil zymography with HR-DGT directly, a consistent/overlapping spatial distribution of P depletion and localized phosphatase maxima was observed (Figure S5 and Figure S6). In addition, P availability and phosphatase activities presented significantly negative relationship as indicated by the Pearson correlation analysis (Table 2), which implied that these P-depletion zones were sufficiently developed to stimulate a biological response, i.e. phosphatase production.

We adopted two indexes, the size and spatial distribution, to quantitatively describe the depletion extent of P flux and hotspots of phosphatase activities and then evaluate their relationships. P flux minima and phosphatase activity maxima were defined as the

zone with intensities lower or higher than the average value of the sampling area[27]. Size of the spot were calculated based on the histogram of phosphatase activities and P flux in the sampling area. Regarding spatial distribution characteristics, the relationships between phosphatase activities and P flux were compared using the Mantel test.

Extracted hotspots of phosphatase activities and depletion of P fluxs were shown in Figure 3 for living roots and Figure 4 for dead roots. Quantitative assessments revealed that the hotspot zones for acid and alkaline phosphatase activities in the sampling area near living root were ~49.4% and 56.0% of the total area, similar to the zone of P minima, accounting for 51.0% of the total area. The Mantel test additionally showed that the spatial distribution characteristics of both acid and alkaline phosphatase were strongly related to P flux with the r value of 0.810 and 0.763 ($p<0.01$), respectively. However, there are some differences between acid and alkaline phosphatase showing that the zone of increased alkaline phosphatase expanded to longer distance from the root surface. For example, the zone of increased alkaline phosphatase expanded out further from the root surface (zone B1, Figure 3), but there was no hotspot for acid phosphatase and no P depletion. The wider distribution of the alkaline phosphatase hotspot compared to acid phosphatase was also reported in previous studies[9, 17]. Microorganisms are the major source of alkaline phosphatase and the high diversity of microbial activities in soils might lead to the widely dispersed alkaline phosphatase hotspots[17, 28].

With respect to the soils with dead roots, the relationship between phosphatase activities and P flux was similar to that in the soils with living roots. Acid and alkaline

phosphatase hotspot zone accounted for 42.8% and 42.8% of the total areas, similar to the P depletion area of 36.4%. The close associations in spatial distribution between phosphatase activities hotspots and depletion of labile P flux were also demonstrated by Mantel test (between acid phosphatase and P flux: 0.610, between alkaline phosphatase and P flux: 0.722l, $p<0.01$).

Discussion

Even though P deficiency in rhizosphere has been well understood for most plants grown in upland soils[6], HR-DGT revealed that in the submerged condition, the depletion of P also occurred in the rice rhizosphere despite that most of P_i can release from Fe (hydr)oxides in anoxic condition. P-flux minima in the rhizosphere would result from various biochemical events, but would be dominated by root uptake, microorganism utilization, and Fe(hydr)oxides adsorption. Rice uptake of P has been reported to be less affected by root structural development and both apical and basal regions of the roots can take up P efficiently[29]. However, after the rice was harvest, the residue of dead root in soils also leads to the depletion of P in the detritusphere zone. The decrease of P flux around dead roots might be due to the relatively higher redox condition in the detrirtusphere zone than bulk soils. In the drained soils, the root-originated biopores will provide channels for O_2 diffusion and lead to better aeration for soils around the detritus[15], which can favor the immobilization of P by Fe (hydr)oxides.

HR-DGT reveals the P chemical flux as a summation of various biogeochemical reactions, but the P chemical flux alone was not sufficient to unravel the underlying

processes related to P regulation. Coupling of multiple 2D high resolution techniques in this study can provide more detailed information of element availability and phosphatase activities to depict an interaction process of P availability regulation, including not only chemical dynamics, but also biological processes. In this study, for the first time, we combined HR-DGT with soil zymography to resolve the chemical P-fluxes with simultaneous biological process detail. The 2D visualization methods at high resolution confirm that chemical flux and biological response align well with each other in spatial distribution characteristics and solute concentration dynamics. Such combined methodology not only supports the robustness of HR-DGT, a chemical and diffusion process based measurement, to quantitatively determine P availability from the perspective of biological ascertainment, but also provides some implications to explain the interactions between P and phosphatase activities. Previous studies using 2D *in-situ* soil zymography to visualize enzyme activities have also revealed higher phosphatase activities in the rhizosphere and detritusphere zone[17, 30, 31]. Some studies related the higher phosphatase activities to the root functions of excreting acid phosphatase and rhizodeposition with high contents of available organic carbon that can promote microorganism to produce extracellular enzyme[17, 32, 33]. However, Spohn and Kuzyakov (2013)[9] combined the ^{14}C image with soil zymography to evaluate the relationship between carbon allocation and phosphatase activities and revealed that rhizodeposition and alkaline phosphatase-producing microorganisms were not directly related. In this study, the overlapping of P depletion and phosphatase activities hotspots in the spatial patterns and their negatively correlated concentrations imply that the

deficiency of labile P near living and dead roots might be a controlling factor on the phosphatase production.

In addition to the biological response of phosphatase production underlying P availability regulation, the interaction between Fe and P is another important chemical process for P mobilization. The high resolution mapping techniques reveal highly heterogeneous interactions between Fe and P covered by the uniform depletion of P in the rhizosphere. To have a better evaluation of the Fe-P relationship, we adopted the ratio of Fe to P flux (Fe/P flux ratio) to indicate the heterogeneity of Fe and P interactions in different soil zones. As shown in Figure S7, the ratio of Fe to P flux varied not only between bulk soils and rhizosphere zone, but also in different rooting zones, such as basal part of roots and root apices, demonstrating the influence of the root activities in creating divergent chemical and biological conditions. First, the ratio of Fe to P flux was generally low in the bulk zone B2 (Fe/P flux ratio: 1.051) than bulk soil zone B1 (Fe/P flux ratio: 1.606). Compared with zone B1, soil zone B2 is more likely to be in oxic condition as a result of the O_2 diffusion from overlying water to soil zone B2[34]. The higher ratio of Fe to P flux in B1 indicates the greater mobilization of Fe than P under reducing condition. Similarly, the O_2 diffusion from overlying water also contributes to a relatively lower Fe/P flux ratio in the basal parts of roots (zone R2)[26]. Second, decoupling between Fe and P in the aerobic rhizosphere zone (R1) leads to the significantly high ratio of Fe to P flux, implying the different trend of Fe and P availability change in the zone. ROL induced oxic conditions can promote Fe(II) oxidation and precipitation, resulting in decreasing Fe(II) concentration in the

rhizosphere. The naturally occurring concentration gradients will favor the movement of Fe(II) from reduced bulk soils to oxidized soils with the coefficient around 10^{-11} m^2 s^{-1} [35]. When Fe(II) reaches the aerobic root zones, soluble Fe(II) can be quickly oxidized to Fe(III) even under sub-oxic conditions[36, 37]. If the precipitation rate of Fe(III) to oxides is lower than the flux of Fe(II), it will form a flux maxima of Fe in the adjacent zone. However, the diffusion of P toward root surfaces is mainly driven by the sink effect of root uptake since root apex is the dominant entry point for nutrient acquisition in plant[18]. In this zone, both the impact of redox change and plant uptake was amplified due to the root function, so their different impacts on Fe and P flux resulted in the decoupling behavior between Fe and P lability. Third, it is interesting that the Fe/P flux ratio in other rhizosphere zone without significant ROL was higher than bulk soil, but lower than the aerobic root zone. It can be speculated that the root uptake of P might lower the P availability and thereby increase the Fe/P flux ratio.

When living roots turn into dead roots that effectively “turn-off” the root activities, the Fe and P flux become consistent in the spatial patterns. However, the significant difference in Fe/P flux ratio can be found between the upper and deeper soil. The ratio of Fe to P flux in the upper soil was about 0.367, much lower than the corresponding value of 1.43 in the deeper soils, which is mainly attributed to the change of redox condition and similar to the difference in Fe/P flux ratio between bulk soils B1 and B2. Interestingly, in the anoxic/oxic transition zone, the ratio of Fe to P flux was slightly higher than the anoxic zone, indicating that the immobilization rate of P is more rapid than Fe when the redox condition turns from anoxic to oxic. Generally, it can be

speculated that the Fe/P flux ratio followed the order: oxic bulk soils < anoxic bulk soils < anoxic/oxic transition zone < oxic rooting zone. The Fe/P flux ratio, being sensitive to the redox change as well as the root activities, is useful to differentiate the soil functional zones and unravel the heterogeneous interactions between Fe and P.

In summary, this study combined three 2D mapping technique, including HR-DGT, O_2 planar optode, and soil zymography, to investigate the P availability regulation processes. It not only reveals the simultaneous biological process with chemical P availability, but also shows the variable Fe regulation process covered by the uniform depletion of P in the rhizosphere. Direct visualization of such heterogeneity mainly benefits from the high-resolution and in-situ mapping technique, which makes us have a new understanding about the P regulation related to rice roots.

Methods

Soil preparation and Rhizotron setup. A loamy soil was collected from the 0–20 cm horizon of an agricultural land located in Shimen, Hunan, China (29° 59' N, 111°35' E), followed by air drying and passing through a 2-mm sieve. Physicochemical properties of the soil were as follows: pH: 5.71, total contents of Fe 2.53 g kg^{-1} and P: 0.81 g kg^{-1}. Perspex rhizotrons with inner dimensions of 40 cm × 10 cm × 1.5 cm (height × width × depth) were filled with the sieved (<2mm) soils. During the rhizotron compaction, soils were tamped in layers to achieve a homogeneous soil structure with a consistent bulk density of 1.3 kg L^{-1}. After that, the detachable front plate of the rhizotrons was opened and the Nuclepore track-etched polycarbonate membrane of 0.2 μm pore size and ~10 μm thickness (Whatman, UK) was placed on

top of the exposed soil layer. The Nuclepore membrane has two functions: i) maintain soil integrity during DGT binding gel deployment and ii) avoid any disturbance to roots and soil when the perspex front plate was removed.

There are two treatments for rhizotrons: (I) living rice cultivated for ~3 weeks and (II) the dead root which was kept in the rhizotron with oxic aging for 30 days. These two treatments simulated the two typical scenarios of flooded condition with rice cropping and drained condition with root detritus. Before rice seedlings were transplanted into rhizotrons, soils in the rhizotrons were saturated slowly and evenly using a water feed system for ~24 h. Then all the rhizotrons were soaked in a water tank (~60 L) and deoxygenated with nitrogen gas to minimize the ingress of O_2. After sterilization in 35% H_2O_2 for 15 min, rice (*Oryza sativa L.*) seeds were soaked in deionized water overnight and germinated at 25 ^{o}C for 4–5 days. Then, rice seedlings were transferred to the prepared rhizotrons for a further 3-week growth in a greenhouse with the temperatures set around 30/20 ^{o}C (day/night) and a 14 h photoperiod. During rice cultivation, all rhizotrons were kept inclined at an angle of 45° with the detachable front plate downward to encourage roots growth alongside the Nuclepore membrane at the detachable front plate. All rooting/soil zones were kept in darkness to prevent microphyte growth. Regarding the treatment of soils with dead roots, rice shoots were cut at the surface of the soil after 3 weeks of rice cultivation, followed by 30 days of dead root oxic aging. During this process soil water content was maintained at ~30%. Rhizotrons were weighed every 3 days and deionized water (DI) (18.2 MΩ·cm) was added to compensate for water loss. During DGT gel deployment (described in the

following section), soils were re-flooded to ~100% water holding capacity to ensure the diffusion of target elements from soils to the gels. Each treatment was carried out in triplicates at the same incubation condition of temperature and photoperiod.

Deployment of O_2 Planar optode. For the living rice roots with radial oxygen loss (ROL), a sandwich sensor combining an ultrathin layer of DGT gel with an O_2 planar optode was used to record the element labile fluxes as well as the O_2 concentration dynamics at the same location. The ultrathin DGT gel layer was exposed directly to the soils and backed by a planar optode as described in Williams et al. (2014)[20]. O_2 images were captured with a fast gateable 12-bit camera modified with a 590 nm long-pass filter.

DGT gel deployment and LA-ICPMS measurement. The DGT gels consist of zirconium hydroxide precipitate and suspended particulate reagent iminodiacetic acid (PZ-SPR-IDA) were prepared following the protocols reported by Kreuzeder et al. (2013)[38]. Briefly, PZ-SPR-IDA were dissolved in the hydrogel material Hydromed D4. Then the mixed hydrogel solution was coated onto a glass plate with a fixed U-shape plastic spacer (50 μm). The glass plate with the freshly coated gel solution was put into an oven at 80 °C until the gel was dry (approximately 10 min). Afterward, the hot glass plate was allowed to cool down to room temperature in a clean laminar hood (Class 100), and the coating process was repeated two more times to achieve a triple coating. Finally, the PZ-SPR-IDA gel was hydrated in Milli-Q water for 24 h.

The PZ-SPR-IDA gel was deployed at the root/soil area for 24 h before soil zymography, to investigate the spatial patterns of labile Fe and P fluxes. The drying

process of PZ-SPR-IDA gel for LA-ICPMS analysis was carried out according to Gao and Lehto (2012)[39]. Briefly, the PZ-SPR-IDA gel was placed on an acid-cleaned 0.45-μm-poresize cellulose nitrate filter membrane with a backing layer of laboratory-grade tissue paper. Gels underwent initial air drying overnight, followed by complete desiccation in a gel dryer (Model 583, Biorad, Hercules, CA) at 50 °C for 2 h before analysis by LA-ICPMS. The accumulated Fe and P in the gel were expressed as the labile fluxes, f_{DGT} (pg cm^{-2} s^{-1}), indicating the availability of Fe and P.

The DGT gel samples including gel standards were laser-ablated by a Nd:YAG solid-state laser ablation system (UP-213, New Wave Research, Fremont, CA), and then transported in the form of an aerosol via helium carrier gas to the ICPMS (NexION 300X, PerkinElmer, USA) for elemental analysis. The laser was set-up first with primary calibrations using the National Institute of Standards and Technology (NIST) glass standard 610. The LA-ICPMS system was optimized principally by adjusting gas flow rates, torch sampling depth, and radio frequency power to achieve the maximum signal intensity and stability of target elements while minimizing interferences[20]. The instrumental parameters for analysis are listed in Table S1.

Sensitivity drift during LA-ICPMS analysis can be caused by matrix differences, defocusing, and the deposition of analyzed materials. It could significantly influence the accuracy and precision of the results. To compensate for the possible deviation in laser focusing and aerosol transport efficiency during analysis, an internal standard (C, m/z 13) was used to calculate the normalized count ratio[20, 39]. Afterwards, the mass loading of target elements on the gel can be quantified from the normalized metal count

rate based on the standard calibration (Figure S1). The fluxes of target elements on the gel in this study were in the quantitative range of standard calibrations.

Soil zymography. Spatial distributions of enzyme activities in the rhizosphere and root-detritusphere zone were measured using an *in situ* technique of soil zymography, according to Spohn et al. (2013)[40] and Razavi et al. (2016)[31]. MUF-phosphate was dissolved in 10 mM MES buffer solution. The buffer was adjusted to pH 6.5 for acid phosphatase activity measurement and pH 11.0 for alkaline phosphatase activity measurement. Polyamide filter membranes (Tao Yuan, China) with a dimension of 130 mm × 130 mm (length × width) and a pore size of 0.45 mm were cut into the size suitable for rhizotrons. The membranes were saturated in the substrate solution for each enzyme. The detachable front plates of rhizotrons were removed and the saturated membranes were applied directly to the soil surface. Soil zymography was performed for each enzyme separately on the same rhizotrons; first acid phosphatase activity and then alkaline phosphatase activity were analyzed. Each membrane containing different enzyme substrates was deployed on the soil surface for 1 h based on previous studies[17, 31, 32]. After that, the membranes were carefully peeled off from the soil surface and any attached soil particles on the membranes were gently removed using tweezers. Enzyme activities were assessed based on fluorescence of the membranes, which were measured using thin-layer chromatography scanning (biostep, DD70, Germany) at the excitation wavelength of 360 nm. Fluorescence visible on the zymograms under UV light shows the areas where substrates have been enzymatically hydrolyzed, and the intensity of fluorescence is proportional to the activity of the enzyme.

To quantify the enzyme activity obtained through soil zymography, a standard calibration was prepared using polyamide membranes that were soaked in solutions of 4-methylumbelliferone (MUF) with different concentrations. The amount of MUF on an area basis was calculated based on the volume of the MUF solution taken up by the membrane, concentration of the MUF solution, and area of the membrane. The membranes for the calibration were imaged under UV light and analyzed in the same way as the sample membrane mentioned above. Then, a standard curve was obtained based on the relationship between the MUF concentrations (pmol mm^{-2}) and the greyscale values of the fluorescence[17, 31], with more intense values implying a higher concentration of MUF.

Image processing was made using ImageJ software. The digital images of zymograms were transferred to 32-bit grayscale image and then the grayscale values of the sample membranes were corrected based on a reference zone. We used the grayscale value obtained from the blank side of the samples as the reference point. After referencing the zymograms, the pixel-wise grayscale values in the zymograms were converted to enzyme activities using the calibration function (Figure S2).

Data analysis. All the statistical analyses were made using R (Ver 3.3.0). Pearson correlation was conducted to evaluate the associations among the spatial patterns of acid phosphatase activity, alkaline phosphatase activity, and P labile flux. Firstly, we selected an image area and obtained the data of the enzyme activities and P labile flux representing the whole targeted area. Data matrixes representing enzyme activities and P labile flux were adjusted to the same size (r: the number of rows, c: the number of

columns) based on matrix linear interpolation. Then, the data matrixes of the enzyme activities and P labile flux were compared via Pearson correlation analysis to evaluate the responses of acid and alkaline phosphatase activity to change of P labile flux.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information file, and from the corresponding author upon reasonable request.

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Author contributions

This work was originally conceived by W.F. and J.L. with additional input from P.W. and H.Z. Laboratory experiments were conducted by W.F., Y.Y., X.S., D.Y., X.H., and Z.L. The manuscript and supporting information were written by W.F., P.W., H.Z., and J.L. with additional input from all other coauthors.

Additional information

Supplementary Information accompanies this paper.

Competing interests: The authors declare no competing financial interests.

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