A genetically encoded biosensor reveals spatiotemporal variation in cellular phosphate content in *Brachypodium distachyon* mycorrhizal roots

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Summary

- Arbuscular mycorrhizal (AM) symbiosis is accompanied by alterations to root cell metabolism and physiology, and to the pathways of orthophosphate (Pi) entry into the root, which increase with Pi delivery to cortical cells via arbuscules. How AM symbiosis influences the Pi content and Pi response dynamics of cells in the root cortex and epidermis is unknown.
- Using fluorescence resonance energy transfer (FRET)-based Pi biosensors, we mapped the relative cytosolic and plastidic Pi content of *Brachypodium distachyon* mycorrhizal root cells, analyzed responses to extracellular Pi and traced extraradical hyphae-mediated Pi transfer to colonized cells.
- Colonized cortical cells had a higher cytosolic Pi content relative to noncolonized cortical and epidermal cells, while plastidic Pi content was highest in cells at the infection front. Pi application to the entire mycorrhizal root resulted in transient changes in cytosolic Pi that differed in direction and magnitude depending on cell type and arbuscule status; cells with mature arbuscules showed a substantial transient increase in cytosolic Pi while those with collapsed arbuscules showed a decrease. Directed Pi application to extraradical hyphae resulted in measurable changes in cytosolic Pi of colonized cells 18 h after application.
- Our experiments reveal that cells within a mycorrhizal root vary in Pi content and Pi response dynamics.

Introduction

Phosphorus (P) is one of the macronutrients essential for plant growth and required by all plant cells. It is obtained, mainly in the form of orthophosphate (Pi), from the soil solution by phosphate transporters in the plasma membranes of root epidermal cells. Subsequent distribution to all cells of the plant and to many sub-cellular compartments, is enabled by several additional families of phosphate transporter proteins (Gu et al., 2016; Versaw & Garcia, 2017). Pi is challenging for plants to obtain as Pi is highly reactive and forms sparingly soluble complexes in the soil. As a consequence, Pi levels in the soil solution are generally low and particularly depleted in the rhizosphere as a result of rapid Pi uptake by roots (Schachtman et al., 1998). Many plants increase their access to Pi through symbiotic associations with arbuscular mycorrhizal (AM) fungi (Chiu & Paszkowski, 2019), a trait that occurs in over 72% of flowering plants (Brundrett, 2017).

During AM symbiosis, the fungal symbiont colonizes the root and also the surrounding soil. This results in hyphal continuity between the soil and the root cortex which enables Pi delivery to the interior of the root. Symbiotic Pi acquisition starts with Pi uptake by the hyphae in the soil and high-affinity Pi transporters are presumed to mediate this step (Harrison & Bauren, 1995; Maldonado-Mendoza et al., 2001; Xie et al., 2013, 2016). The current models propose that Pi is then assimilated into polyphosphates in vacuoles and translocated through the hyphae to specialized, highly branched hyphae called arbuscules, which are located in the cortical cells (Cox et al., 1980; Rasmussen et al., 2000; Hijikata et al., 2010). Polyphosphate breakdown in the arbuscules is followed by Pi efflux from the fungus. Each arbuscule is surrounded by a plant membrane, the periarbuscular membrane, which contains Pi transporters that transport the fungal-delivered Pi into the root cortical cell (Javot et al., 2007; Yang et al., 2012). In some situations, plants can obtain the majority of their Pi via their fungal symbiont (Smith et al., 2003). The plant supports the AM fungus with lipids and sugar which are synthesized in the colonized cell and exported across the periarbuscular membrane (Bravo et al., 2017; Jiang et al., 2017; Keymer et al., 2017; An et al., 2019). Thus the colonized cortical cells show substantial alterations to their metabolism, cellular organization and physiology.

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In *Brachypodium distachyon* roots, AM fungal hyphae follow an intracellular growth pattern in which thick linear hyphae grow cell to cell along the longitudinal axis of the root (Hong *et al.*, 2012). Arbuscules develop on branches that arise from these thick linear hyphae. Distinct stages can be observed, including arbuscules with a few thick branches, mature arbuscules with many fine branches, and degenerating arbuscules that ultimately form clumps (Gutjahr & Parniske, 2013). These stages can generally be observed consecutively behind the growing hyphal front (Cox & Sanders, 1974; Walker & Smith, 1984). As arbuscules and the periarbuscular membrane develop, the location and morphology of several plant cells organelles are altered. In particular, plastids become highly stromulated, a morphology that has been suggested to be associated with increased metabolic activity (Kwok & Hanson, 2004; Waters *et al.*, 2004), potentially the result of extensive fatty acid biosynthesis.

Several techniques have been used to measure or image Pi levels in plants, such as phosphorus-31 nuclear magnetic resonance ($^{31}$P-NMR), X-ray spectrometry, and use of Pi radioisotopes (Kanno *et al.*, 2016). However, none of these techniques allow *in vivo* monitoring of Pi profiles with cellular resolution in real-time. In contrast, genetically-encoded fluorescent biosensors provide an opportunity to report ion levels as measurable fluorescent signals (Lalonde *et al.*, 2005; Okumoto *et al.*, 2012). Fluorescence resonance energy transfer (FRET)-based biosensors have been widely used to measure ions, such as calcium ion ($Ca^{2+}$) and zinc ion ($Zn^{2+}$), and provided new insights that could not have been obtained by other means, such as the discovery of salt stress-induced $Ca^{2+}$ waves in *Arabidopsis* roots (Choi *et al.*, 2014). With the development of a series of FRET-based Pi sensors (FLIPPi), it became possible to monitor Pi in real-time as demonstrated in Cos-7 cells (Gu *et al.*, 2006). Mukherjee *et al.* (2015) improved the dynamic range of these sensors by substituting enhanced yellow fluorescent protein (eYFP) with circularly permuted Venus (cpVenus) and further mutating the Pi binding domain to generate a series of biosensors, cpFLIPPis with an affinity range (80 $\mu$M to 11 mM) suitable for use in plants. To further increase the robustness of cpFLIPPi measurements, Banerjee *et al.* (2016) created cpFLIPPi-Null, which is insensitive to Pi and therefore serves as an important control for nonspecific changes in FRET or fluorescence. Recently, these sensors were used to generate a high resolution map of cytosolic Pi distribution in *Arabidopsis* roots, which revealed that cytosolic Pi levels vary across root development zones but were consistent across cell and tissue types within one zone (Sahu *et al.*, 2020).

Here, we used *B. distachyon* plants expressing cpFLIPPi sensors to monitor the relative Pi levels in the cytosol and plastids of individual cells within a mycorrhizal root and responses to external Pi application.

**Materials and Methods**

**Plant material and growth conditions**

*Brachypodium distachyon* (line Bd21-3) was used in these experiments. *B. distachyon* seed sterilization and germination were described previously (Hong *et al.*, 2012). Surface sterilization of *Diversispora epigeta* spores (formerly *Glomus versiforme*) and *Gigaspora gigantea* spores followed protocols described in Liu *et al.* (2004). Two growth systems were used in this study. In the first system, *B. distachyon* plants were grown in 20-cm-long plastic cones filled with a sand-gravel mix of sterile play sand : filter sand : gravel in a 2 : 2 : 1 ratio as previously described (Floss *et al.*, 2017). Plants were inoculated with 100 *D. epigeta* spores or 50 *G. gigantea* spores placed in the substrate 7 cm below the top of the cones.

Plants were grown in a growth chamber with a 12 h : 12 h, 24°C : 22°C, light : dark cycle. Plants were maintained with deionized water and fertilized once per week with 10 ml of $\frac{1}{4}$ Hoagland’s solution containing 20 $\mu$M potassium phosphate. One day prior to imaging, the plants received 10 ml of $\frac{1}{4}$ Hoagland’s solution supplemented with 2 $\mu$M potassium phosphate. For experiments involving a high Pi treatment, the plants received 10 ml $\frac{1}{4}$ Hoagland’s solution containing 200 $\mu$M Pi at time zero, and were then imaged 0.5, 1, 2 or 18 h later. We chose 200 $\mu$M Pi based on results of a pilot experiment (data not shown).

The second growth system was adapted from Giovannetti *et al.* (1993) with modifications. A 10-d-old *B. distachyon* plant and two *Gigaspora gigantea* spores were sandwiched between two-cellulose membranes (HAWP09000; Millipore). The spores were placed on each side of the root. Membranes containing the seedlings and spores were planted vertically in the earlier-mentioned sand : gravel mix in an 11 cm diameter pot and grown in the growth chamber in the conditions described for growth system 1.

Roots were used for imaging or assays 4 wk after being transplanted to cones or pots.

**Plasmid generation**

All vectors were generated using the multi-site gateway cloning system (Invitrogen). The *BdPT7* (1171 bp upstream of *Bradi2g45520* and ZmUbi1 promoters were amplified from *Brachypodium distachyon* genomic DNA and the vector pHB7GW1-WG-UBIL (Karimi *et al.*, 2007; VIB, Ghent, Belgium) respectively, using primers flanked by attB4L and attB1R recombination sites (Table S1). To create *pDONRp4p1r* vectors containing either the *BdPT7* promoter or the ZmUbi1 promoter, PCR fragments were recombined into *pDONRp4p1r* using BP recombinase (Invitrogen). *cpFLIPPi-5.3m* (Pi Sensor), *cpFLIPPi-Null* (Control Sensor), *eCFP* and *cpVenus* coding sequences with or without a chloroplast transit peptide (*AtRbcS*), were excised from original vectors, *cpFLIPPi-5.3m*, *cpFLIPPi-Null*, *eCFP* and *cpVenus* (Mukherjee *et al.*, 2015; Banerjee *et al.*, 2016), using XbaI and HindIII restriction enzymes, respectively. To generate gateway-compatible *pENTR1-2* clones, digested DNA fragments were ligated into an XbaI-HindIII-digested *pENTR221* vector containing a multiple cloning site from *pUC19*. A *pENTR2-3* plasmid containing the *CaMV35S* terminator was cloned previously (Ivanov & Harrison, 2014). To generate binary vectors for transformation, different combinations of four vectors (*pDONRp4p1r*...
containing the BdPT7 promoter or the ZmUbi1 promoter; pENTRI-2 containing one of the four coding sequences; pENTR2-3 CaMV35S terminator; and the destination vector pHb7m34GW (Karimi et al., 2005) were assembled using LR recombination. All constructs were confirmed by Sanger sequencing.

**Generation of Brachypodium distachyon transfectants**

Constructs with cpFLIPPi-5.3m, cpFLIPPi-Null, CFP and cpVenus targeted to the cytosol or the plastid expressed from the BdPT7 or ZmUbi1 promoters, were introduced into B. distachyon (accession no. Bd21-3) via *Agrobacterium tumefaciens*-mediated transformation (Bragg et al., 2015). Hygromycin was used to select transformed calli. Primary transformants were evaluated for the presence of the appropriate fluorescent signal and genotyped to confirm the presence of the constructs. Multiple independent transgenic lines for each construct were obtained, and 3–4 lines of T2 and T3 generations with equivalent fluorescent intensity were used in the experiments (Table S2). The transgenic lines showed normal growth, and root system development was typical for our experimental conditions. Arbuscule development and infection unit appearance were wild-type in appearance as illustrated in several wheat-germ agglutinin (WGA)-stained images presented in the figures. Unfortunately, transformation failed for the ZmUbi1::cpVenus and the plastid-targeted sensors expressed from the ZmUbi1 promoter. In previous studies, the plastid location of these sensor constructs has been validated (Mukherjee et al., 2015). Here, transgenic lines with plastid-targeted sensors expressed from the BdPT7 promoter showed the expected discrete location and morphology associated with plastids (Supporting Information Fig. S1).

**Radionuclide $^{32}$Pi uptake assay**

*Brachypodium distachyon* plants grown between cellulose membranes were used in this assay. Radionuclide $^{32}$Pi in the form of orthophosphoric acid in water was used (PerkinElmer, Waltham, MA, USA). The ¼ Hoagland’s medium containing 0.01 μCi H$_3$[32]PO$_4$ and 1% agar (Sigma) was freshly made immediately before the experiment and pipetted in 10 μl droplets that were allowed to solidify. The membrane sandwich was opened and one of the membranes was removed. The mycorrhizal roots colonized with *Gigaspora gigantea* were gently rinsed to remove sand particles. Colonized root regions and their connecting fungal hyphae were screened using an Olympus SZX-12 Stereomicroscope to identify hyphae and to track their connections to roots. Infected root zones were readily identified by their density and color. Sites at which to apply the Pi-containing agar blocks were chosen and marked. To allow calculations of the distance between the root and $^{32}$Pi agar block sites, photographs with distance scales were taken and marked sites were labeled. The 10 μl $^{32}$Pi agar blocks were then placed over the fungal hyphae at the marked sites. The root system on the membrane was enclosed in a 20 cm Petri dish (leaves were outside) and sprayed with ¼ Hoagland’s solution without Pi to maintain a moist environment. The plates were incubated in dark for either 0.5, 1, 6 or 18 h.

Following incubation, roots, that were either colonized or not, within a 5 mm radius of the agar block were collected in c. 5 mm pieces for scintillation counting and their locations were marked on the photographs. Root samples were immediately dried with paper towels and transferred into scintillation vials filled with 3 ml of Ecoscint H scintillation fluid (National Diagnostics, Atlanta, GA, USA). Each vial was counted three times for 1 min per count (LSC LS6500 scintillation counter; Beckman, Brea, CA, USA) and a mean count value was used for statistical analysis.

To test the rate of $^{32}$Pi diffusion through the membrane, a 10 μl $^{32}$Pi agar block was placed onto a blank piece of membrane and incubated for 18 h. The membrane was sampled at distances of 0, 4 and 8 mm from the agar block and analyzed by scintillation counting.

For experiments involving a Benomyl pre-treatment, 1 ml of 100 μg μl$^{-1}$ 1-methyl-1-(butylcarbamoyl)-2-benzimidazolcarbamate (Benomyl; Millipore Sigma, Bedford, MA, USA) was applied to the mycorrhizal root system 1 d before the $^{32}$Pi tracing experiment.

**Pi imaging and image analysis**

The preparation of roots for Pi imaging differed slightly for the two growth systems. For growth system 1, the plants were removed from the cones and the roots were placed in a 20 cm Petri dish and rinsed gently with running deionized water to remove the sand. They were then immersed in ¼ Hoagland’s solutions with a designated Pi concentration of either 2 μM Pi or 200 μM Pi in the Petri dish. Using a stereomicroscope, a colonized root region was located and moved onto a microscope slide (75 mm × 25 mm) and covered with a coverslip (20 mm × 20 mm) to keep it static and flat during imaging. The rest of the root remained attached and was gathered around the edge of the slide and frequently sprayed with the specified Hoagland’s solution to keep it moist. The shoot remained attached.

For plants in growth system 2, the cellulose membrane sandwich was opened, and colonized root regions were located and marked using the stereomicroscope. The membrane containing the plant was then transferred onto a glass slide (75 mm × 25 mm) with the marked region of interest at the center of the slide. A coverslip (20 mm × 20 mm) was placed on top of the region of interest. During imaging, the membrane was kept wet by spraying with the specified Hoagland solution. To find the same cells before and after local Pi feeding, imaged sites were recorded. The mounting process for both growth systems took c. 5 min and it was counted as part of the incubation time when local Pi feeding was used.

Roots were imaged with a Leica TCS SP5 Confocal Microscope equipped with an Argon laser and two HyD detectors using settings described in Table S3. The same optimized confocal settings were used for all experiments (Table S3). Donor (enhanced cyan fluorescent protein (eCFP)) bleedthrough and acceptor (cpVenus) cross-excitation were measured to calculate sensitized FRET (Rincón-Zachary et al., 2010; Banerjee et al., 2016). For each experiment this requires imaging of individual plants expressing the Pi sensor, the Pi-insensitive control sensor, and the...
two single fluorophore controls (B. distachyon roots expressing eCFP and cpVenus separately). As we lacked plants expressing ZmUbi1::cpVenus, plants expressing BdPT7::cpVenus were used as a control. Any background autofluorescence, including that arising from the AM fungi (an example shown in Fig. S2) is accounted for in the sensitized FRET calculation and through the use of the Pi-insensitive control sensor (Table S4).

Image processing and analysis were as previously described (Mukherjee et al., 2015; Banerjee et al., 2016), using a customized macro in Fiji (ImageJ) (script in Methods S1). Sensitized FRET (FRET : eCFP) ratios were calculated as described in Mukherjee et al. (2015) and shown in the figures as FRET ratio (Table S4 provides the equation and an example of the calculations). The calculated sensitized FRET was displayed as pseudo-color images generated using Fiji (script in Methods S2). Z-stacks were also generated in Fiji using Z project. As reported previously, assay linearity was confirmed for each experiment (Table S4; Banerjee et al., 2016). If changes in FRET ratios were detected for both Pi and control sensors, delta FRET ratios were calculated as reported previously (Banerjee et al., 2016). A delta FRET ratio for the control sensor indicates a non-Pi-specific effect, whereas a delta FRET ratio for the Pi sensor reflects the combination of both specific and nonspecific effects, and the Pi-specific effect is the difference between the two.

Visualization of arbuscules with WGA-Alexa Fluor 488 staining

After Pi imaging, roots were immediately stained in situ with wheat-germ agglutinin-Alexa Fluor 488 (WGA-AF488; Invitrogen) to detect arbuscules. Roots were immobilized under a thin layer of 1% agar (Sigma) between the coverslip and the glass slides by pipetting 0.5 ml of molten agar into the gap (Brown & Lemmon, 1995; Blancaflor et al., 2001). The immobilized root was then excised and separated from the plant. Root segments were fixed and stained according to Hong et al. (2012) with the following modifications. Roots were fixed in 50% ethanol for 3 d and then cleared in 20% (w/v) potassium hydroxide for 5 d. Roots were stained with 0.2 µg ml−1 WGA for 2 d at 4°C. FRET images were used as references to locate arbuscules. WGA-AF488 was excited with an argon laser (488 nm) and emission was collected from 500 to 550 nm. Then, 10–20 Z-stack images (slice distance: 1 µm) were collected to visualize arbuscules.

Statistical analyses

All experiments were performed using at least six biological replicates except for the 32Pi uptake experiment, which had at least three biological replicates. All experiments were repeated twice to three times. All statistical analyses were performed using R. The normality of every data set was tested using the Shapiro–Wilk test. Pairwise comparisons were made using two-tailed Student’s t-tests for normally distributed data sets; otherwise, nonparametric Mann–Whitney U-tests were used. For multiple comparisons, if a data set was normally distributed, ANOVA followed by a Tukey’s HSD post hoc test was performed on the raw data. Otherwise, a Kruskal–Wallis test followed by a two-tailed Dunn’s post hoc test was performed.

Results

Brachypodium distachyon transgenic lines expressing Pi sensors for live imaging of Pi in the cytosol and plastids

To image Pi in the cytosol of B. distachyon mycorrhizal root cells, we generated transgenic lines expressing a second-generation Pi sensor (cpFLIPPi-5.3m), single fluorescent protein controls, and a Pi-insensitive control sensor (cpFLIPPi-Null), from either the constitutive ZmUbi1 promoter or a mycorrhiza-inducible, cell-type specific BdPT7 promoter (Table S2). BdPT7 is the ortholog of Medicago truncatula PT4 and Orzya sativa PT11 (Harrison et al., 2002; Paszkowski et al., 2002; Hong et al., 2012). Similarly, to image Pi in plastids of mycorrhizal root cells, transgenic lines expressing plastid-targeted versions of the sensors (cpFLIPPi-5.3m and cpFLIPPi-Null) (Mukherjee et al., 2015; Banerjee et al., 2016) under control of the BdPT7 promoter were generated (Table S2). Despite multiple attempts, we failed to regenerate lines constitutively expressing plastid-targeted sensors. This may be a consequence of the tissue culture-based transformation approach because constitutively expressed, plastid-targeted sensors lines have been generated in Arabidopsis (Mukherjee et al., 2015).

Cytosolic Pi levels are higher in Diversispora epigaea-colonized cells than in adjacent epidermal cells

We first wanted to ask whether cytosolic Pi levels differ between colonized cells and noncolonized cells in a mycorrhizal root. To make this comparison, B. distachyon lines constitutively expressing the Pi sensor (cpFLIPPi-5.3m) and Pi-insensitive control sensor (cpFLIPPi-Null) were inoculated with D. epigaea and then imaged 4 wk post-inoculation. Emission from direct excitation of cpVenus indicated that sensor abundance in these cell types was similar (Fig. 1a). Ratiometric imaging (FRET : eCFP), referred to as FRET ratio hereafter, was then used to assess relative Pi levels (Mukherjee et al., 2015; Banerjee et al., 2016). The B. distachyon roots that we imaged here had only one layer of cortical cells, so for this initial comparison, we compared colonized cells containing fully developed, mature arbuscules with adjacent, noncolonized epidermal cells. As shown in the representative pseudo-color images and by quantitative analysis, Pi-dependent FRET ratios were lower, indicating higher Pi levels, in colonized cells than in epidermal cells (Fig. 1a,b). In contrast, FRET ratios for the Pi-insensitive control sensor did not differ between these two cell types, indicating that differences detected with the Pi sensor can be attributed to differences in relative cytosolic Pi levels. These findings were further confirmed in a parallel experiment, which included colonized roots and also noncolonized roots (from mock-inoculated plants) (Fig. S3). In colonized roots, the Pi content of the colonized cortical cells was higher than that of the epidermal cells, while in noncolonized roots, the
Pi content of cortical and epidermal cells did not differ from each other (Fig. S3).

Hong et al. (2012) showed that B. distachyon shoot P content was higher when associated with D. epigaea than with Gigaspora gigantea. We therefore investigated the relative cytosolic Pi levels in roots colonized with these two AM fungi. As shown in Fig. 1 (c), FRET ratios from the Pi sensor were similar in epidermal cells in the two mycorrhizal root systems. In contrast, the Pi sensor showed a lower ratio in cells colonized by D. epigaea than cells colonized by Gigaspora gigantea (Fig. 1d). FRET ratios from the control sensor did not differ in either comparison. Consequently, we conclude that cytosolic Pi levels are higher in root cells colonized by D. epigaea than Gigaspora gigantea, which aligns with earlier observations of shoot P content.

Next, we sought to determine whether Pi levels differ between cells containing distinct fungal structures. We imaged cells with intracellular hyphae (IH), cells in which arbuscules filled the cell (mature arbuscules, Aₘ), and cells with arbuscules that had degenerated to clumps (collapsed arbuscules, Aₐ) (Fig. 1e). Regions of interest (ROIs) were applied to cytosolic regions associated with the hypha, arbuscule branches, or arbuscule clumps, respectively. Quantitative analyses showed that FRET ratios from the Pi sensor did not differ between these cells. This result was unexpected as we anticipated that the Aₘ cells would have the highest Pi levels. However, if differences between these cells exist, they are below the limits of detection with this Pi sensor.

Plasticid Pi levels are highest in cells at the growing front of an infection unit

Given that development and functioning of AM symbiosis requires several products of plastid metabolism (MacLean et al., 2017), we first asked whether plastids within a single colonized cell show any variation in Pi levels. Transgenic lines with plastid-localized Pi and control sensors expressed from the BdPT7 promoter were colonized with D. epigaea and then FRET ratios were quantified in plastids in 10 randomly selected cells that contained mature arbuscules. Comparisons of FRET ratios in six ROIs across three optical sections in each of 10 cells indicated that plastids within a single cell have the same relative Pi level (Fig. S4).

Next, we imaged plastids in cells across an entire infection unit. This enabled a systematic comparison of plastids in cells containing arbuscules at different developmental stages. For ease of comparison, the infection units were divided into three regions: the region proximal to the growing hyphal front (1), the middle region (2), and the region distal to the hyphal front (3) (Fig. 2a). Each region covered 3–5 longitudinally-positioned cells. The pseudo-color images (Fig. 2b) from the Pi sensor suggested that within the region 1, FRET ratios in cells at the growing hyphal front (region 1ₐ in Fig. 2a) were lower than those a few cells behind (region 1ₜ in Fig. 2a) and potentially lower than those in region 2. Quantitative analyses of roots expressing the Pi sensor revealed that FRET ratios in cells in position 1ₐ were lower than those in 1ₜ and lower than cells in region 2 (Fig. 2c). However, roots expressing the control sensor also showed differences in FRET ratios between cells in these regions indicating that Pi sensor responses cannot be attributed solely to differences in Pi levels. To address this issue, we compared delta FRET ratios for both sensors in cells at position 1_b vs 1_t and for cells at position 1_b vs cells in region 2 (Fig. 2d). For cells in 1_b vs 1_t, the delta values for the Pi sensor were larger than those of the control sensor, and the difference was statistically significant. Therefore, there is a statistically significant difference in Pi in the plastids of cells in region 1_b vs 1_t with higher Pi levels in the plastids of cells in 1_a. In contrast, differences were not significant for cell types in the middle region vs cells in 1_b (Fig. 2d).

Arbuscules in cells at the growing front of the infection unit (position 1_a) are typically in the earliest stages of development, while those in position 1_b are typically more advanced in their development. To overlay the developmental status of the arbuscules with Pi imaging, arbuscules were visualized by staining in situ with WGA-AF488 (Fig. 2b). There were differences in the arbuscules in position 1ₜ vs 1ₐ (Fig. 2b). Cells at position 1ₚ, contained arbuscules with many thick branches, i.e. developing arbuscules, while cells at position 1_b, had arbuscules that contained more thin branches, i.e. in their late stages of development (Fig. 2b). This suggests that in colonized cells, plastidic Pi levels decrease as the arbuscules develop, likely as a result of changes in their metabolic activity.

Plastid morphology varies in colonized root cells; the plastids are stromulated in cells with developing and mature arbuscules, while they are typically lens-shaped in cells in which arbuscules have collapsed (Ivanov & Harrison, 2014). We hypothesized that relative plastidic Pi levels would vary between the two shapes of plastids. In region 3, cells with lens-shaped plastids (L) can be found directly adjacent to cells with stromulated plastids (S) which allows comparisons of the effects of shape without any confounding effects of longitudinal position (Figs 2b, S5). Quantitative analysis showed no difference in plastidic Pi levels in these two cell types (Fig. 2c), indicating the relative Pi levels in plastids of different shapes remain the same, at least within the detection range of this sensor. Furthermore, the relative Pi levels of plastids in cells in region 2 did not differ from those in cells in region 3 (P = 0.34, n ≥ 29, Student’s t-test).

Pi application to the root system elicits a transient increase in cytosolic Pi in arbuscule-containing cells

To determine if colonized cells show temporal responses to a Pi increase in the external environment, plants expressing the symbiosis-specific cytosol-localized sensor (BdPT7::xFLIPPi-5.3m) and the respective control sensor (BdPT7::xFLIPPi-Null), each colonized with D. epigaea were treated with a single application of ¼ Hoagland’s solution containing 200 µM Pi. Focusing on cells that contained mature arbuscules, Pi imaging was carried out before external Pi application (0 h) and again after 0.5, 1, 2 and 18 h. FRET ratios for the Pi sensor decreased 0.5 h after Pi application but returned to the pre-treatment levels by 1 h and then remained relatively constant (Fig. 3a,b). In contrast, FRET ratios for the control sensor remained constant during this period. These results indicate a transient increase in cytosolic Pi in colonized cells. This
finding was supported by a similar experiment using plant lines expressing the constitutively expressed Pi sensor. Again, in the colonized cortical cells, FRET ratios for the Pi sensor showed a transient decrease 0.5 h post-Pi application (Fig. 3c). These data indicate a transient increase in cytosolic Pi levels of cells containing arbuscules following Pi application to the mycorrhizal root. Considering the speed of the response, we speculate that the observed changes result from Pi that was taken up by the root directly.

Using *B. distachyon* lines expressing plastid-targeted sensors, we carried out a similar experiment to monitor plastidic Pi...
responses. In contrast to the cytosolic Pi sensor, the FRET ratios of the plastid Pi sensor did not change (Fig. 3d), indicating that plastidic Pi levels are not influenced by this external Pi treatment.

Cytosolic Pi levels and cytosolic Pi response dynamics vary across cell types within a mycorrhizal root

Having observed temporal changes in cytosolic Pi accumulation in cells containing mature arbuscuses, we asked how other cells within the mycorrhizal root respond to a high external Pi treatment. Using colonized B. distachyon plants with constitutively expressed sensors, five cell types were imaged before and after Pi application: epidermal cells (E), noncolonized cortical cells (N), cells containing IH, cells containing AM and cells containing AC (Fig. S6). Visualization of responses in individual cell types over time and comparisons of relative Pi levels across cell types are displayed through a heat map (Fig. 4a) as well as graphs that provide details of FRET ratios in cell types and over time (Figs 4b, S7; Table S5). The data are consistent with earlier experiments in that the three colonized cell types did not differ in relative cytosolic Pi levels prior to treatment and, as observed previously, AM cells showed a transient decrease in FRET ratios, consistent with an increase in cytosolic Pi, at 0.5 h. Surprisingly, FRET ratios in AC cells increased at 0.5 h post-Pi treatment (Figs 4, S7), indicating that relative cytosolic Pi levels decrease transiently in these cells in response to external Pi. The IH cell type did not show a significant response to Pi treatment.

Noncolonized cortical cells have a lower relative cytosolic Pi level than colonized cells and also respond to Pi treatment differently with no significant change at 0.5 h, but Pi levels increase at 1 h post-Pi treatment (Figs 4, S7). Despite this increase, relative Pi levels in this cell type remain much lower than those in AM cells. Prior to Pi treatment, epidermal cells have higher cytosolic Pi levels than noncolonized cortical cells, but not as high as colonized cortical cells. The epidermal cell response to Pi treatment is similar in trajectory to that of the AC cells in that they show a substantial transient decrease in Pi level 0.5 h post-Pi treatment followed by a slight increase 1 h post-treatment. At 0.5 h post-Pi treatment the epidermal cells showed the lowest Pi level while AM cells showed the highest Pi level.

In summary, these data indicate that cells of a mycorrhizal root differ in their relative cytosolic Pi levels and also in the dynamics of their responses to Pi treatment (Figs 4, S7).

Pi levels in colonized cortical cells increase 18 h after Pi application to Gigaspora gigantea extraradical hyphae

Pi sensors provide the means to monitor Pi delivery to the cortical cells via the fungal symbiont; however, such experiments require an experimental setup in which Pi can be applied specifically to the extraradical hyphae (ERH) rather than to the entire mycorrhizal root system. To achieve localized feeding of Pi directly to the ERH, we adapted a membrane sandwich system from Giovannetti et al. (1993) and Novero et al. (2002) and then evaluated the time frame for symbiotic Pi transfer using radioisotope tracing. Briefly, B. distachyon roots and Gigaspora gigantea spores were sandwiched between two pieces of cellulose membranes, which were then placed in pots to allow the association to develop (Fig. 5a–d). Membrane sandwiches were then removed from the pots, opened, and small blocks of agar impregnated with $^{32}$Pi were placed on the ERH. Radioactivity in both colonized and noncolonized regions of the root, within a 5 mm radius of the source, was monitored at 0.5, 1, 6 and 18 h post-application. Statistically significant increases in radioactivity in the colonized root regions were detected at 18 h (Fig. 5e). Measurements from the noncolonized regions indicate that $^{32}$Pi diffusion across the membrane surface was low (Table S6), therefore supporting the conclusion that colonized regions received $^{32}$Pi via the fungus. To further confirm that the Pi was transferred by the AM fungus, a Benomyl pretreatment was applied to kill the fungus. At 18 h, colonized root regions (C) had twice the radioactivity compared to the Benomyl-pretreated colonized regions (BC) and also to the noncolonized (NC) and Benomyl-pretreated noncolonized (BNC) regions (Fig. 5f). Together, these results support active transfer of Pi to the roots via the ERH and indicate that statistically significant increases are detectable 18 h following application.

These $^{32}$Pi tracing experiments provide a time frame for transfer through the hyphae to the root system but lack the cellular resolution needed to determine whether Pi enters the root cytosol. To address this, we repeated the experiment using plants expressing the sensors driven from the BdPT7 promoter (Fig. 5d) and omitting the radiotracer. FRET ratios were measured in individual cells containing mature arbuscules before Pi application and then in the same cells 18 h after local Pi feeding to the ERH. Quantitative analyses showed changes in FRET ratios for the Pi sensor and a smaller change from the control sensor (Fig. 6). Delta FRET ratios indicate an increase in cytosolic Pi levels in
the colonized cells 18 h post-Pi treatment suggesting that Pi is transferred from the arbuscule and can be detected in the root cell within this time frame. In summary, using both radiotracing and Pi biosensors provides a means to monitor Pi transfer through the ERH and delivery to the root cell cytosol.

Discussion

Development of endosymbiosis with AM fungi results in substantial changes in the cellular structure and metabolism of the colonized cortical cells as well as a new pathway for Pi entry to the root. While much research has focused on Pi transporters and their roles in symbiotic Pi transfer, we have little knowledge of how the association affects cellular Pi content. Here, spatiotemporal comparisons of intracellular Pi across cells of a mycorrhizal root showed that steady-state cytosolic Pi levels differ between cell types. Furthermore, cytosolic Pi content varies in response to Pi application to the root and individual cell types differ in the trajectory and dynamics of this response.

Using *B. distachyon*, which was chosen because its roots are thin enough to allow direct imaging of the cortical cell layer, a series of experiments revealed that the cytosolic Pi content of cells with mature arbuscules (A_M) cells) is higher than that of noncolonized cortical cells and also higher than epidermal cells. Colonized cells are unique in that they receive Pi via the arbuscule, with estimated flux rates of $1.3 \times 10^{-12}$ mol mm$^{-2}$ s$^{-1}$ (Cox & Tinker, 1976), which may explain their higher cytosolic Pi levels. Somewhat surprisingly, cells containing intracellular hyphae (referred to IH cells) showed relative cytosolic Pi levels similar to that of colonized cells. It is generally assumed that in the intracellular hyphae do not contribute Pi transfer to the plant. However, this assumption is based on the location of the plant mycorrhiza-induced Pi transporters on the peribasal membrane (Harrison et al., 2002; Kobae & Hata, 2010). The possibility that Pi transfer occurs across other fungal–plant interfaces, with uptake mediated by constitutively expressed Pi transporters, has not been investigated. Alternatively, intracellular hyphal continuity between IH cells and adjacent arbuscule-containing cells within the same cell file might influence Pi distribution between these cells.

Following Pi application to the root system, colonized and noncolonized cells differed in the dynamics and trajectory of their cytosolic Pi response. This suggests that these cell types differ in some, or all, of the typical factors that influence cytosolic Pi levels, including Pi transport, vacuolar sequestration, cellular structure and metabolic transformations (Plaxton & Tran, 2011; Versaw & Garcia, 2017; Dissanayaka et al., 2021). That A_M cells and noncolonized cells differ in the latter two factors is well established. For example, in A_M cells the vacuole volume decreases as the arbuscule occupies space within the cell but the tonoplast surface area increases (Cox & Tinker, 1976; Bonfante-Fasolo, 1984). These alterations could influence Pi transport across the tonoplast as well as the potential Pi buffering capacity of the vacuole, which serves as the main Pi store in plant cells. In Arabidopsis root cells, vacuole Pi loading was largely independent of vacuole size, but vacuolar Pi transport influenced cytosolic Pi levels differentially across the transition, elongation and mature zones (Sahu et al., 2020). Currently, we lack information about Pi transport activities on the tonoplast of A_M cells, but it is possible that this parameter differs between A_M and noncolonized cortical and epidermal cells. Cellular metabolism, including fatty acid biosynthesis and membrane production, in A_M cells differs from that of noncolonized cells and may also contribute to differing cytosolic Pi responses (Bravo et al., 2017; Keymer et al., 2017).

The contrasting Pi response dynamics in the A_M cells vs epidermal cells could also arise from differences in Pi transport and Pi content. It is well documented that mycorrhizal roots downregulate Pi uptake transporters in the root epidermis which may partly explain this low cytosolic Pi content relative to that of A_M cells (Paszkowski et al., 2002; Liu et al., 2008, 2016) (Fig. 4). These epidermal cells may be Pi-starved relative to A_M cells, a physiological difference that could also influence Pi response dynamics. The decrease in cytosolic Pi levels in the epidermal cells at 30 min post-Pi application may reflect Pi transfer to the vacuole and/or incorporation into adenosine triphosphate (ATP) and other organic compounds (Arisz et al., 2009; Dissanayaka et al., 2021), both of which are known to occur within minutes of Pi uptake into plant cells (Sahu et al., 2020). Pi transfer to the vacuole occurs even more rapidly in Pi-deprived cells (F. Liu et al., 2016; T-Y. Liu et al., 2022).
so the 30 min time point in our experiments was likely too slow to detect any initial increase in cytosolic Pi in the epidermal cells. Our measurements reflect steady-state levels as the cells reset their Pi homeostasis and, as noted earlier, differences in cell structure and metabolism influence the steady-state cytosolic Pi levels.

Also notable, cells with collapsed arbuscules (referred to as AC cells) showed a cytosolic Pi response similar in trajectory to the...
noncolonized cells with a transient decrease in cytosolic Pi and a subsequent increase at 1 h. Perhaps this reflects the cellular transition of AC cells back to their noncolonized state, and the reestablishment of the large central vacuole which occurs once the arbuscule has collapsed (Bonfante-Fasolo, 1984).

Pi efflux from cells may also influence cytosolic Pi content. In Arabidopsis, tracer experiments reveal that Pi distribution through the root and even to the shoot, can be detected within 30 min of Pi application (Kanno et al., 2016) suggesting Pi uptake and efflux activities operating within this time frame.
Currently, we have little knowledge of Pi efflux systems in cells in a mycorrhizal root. While mechanistic explanations require further experimentation, our measurements reveal considerable differences in relative cytosolic Pi content and response dynamics across mycorrhizal root cell types.

At the subcellular level, the relative Pi content of the plastids was uniform across cells and in response to changes in external Pi. However, we could detect a short gradient in the plastidic Pi levels in cells at the active growing front of an infection unit, with a higher Pi level in cells with younger (less branched) arbuscules.
Higher plastidic Pi in these cells could be a result of very active plastid metabolism, including fatty acid biosynthesis to support peri-arbuscular membrane development and to supply lipids to the arbuscule (Bravo et al., 2017; MacLean et al., 2017). A large amount of Pi is produced as a byproduct of ATP hydrolysis during the synthesis of a key precursor Malonyl-CoA (Buchanan et al., 2016). In the majority of our experiments, the control sensor did not fluctuate between treatments or cell types but in a few cases the control sensor registered a significant FRET change, as seen for the epidermal cells following Pi treatment (Fig. 4) indicating a background effect on the sensors. As the FRET change for the Pi sensor is larger than that of the control sensor, we infer that there is a Pi-dependent component to the FRET response which can be revealed by calculating the difference between the FRET measured by the two sensors. This approach assumes that the unknown background effects on the two sensors are the same, which seems likely given the minor change in protein sequence and the examples of known perturbations pH and ionic strength (Banerjee et al., 2016). Nevertheless, this particular point cannot be proven. Analyse-insensitive control sensors are available only for a few FRET-based sensors but where available, they add considerable experimental value. Display of the control data provides an instant view of background effects for any cell type or treatment comparison and increase confidence in measurements from the sensor (Imamura et al., 2009; Banerjee et al., 2016).

In order to image cells during Pi delivery via the AM fungus, we modified a membrane sandwich growth system (Giovannetti et al., 1993) which constrains mycorrhizal root growth to two dimensions and provides access to the root and hyphae. Using this system, Pi application can be restricted to the ERH and

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**Fig. 5** Tracing $^{32}$Pi to Brachypodium distachyon roots by Gigaspora gigantea. (a, b) Schematic diagrams of the experimental system used for local application of $^{32}$Pi to Gigaspora gigantea extraradical hyphae. (a) Depicts a B. distachyon plant and two Gigaspora gigantea spores (yellow dots) between two cellulose membranes. (b) an agar block (blue circle) containing $^{32}$Pi was placed over the extraradical hyphae (yellow lines). (C) An example of Pi-containing agar placement (blue circle) over the extraradical hyphae and the root regions collected for radioactivity counting (white boxes). Bar, 5 mm. (d) A representative stereo microscope image showing fluorescence from the cytosolic Pi sensor and the positions at which the extraradical hyphae attach to the root (arrowheads). (e) Counts per minute (cpm) from noncolonized root regions (NC, white bars) and colonized root regions (C, yellow bars) at 0.5, 1, 6, and 18 h after $^{32}$Pi application. Pairwise Mann–Whitney tests were performed separately for each time point, and significant differences are indicated (***, $P < 0.001$). (f) Counts per minute (cpm) root regions collected from Benomyl pre-treated mycorrhizal root systems 6 and 18 h post-$^{32}$Pi application. NC, noncolonized root regions (white bars); BNC, Benomyl-treated noncolonized root region (pink bars); C, colonized root region (yellow bars); BC, Benomyl-treated colonized root region (green bars). Different letters and letters with primes indicate significant differences as determined using Kruskal–Wallis test followed by Dunn’s *post hoc* test ($P < 0.05$). The box plots in (e, f) show the lower and upper quartiles, and the minimum and maximum values. The bar in the box represents the median. Each gray point represents the average cpm from one root segment. Measurements at each timepoint and root type combination were collected from more than three root segments.
Measurements were made on more than 46 cells. Each bar in the box represents the median. Each gray point in the boxplots indicates the lower and upper quartiles, and the minimum and maximum values. The associations, 32Pi applied to the ERH was significantly greater than the control sensor (Student’s t-test; **, P < 0.01). The box plots in (b, c) show the lower and upper quartiles, and the minimum and maximum values. The bar in the box represents the median. Each gray point in the boxplots represents a measurement from one region of interest (ROI) per cell. Measurements were made on more than 46 cells.

Followed by radiotracing and Pi imaging in the colonized cell. The radiotracer experiments indicated significant Pi transfer through the ERH to the root 18 h post-Pi application. Furthermore, the relative Pi content of colonized cells was higher 18 h post-Pi application relative to the pre-Pi application measurement of the same cells. This time frame is similar to previous radiotracer experiments. For example, in vitro carrot root-Glomus intraradices associations, 32Pi applied to the ERH was detected under mycorrhizal roots after 14 h (Nielsen et al., 2002), although these experiments do not resolve the cellular location of the Pi. In this same growth system, Glomus intraradices Pi transporter transcripts were constitutively present but increased 25-fold within 6 h of Pi application (Maldonado-Mendoza et al., 2001) indicating the potential time frame for increases in transport capacity. Polyphosphate accumulation in the ERH of Arbuscular Mycorrhiza was detected just 1 h after P addition to the medium, suggesting that polyphosphate synthesis initiates very quickly (Ezawa et al., 2004). Using 31P-NMR, polyphosphate signals were detected in the ERH of Glomus intraradices 5 h after the addition of P (Viereck et al., 2004), while polyphosphate was estimated to move through the ERH of Glomus sp. HR1 at a rate of 2.0 mm h⁻¹ (Hijikata et al., 2010). While there are differences in the experimental systems as well as the plant–fungus combinations, they are consistent in suggesting that it takes several hours for P movement through the hyphae to the root. Transfer will be influenced by factors in both symbionts, so variation between systems is not surprising (Smith et al., 2004, 2011; Hong et al., 2012). In summary, this experimental setup coupled with plants expressing the Pi biosensor, offers an approach for future exploration of cellular Pi dynamics during symbiosis. Future improvements, including calibration of the Pi sensor in B. distachyon, a challenge that has been achieved in Arabidopsis (Sahu et al., 2020), would enable absolute measurements of Pi content, and reveal the magnitude of these cellular Pi responses.

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Author contributions

SZ, WKV and MJH designed research; SZ performed research; SZ and MJH analyzed data: DAD, SI, LMM, contributed materials and preliminary imaging method development; LJ, SI and SZ contributed to the IMAGEJ FRET analyzer; SZ and MJH wrote the article; WKV edited the manuscript. DAD, LMM, LJ and SI made equal contributions and are considered equal second co-authors with names listed in alphabetical order.

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Data availability

Data available on request from the authors.
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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Overlay image of a root expressing a plastid-targeted sensor expressed from the BdPT7 promoter.

Fig. S2 Representative CFP, FRET and Brightfield images showing autofluorescence from Diversispora epigaea and Gigaspora gigantea.

Fig. S3 FRET ratios from roots expressing either the control sensor or the Pi sensor in colonized and noncolonized roots.

Fig. S4 Plastsids within a single Diversispora epigaea-colonized cell show consistent FRET ratios.

Fig. S5 A higher magnification view of Fig. 2(b), zone 3 showing morphological differences between stromulated (S) and lens-shaped (L) plastsids.

Fig. S6 An illustration of cells containing distinct arbuscle stages chosen for analyses in Fig. 4.

Fig. S7 Temporal changes in cytosolic FRET ratios in five cell types following Pi application to Brachypodium distachyon/Diversispora epigaea mycorrhizal roots.

Methods S1 IMAGEJ scripts for sensitized FRET analysis.

Methods S2 IMAGEJ scripts for pseudocolor FRET image generation.

Table S1 Primers used in this study.

Table S2 Transgenic lines.

Table S3 Leica TCS SP5 confocal microscope configurations for Pi imaging.

Table S4 (a, b) A set of sample data (from Fig. 1a) to demonstrate image analysis procedure.

Table S5 Significance levels for each time/cell type combination shown in Fig. 4.

Table S6 32Pi diffusion on a wet cellulose membrane.

Table S7 Biochemical characteristics of cpFLIPPi sensors.

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