Live Imaging of Inorganic Phosphate in Plants with Cellular and Subcellular Resolution

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Despite variable and often scarce supplies of inorganic phosphate (Pi) from soils, plants must distribute appropriate amounts of Pi to each cell and subcellular compartment to sustain essential metabolic activities. The ability to monitor Pi dynamics with subcellular resolution in live plants is, therefore, critical for understanding how this essential nutrient is acquired, mobilized, recycled, and stored. Fluorescence indicator protein for inorganic phosphate (FLIPPi) sensors are genetically encoded fluorescence resonance energy transfer-based sensors that have been used to monitor Pi dynamics in cultured animal cells. Here, we present a series of Pi sensors optimized for use in plants. Substitution of the enhanced yellow fluorescent protein component of a FLIPPi sensor with a circularly permuted version of Venus enhanced sensor dynamic range nearly 2.5-fold. The resulting circularly permuted FLIPPi sensor was subjected to a high-efficiency mutagenesis strategy that relied on statistical coupling analysis to identify regions of the protein likely to influence Pi affinity. A series of affinity mutants was selected with dissociation constant values of 0.08 to 11 μM, which span the range for most plant cell compartments. The sensors were expressed in Arabidopsis (Arabidopsis thaliana), and ratiometric imaging was used to monitor cytosolic Pi dynamics in root cells in response to Pi deprivation and resupply. Moreover, plastid-targeted versions of the sensors expressed in the wild type and a mutant lacking the PHOSPHATE TRANSPORT4;2 plastidic Pi transporter confirmed a physiological role for this transporter in Pi export from root plastids. These circularly permuted FLIPPi sensors, therefore, enable detailed analysis of Pi dynamics with subcellular resolution in live plants.

Phosphorus is an essential element that plants acquire and assimilate in the form of inorganic phosphate (Pi). This macronutrient is a component of numerous metabolites and macromolecules, including ATP, nucleic acids, and phospholipids, and serves key roles in energy transfer reactions, signal transduction processes, and regulation of enzyme activities. Of fundamental importance to plants, Pi also serves critical roles in photosynthesis as both a substrate for ATP synthesis through photophosphorylation and a regulator in the partitioning of fixed carbon between the starch and Suc biosynthetic pathways.

In many soils, particularly those used for low-input agriculture, the amounts of Pi available to plants are limiting for growth and productivity (Vance et al., 2003). Most of the Pi in soils is unavailable, because it is immobilized through formation of insoluble complexes or exists in organic forms, such as phytate, that plants cannot use directly (Schachtman et al., 1998). As a result, concentrations of free Pi in soil solution range from 1 to 10 μM, whereas cells require Pi in the millimolar range (Bieleski, 1973).

1 This work was supported by the National Science Foundation (grant no. IOS-0956486 to W.K.V.), the Welch Foundation (grant no. A-1742 to S.W.L.), and the Howard Hughes Medical Institute (to L.R.G.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.114.254003

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changes in conformation as changes in fluorescence intensity, whereas sensors with two fluorescent proteins can yield changes in fluorescence resonance energy transfer (FRET), which can be quantified through ratiometric imaging. FRET-based sensors have been used in live plants to assess a variety of analytes, including Glc, maltose, Suc, Gln, calcium, zinc, and pH (Deuschle et al., 2006; Chaudhuri et al., 2008, 2011; Kaper et al., 2008; Rincón-Zachary et al., 2010; Adams et al., 2012; Gjetting et al., 2012, 2013; Krebs et al., 2012).

Gu et al. (2006) engineered a FRET-based Pi sensor named fluorescence indicator protein for inorganic phosphate (FLIPPi) that consists of a cyanobacterial inorganic phosphate binding protein (PiBP) fused to enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP) and showed the use of one of these sensors for monitoring cytosolic Pi in cultured animal cells. In this study, we generated a series of second generation FLIPPi sensors that were modified and optimized for use in live plants. Substitution of eYFP with a circularly permuted (cp) form of the fluorescent protein Venus (cpVenus; Nagai et al., 2002, 2004) greatly increased the magnitude of Pi-dependent FRET responses. In keeping with the initial nomenclature, Pi sensors constructed with cpVenus were designated cpFLIPPi. We also used a targeted mutagenesis approach to obtain cpFLIPPi sensors with Pi binding affinities that spanned the physiological range of most cell compartments and expressed these in Arabidopsis (Arabidopsis thaliana). Confocal microscopy coupled with ratiometric analysis or acceptor photobleaching detected changes in cytosolic Pi levels in root epidermal cells in response to Pi starvation, and these changes were fully reversed by Pi replenishment. Plastid-localized versions of the same sensors expressed in wild-type plants and mutants lacking the PHOSPHATE TRANSPORTER4;2 (PHT4;2) plastidic Pi transporter (Irigoyen et al., 2011) were used to confirm a role for this transporter in the export of Pi from root plastids. These results show the use of cpFLIPPi sensors for monitoring Pi distributions with both cellular and subcellular resolutions in live plants.

RESULTS

Efficacy of FLIPPi Sensors in Plants

In an initial attempt to monitor Pi distributions in live Arabidopsis plants, we generated stable transgenic lines for two of the previously described Pi sensors: FLIPPi-200μ and FLIPPi-30m (Gu et al., 2006). When assayed in vitro, these sensors yielded robust Pi-dependent FRET responses with dissociation constant ($K_d$) values for Pi binding of 200 μM and 30 μM, respectively. The FLIPPi-30m sensor was also used to report changes in cytosolic Pi concentrations in cultured animal cells (Gu et al., 2006). It should be noted that these sensors exhibit a Pi-dependent decrease in FRET (Fig. 1A).

Constitutive expression of the sensor genes was directed from the 35S promoter, and because no organellar targeting sequences were incorporated in the constructs, the proteins were restricted to the cytosol. Independent transgenic lines for both sensors exhibited weak or highly variable fluorescence that diminished rapidly with plant age, suggestive of transgene silencing (Deuschle et al., 2006). Although signal intensity and stability improved when the sensors were expressed in a small interfering RNA (siRNA)-defective mutant host, suppressor of gene silencing3-13 (sgs3-13; Kumakura et al., 2009), no changes in FRET were detected when plants were starved for Pi.

We hypothesized that one or more attributes of the FLIPPi sensors limited their efficacy when used in plants. For example, if the magnitudes of Pi-dependent responses were small, then subtle changes in cytosolic Pi concentrations would not be detected. Similarly, no change in FRET would be expected if binding affinities for Pi were too far above or below the cytosolic concentrations. To explore these possibilities, we first confirmed that purified FLIPPi-200μ and FLIPPi-30m sensor proteins yielded Pi-dependent FRET responses equivalent to those reported previously (Gu et al., 2006). We then examined FRET responses for the purified proteins in a pseudocytosol medium (Messerli and Robinson, 1998; Feijó et al., 1999; Cárdenas et al., 2008) to simulate in vivo conditions more closely than the buffer solution used previously (Gu et al., 2006). In this case, the FLIPPi-30m sensor yielded erratic FRET signals regardless of

![Figure 1. Pi-dependent changes in FRET ratios of purified Pi sensors. A, Schematic of Pi sensor indicating high FRET in the absence of Pi and reduced FRET when bound to Pi. Acceptor is eYFP for FLIPPi sensors and cpVenus for cpFLIPPi sensors. B, Values shown are means ± se for three independent protein preparations. Error bars smaller than the symbols are not shown.](Image 338x131 to 523x392)
Pi concentration, which may explain the absence of Pi-dependent FRET responses in the corresponding transgenic plants. In contrast, FLIPPi-200μ exhibited the expected Pi-dependent reduction in FRET, and the 190 μM ₀ was nearly identical to that measured in buffer (Fig. 1B). However, the dynamic range (i.e. the maximum change in FRET ratio \(ΔR_{\text{max}}\)) was relatively small (Fig. 1B), and the change was very small over the 2.5- to 10-fold concentration range that encompasses most estimates for cytosolic Pi in plants grown in Pi-replete conditions. (Rebeille et al., 1984; Stitt et al., 1988; Lee and Ratcliffe, 1993; Copeland and Zammit, 1994; Gout et al., 2011).

Based on these results, we chose to optimize FLIPPi-200μ.

**Expanded Dynamic Range of a Pi Sensor with a cpVenus**

Substitution of the YFP acceptor component of a FRET-based sensor for Ca\(^{2+}\) with a cpVenus improved dynamic range and stability and reduced sensitivity to acidic pH (Nagai et al., 2004). This modified sensor has been effective for monitoring Ca\(^{2+}\) dynamics in plants (Rincón-Zachary et al., 2010; Krebs et al., 2012). To test whether this modification would also increase the dynamic range of a Pi sensor, we replaced the eYFP portion of FLIPPi-200μ with cp173Venus, the same cpVenus variant used to improve the YC3.60 Ca\(^{2+}\) sensor (Nagai et al., 2004). As shown in Figure 1, the modified Pi sensor, cpFLIPPi-200μ, exhibited greater FRET ratios in the absence of Pi and at all concentrations tested. More importantly, the dynamic range of the modified sensor (\(ΔR_{\text{max}} = -1.08\)) was nearly 2.5-fold greater than that of the original sensor (\(ΔR_{\text{max}} = -0.44\)). The emission spectra for cpFLIPPi-200μ (with eCFP excitation) indicated that the Pi-dependent response of this sensor was caused by a change in FRET (increase in eCFP emission coupled to a decrease in cpVenus emission) rather than unrelated changes in the fluorescence intensities of either eCFP or cpVenus alone (Supplemental Fig. 1).

**Mutant cpFLIPPi Sensor Variants with Altered Affinities for Pi**

Although the use of cpVenus increased sensor dynamic range, it had no significant effect on affinity for Pi. Consequently, the magnitude of the response change over the target concentration range of 2.5 to 10 mM remained small for cpFLIPPi-200μ (Fig. 1). This situation necessitated a mutant screen to obtain cpFLIPPi variants with altered binding affinities.

To limit the scope of mutagenesis, we used statistical coupling analysis (SCA), a sequence-based method for identifying protein residues or regions responsible for ligand binding affinity and potential allosteric sites (Lockless and Ranganathan, 1999; Socolich et al., 2005). SCA was conducted with an alignment of the *Synechococcus* spp. PIBP sequence with 1,020 of the most closely related protein sequences (PSI-BLAST e score < 1e−10) available from GenBank. We found that the region spanning amino acids 18 to 23 contained multiple coupled sites that mapped near the predicted Pi binding site (Supplemental Figs. 2 and 3). We predicted that changes in this region would affect the Pi binding affinity of the sensor protein.

We used PCR to introduce random mutations in the corresponding region of the cpFLIPPi-200μ plasmid clone. The mutant library was transformed into *Escherichia coli*, and lysates of individual clones were screened for Pi-dependent FRET. The mutagenesis strategy proved to be remarkably effective. Of nearly 600 mutants screened, less than 1% resembled the parent sensor, and 80% of the mutants exhibited Pi-dependent FRET with altered affinity. The remaining 20% of the mutant population were either unresponsive to Pi or had ratemetric signals that could not be accurately fit to a binding isotherm. Eight candidate sensor mutants were selected, and their Pi-dependent FRET responses were verified using the purified proteins (Table I). The ₀ values ranged from 0.08 to 11 mM, which span the predicted physiological range for the cytosol and other cell compartments (Rebeille et al., 1984; Stitt et al., 1988; Sharkey and Vanderveer, 1989; Lee and Ratcliffe, 1993; Copeland and Zammit, 1994; Gout et al., 2011; Tiessen et al., 2012).

**Specificity and pH Sensitivity of cpFLIPPi Sensors**

To determine whether cpFLIPPi mutations that altered affinity for Pi also affected specificity, we examined the effect of other oxygenations, including organophosphates, on FRET responses. None of the sensors showed responses indicative of binding to sulfate or nitrate, and results for one sensor, cpFLIPPi-6.4m, are shown in Supplemental Figure S4A. Similarly, treatment with glycerol-2-P and pyrophosphate, a phosphate ester and anhydride, respectively, yielded little or no FRET responses (Supplemental Fig. S4B). These results suggest that the cpFLIPPi sensors have retained high specificity for Pi.

Our in vitro calibration assays and related tests for specificity were conducted at pH 7.5 to simulate the cytosol (Fratt et al., 2009; Gout et al., 2011). Although the cytosol is tightly buffered at this pH, the chloroplast stroma can vary from pH 7 to pH 8 in response to light (Dennig and Gimmler, 1983). Because one of our goals was to monitor plastidic Pi concentrations, we examined Pi-dependent FRET responses over this pH range for each of the cpFLIPPi sensors. We observed a consistent trend, in which affinity decreased with increasing pH, and results for one representative sensor, cpFLIPPi-6.4m, are shown in Figure 2. The apparent differences in affinity over this pH range can be largely attributed to the effect of pH on ionization of Pi with specificity for the monobasic form. Unfortunately, these sensors had very low dynamic ranges when assayed at pH 5.5, which would limit their use in acidic compartments, like the vacuole and apoplast.

**Subcellular Targeting of cpFLIPPi Sensors in Arabidopsis**

To test the fidelity of targeting cpFLIPPi sensors to different compartments of plant cells, we constructed
expression clones for each of eight different cpFLIPPi sensors to yield proteins with and without an N-terminal ribulose bisphosphate carboxylase small subunit (RbcS) chloroplast transit peptide (Lee et al., 2006). To avoid potential complications associated with the 35S promoter, the Arabidopsis UBQUITIN10 (UBQ10) promoter was used to drive constitutive expression at moderate levels (Norris et al., 1993; Geldner et al., 2009; Grefen et al., 2010). Constructs were introduced into the siRNA mutant sgs3-13 (Kumakura et al., 2009) to minimize potential loss of fluorescent signals caused by post-transcriptional gene silencing (Deuschle et al., 2006). Protoplasts were prepared from leaves of 5-week-old plants, and fluorescent signals were viewed with confocal microscopy. Colocalization with chlorophyll autofluorescence shown in Figure 3 confirmed that addition of the RbcS chloroplast transit peptide was sufficient to target sensors to plastids, whereas unmodified sensors were excluded from plastids and appeared to be restricted to the cytosol. FRET emission was detected with the same localization patterns in palisade mesophyll cells of intact leaves (Supplemental Fig. S5), suggesting that live Pi imaging could be conducted in aerial tissues.

Live Imaging of Cytosolic Pi in Root Epidermal Cells

Live-cell imaging using a spinning disc confocal microscope equipped with a beam splitter for simultaneous dual-emission fluorescence was used to test the functionality of cpFLIPPi sensors for reporting changes in cytosolic Pi concentrations. Untransformed plants and transgenic plants expressing eCFP and cpVenus separately were imaged each day to correct for fluorescence background, spectral bleed through, and cross excitation. T2 generation transgenic seedlings were grown for 5 d in hydroponic medium containing 0.5 mM Pi and then placed in an imaging chamber containing the same medium.

Images for multiple epidermal cells within the root differentiation zone were captured from at least six independent seedlings of each genotype to establish the steady-state baseline for the FRET ratio under this Pi-sufficient growth condition. The same seedlings were then transferred to medium that lacked Pi and imaged again after 24 h of Pi starvation. This duration of Pi starvation was chosen to avoid morphological changes or cell death associated with more severe Pi deprivation (Sánchez-Calderón et al., 2005). Specifically, cell viability tests with SYTOX orange (Truernit and Haseloff, 2008) consistently revealed a substantial number of nonviable cells when plants were Pi starved for 72 h (Supplemental Fig. S6), but none or very few were detected when plants were held in Pi-replete conditions or starved for 24 h. Sensitivity to prolonged Pi deprivation is undoubtedly a function of growth conditions and the age of the plant when deprivation is initiated.

Separate experiments showed that our Pi starvation regime was sufficient to reduce the spatially undefined Pi contents of root extracts by 19% (from 5.3 ± 0.2 nmol Table 1. Pi sensor affinity mutants

The cpFLIPPi-200μ sensor plasmid was subjected to a PCR-based mutagenesis. The sequences shown correspond to the mutated region of the sensor. Pi binding constants were determined in vitro using three independent protein preparations for each sensor. ΔR_{max} indicates the maximum change in FRET ratio between Pi-free and -saturated sensors.

| Sensor         | K_d (mM) | ΔR_{max} | DNA Sequence of PiBP cdS | Protein Sequence       |
|----------------|----------|----------|--------------------------|------------------------|
| cpFLIPPi-80μ   | 0.08     | −1.06    | 49 TCCGtcGtcGGGccGCACcGCG 17 SVAGSTTA |
| cpFLIPPi-200μ  | 0.2      | −1.08    | 49 TCCGCGGGCCGGCCGCACTTTCG 17 SGACGATFA |
| cpFLIPPi-1.6m  | 1.6      | −0.91    | 49 TCCGgcGtcGGGccGCACcGCG 17 SADGSTIA |
| cpFLIPPi-3.5m  | 3.5      | −0.43    | 49 TCCGCGGCGGCGCGGAAcGtcGTCGG 17 SGACGSKIA |
| cpFLIPPi-4.8m  | 4.8      | −0.76    | 49 TCCGCGGCGGCGGCAcCAGcGCG 17 STDGATTAA |
| cpFLIPPi-5.3m  | 5.3      | −0.71    | 49 TCCGcGtcGtcGGGccGCACcGCG 17 SSDGSSVA |
| cpFLIPPi-6.4m  | 6.4      | −0.75    | 49 TCCGCGGCGGCGGCGCGGCAcCAGcGCG 17 SGACGAAFA |
| cpFLIPPi-11m   | 11.0     | −0.45    | 49 TCCAtctCAGGCGGACGcGCAcGCG 17 SISGSSIA |

Figure 2. Effect of pH on Pi-dependent FRET responses. Pi binding of cpFLIPPi-6.4m was tested in pseudocytosol medium adjusted to pH 7.0, 7.5, and 8.0, and Pi was adjusted to the same pH. Binding affinity (K_d) values were estimated from nonlinear regression analysis. Plotted values are means ± se for three independent protein preparations. Error bars smaller than the symbols are not shown.
of Pi per fed seedling to 4.3 ± 0.2 nmol of Pi per starved seedling; mean ± se; n = 3) and also, sufficient to elicit a 20% increase in secreted acid phosphatase activity (from 60 ± 4 milliunits per fed seedling to 72 ± 5 milliunits per starved seedling; mean ± se; n = 12), which is indicative of Pi starvation (Robinson et al., 2012). Both of these changes were statistically significant as determined by Student’s t test (P < 0.05). Moreover, secreted acid phosphatase activities were identical in wild-type and sgs3-13 plants, indicating that this mutation has no obvious effect on normal responses to Pi starvation. Consequently, sgs3-13 was used as the wild-type background for all subsequent experiments.

We screened transgenic lines for cpFLIPPi-80m, -200m, -1.6m, -4.8m, -5.3m, and -6.4m under Pi-replete and 24-h Pi starvation conditions and found that only those with Kd values of 4.8 mM and greater showed a significant change in FRET ratio, and in each of these cases, the ratio increased in response to Pi starvation, consistent with decreased cytosolic Pi concentrations. Transgenic lines expressing the cpFLIPPi-6.4m sensor showed the largest response, and representative FRET ratio images and quantitation derived from six independent seedlings are shown in Figure 4. We attributed this response to altered Pi concentrations rather than unrelated changes in the emission of one or both fluorophores, because no differences in FRET ratios were detected in seedlings that express a high-affinity sensor (i.e. cpFLIPPi-80m; Supplemental Fig. S7A).

Comparisons of fluorescence intensities in cpFLIPPi-6.4m seedlings with those of purified sensor protein standards imaged under identical conditions indicated that the cytosolic concentrations of this sensor were only approximately 1 μM (Supplemental Fig. S8). Consequently, we suggest that the fraction of cytosolic Pi sequestered by this sensor is negligible.

To test whether FRET ratios in Pi-starved plants could be reversed by replenishment with Pi, we added 0.5 mM Pi to 24-h starved cpFLIPPi-6.4 lines and monitored FRET over time (Fig. 5). No changes were detected when FRET was monitored for the first few minutes. However, changes in FRET ratios indicative of replenishment were detected after 1 h, and FRET signals were nearly equivalent to the initial fed state within 8 h (Fig. 5). These results strongly indicate that the in vivo FRET responses are specific for Pi.

It was possible that, within limits, the steady-state concentration of Pi in the cytosol is a variable continuum dependent on the magnitude of Pi supply. Alternatively, if other cellular pools buffer cytosolic Pi (Mimura, 1999), then the concentration would be relatively constant until buffering capacity is exceeded. To distinguish these possibilities, we grew transgenic cpFLIPPi-6.4 lines in hydroponic medium containing 0.5 mM Pi for 5 d and then replaced the medium with fresh media containing the same or reduced concentrations of Pi. Plants were grown in the new condition for another 24 h and then imaged in the same final growth medium. As shown in Figure 6, FRET ratios increased when supplied with 25 μM or less Pi but remained nearly constant when
plants were supplied with Pi concentrations greater than 25 μM. FRET ratios reached a maximum when plants were supplied with 10 μM Pi, but the values were not significantly different from those measured with plants completely deprived of Pi. These results support the ideas that cytosolic Pi is buffered over a broad range of external Pi concentrations and that this buffering capacity is overwhelmed when Pi supply drops below a critical threshold.

It was formally possible that the Pi-dependent changes in FRET ratios that we detected were caused by a mechanism other than FRET. Because it was not possible to address this issue through analysis of the emission spectrum in vivo, which was done for our in vitro studies, we used partial acceptor photobleaching as an alternative approach (Roszik et al., 2008). Evidence for FRET would be revealed by this method if photodestruction of the acceptor cpVenus resulted in an increase in donor eCFP emission caused by dequenching of the donor. Moreover, the increase in eCFP emission would be proportional to FRET efficiency, which would be dependent on Pi concentration. No consistent change in eCFP emission would be expected if sensor responses were unrelated to FRET.

We conducted partial acceptor photobleaching with each of the same plants used to evaluate the effect of varied Pi supply on its accumulation in the cytosol (Fig. 6). In each case, eCFP emission clearly increased as cpVenus emission diminished, indicating a FRET response (Supplemental Fig. S9A). FRET efficiencies were calculated from the photobleach responses as described previously (Roszik et al., 2008). The FRET efficiency values calculated for each concentration of supplied Pi correlated with the corresponding FRET ratios (Supplemental Fig. S9B) and thus, further validated the efficacy of the cpFLIPI-6.4m sensor for monitoring cytosolic Pi.

Live Imaging of Pi in Root Plastids of the Wild Type and a Plastidic Pi Transport Mutant

Nonphotosynthetic plastids import ATP from the cytosol in stoichiometric exchange for stromal ADP (Reiser et al., 2004; Reinhold et al., 2007). A consequence of the unbalanced phosphate moieties associated with this exchange is that Pi would accumulate to deleterious levels if not balanced by export. We proposed previously that the plastidic Pi transporter PHT4;2 confers this export activity in root plastids (Irigoyen et al., 2011). Transport activities measured with root plastids isolated from the wild type and a pht4;2 mutant support this idea. However, the mutation also reduced Pi import. It was possible that the import activity simply reflected the reversibility of the transport process, because maintenance of relevant electrochemical gradients cannot be assured with isolated organelles. Alternatively, PHT4;2 may have an undefined physiological role in Pi import.

To directly assess the role of PHT4;2 in plastidic Pi transport within the context of an intact plant, we introduced the plastid-targeted version of the cpFLIPPi-6.4m sensor into the pht4;2 background and then compared FRET ratios in this line with those of the same sensor in the wild type. Plants were grown for 5 d in medium containing 0.5 mM Pi to establish uniform growth and then imaged in the same medium. FRET ratios were significantly lower in mutant plastids, which is consistent with increased accumulation of Pi. Representative ratio images and quantitation from six

Figure 5. Replenishment of cytosolic Pi after 24 h of starvation. Seedlings expressing cpFLIPI-6.4m were grown with sufficient Pi (0.5 mM; +Pi) or starved for 24 h (−Pi); then, Pi was added to 0.5 mM, and FRET ratios were monitored over time. The plotted values are means ± se for six independent seedlings. The first time point shown after replenishment is 1 h. *, Significant difference (P < 0.05, Student’s t test) from the −Pi treatment.

Figure 6. Effect of Pi supply on cytosolic contents of root epidermal cells. Seedlings expressing cpFLIPI-6.4m were grown with a sufficient supply of Pi (500 μM), transferred to medium containing the same or less Pi as indicated, and grown for an additional 24 h before imaging. Plotted FRET ratio values are means ± se for at least six independent seedlings. *, Significant difference (P < 0.05, Student’s t test) from the 500 μM treatment.
independent seedlings of each genotype are shown in Figure 7. The FRET ratio images were merged with differential interference contrast images to distinguish individual cells, and this revealed that there is little variation in the amounts of Pi accumulated in plastids within the same cell or in different epidermal cells (Fig. 7A). The differences in FRET ratios can be attributed to altered Pi concentrations rather than unrelated effects of the pht4;2 mutation, because no differences in FRET ratios were detected when the high-affinity plastid cpFLIPPi-80μ sensor was used in these same genotypes (Supplemental Fig. S7B). These results strongly support the hypothesis that the physiologically relevant role of PHT4;2 is Pi export from root plastids.

DISCUSSION

Despite variable and often scarce supplies of Pi from soils, plants must distribute appropriate amounts of Pi to each cell and subcellular compartment to sustain essential metabolic activities. Because its concentrations in different locations can vary widely and in some cases, may also serve as a signal of Pi sufficiency (Carswell et al., 1996; Ticconi et al., 2001; Varadarajan et al., 2002; Kanno et al., 2012), dynamic assessment of these concentrations in live plants is critical for understanding the complex mechanisms underlying Pi homeostasis. Nonaqueous fractionation and 31P-NMR have been used to probe Pi contents of some cell compartments, but these methods lack cellular resolution and have no or limited temporal resolution (Rebeille et al., 1984; Sharkey and Vanderveer, 1989; Stitt et al., 1989; Pratt et al., 2009; Gout et al., 2011). Therefore, to achieve the necessary spatial and temporal resolution, we chose to adapt for use in plants a genetically encoded Pi sensor (FLIPPi) that had been used previously to track Pi in cultured animal cells (Gu et al., 2006).

Our efforts to adapt FLIPPi sensors for use in plants included modification of the sensor proteins first to enhance dynamic range and then, to optimize affinity. To enhance dynamic range (i.e. the maximum change in Pi-dependent FRET), we used a strategy that had proven successful for improvement of yellow chameleon Ca2+ sensors (Nagai et al., 2004). Specifically, we substituted the eYFP component of a FLIPPi sensor with a cpVenus. The resulting cpFLIPPi sensor exhibited increased FRET, even in the absence of Pi. More importantly, its dynamic range was more than 2 times that of the parent sensor. This enhancement is presumably a function of the relative orientation of the two chromophores in the sensor rather than their distance, because the chromophores remain equally separated by the PiBP (Nagai et al., 2004). Regardless, it is unlikely that cpVenus and eYFP interact differently with the PiBP component of the sensor, because binding affinity was unaffected.

Most estimates of cellular Pi concentrations in Pi-replete plants range from 2.5 to 10 mM and are derived from enzyme kinetic parameters, nonaqueous fractionation, and 31P-NMR (Rebeille et al., 1984; Stitt et al., 1988; Sharkey and Vanderveer, 1989; Lee and Ratcliffe, 1993; Copeland and Zammit, 1994; Gout et al., 2011; Tiessen et al., 2012), although cytosolic concentrations as low as 60 to 80 μM have also been reported (Pratt et al., 2009). We used these estimates as a guide for selecting cpFLIPPi affinity mutants with a goal of encompassing the entire range. Our mutagenesis strategy involved a unique use of SCA (Lockless and Ranganathan, 1999; Socolich et al., 2005) as a means to enhance the efficiency of the screen. The premise of SCA is that coupling represents an evolutionary constraint for structural or functional properties (e.g. ligand binding, protein-protein interactions, and allosteric sites). We conducted SCA with a set of 1,020 conserved PiBPs and identified a six-amino acid region that contained coupled residues that also map near the predicted Pi binding site. By limiting our mutagenesis to

Figure 7. Live imaging of Pi in plastids of root epidermal cells. A, Representative FRET ratio images for cpFLIPPi-6.4m stably expressed in wild-type and pht4;2 seedlings. The images were merged with the corresponding differential interference contrast images to distinguish cell boundaries. The same color scale was applied to ratio values for both images. Bar = 5 μm. B, Plotted FRET ratio values are means ± SD for six independent seedlings. The difference between the two genotypes is significant (P < 0.05, Student's t test). WT, Wild type.
alter only this 6-amino acid region rather than exploring all of the 340 amino acids of the PiBP included in the cpFLIPPi sensor, we reduced the scope of mutagenesis numerically and also, greatly biased our screen for those likely to alter Pi binding properties.

The mutant screen was highly efficient, with nearly 80% of the individuals screened showing altered affinity. Eight of these mutants were selected for additional analysis. Each of these was highly specific for Pi and had $K_v$ values for Pi binding that ranged from 0.08 to 11 mm, which spanned the entire target range. Although the in vitro binding assays were conducted in a medium that approximated cellular viscosity and primary ionic composition (Messerli and Robinson, 1998; Feijó et al., 1999), we recognize that the binding affinities may differ in vivo. Nevertheless, the large (>100-fold) concentration range collectively probed by the cpFLIPPi sensors was more than sufficient to monitor relative changes in the Pi contents of cytosol and plastid stroma of Arabidopsis root epidermal cells and is likely to also be useful for monitoring Pi contents in other cells and other cell compartments with near-neutral pH.

The cpFLIPPi sensors yielded weak or unstable fluorescent signals when expressed in wild-type plants, suggesting that they were subject to transgene silencing. Silencing was also reported for related FRET-based sensors expressed in siRNA-Silencing was also reported for related FRET-based sensors expressed in wild-type plants, suggesting that they were subject to transgene silencing. Despite this, a related Ca$^{2+}$ sensor that contained the ligand binding portions of these sensors promotes their silencing, because a related Ca$^{2+}$ sensor that contains the same fluorophores as cpFLIPPi and is expressed from the same Arabidopsis UBQ10 promoter is not subject to silencing (Krebs et al., 2012). Regardless, we attained strong, stable fluorescence when cpFLIPPi sensors were expressed in the sgs3-13 background. It should be noted that, although $rdr6$ mutations are more effective at eliminating transgene silencing than $sgs3$ mutations (Deuschle et al., 2006), we chose to use $sgs3$-13 for our studies, because unlike $rdr6$ mutations, it had little effect on plant growth and morphology. Moreover, secreted acid phosphatase activities measured in Pi-sufficient and -starved $sgs3$-13 seedlings were equivalent to those of wild-type seedlings grown under the same conditions, suggesting that this mutation has no significant effect on normal responses to Pi starvation.

Analysis of root extracts revealed that depriving 5-d-old seedlings of Pi for 24 h was sufficient to reduce free, cellular-undefined, and subcellular-undefined Pi contents by approximately 19%. Live imaging of cpFLIPPi sensors allowed us to unambiguously monitor the fraction attributed to cytosolic Pi contents of epidermal cells. A significant change in FRET ratio was detected only for sensors with $K_v$ values of 4.8 mM or greater, which suggests that sensors with higher affinity remained effectively saturated under this relatively mild starvation regime. Transgenic lines with the cpFLIPPi-6.4m sensor yielded the largest FRET response upon Pi deprivation, which may reflect an optimal pairing of the binding affinity of this sensor with cytosolic Pi concentrations.

Multiple lines of evidence indicate that the changes in FRET ratios that we measured in epidermal cells represent genuine Pi-dependent responses. First, FRET responses elicited by Pi deprivation were completely reversed by Pi replenishment. Second, high-affinity sensors exhibited no detectable FRET responses, suggesting little or no change in Pi-independent factors that could influence sensor properties. Third, Pi-dependent FRET efficiencies determined from acceptor photobleaching experiments directly correlated with the corresponding FRET ratios. Consequently, we conclude that the cpFLIPPi-6.4m sensor reports changes in cytosolic Pi concentrations with high specificity. Our results also suggest that the concentration of Pi in the cytosol is relatively constant, despite large differences in the amount of Pi supplied in the growth medium, presumably because of buffering by vacuolar pools (Mimura, 1999). This buffering, however, can be overwhelmed when Pi supply falls below a critical threshold. For our growth and assay conditions, this threshold was approximately 25 μM.

In addition to cytosolic Pi, the cpFLIPPi-6.4 sensor also enabled monitoring of Pi at the subcellular level. We proposed previously that the physiologically relevant role of PHT4;2, a plastidic Pi transporter, was export of Pi from root plastids (Irigoyen et al., 2011). This hypothesis was based on transport assays conducted with isolated root plastids, but the assays also revealed an import activity, which we viewed as a consequence of transport reversibility under our assay conditions. However, it was formally possible that Pi import was the relevant biological activity. Ratiometric imaging of a plastid-targeted version of cpFLIPPi-6.4m expressed in wild-type and pht4;2 plants distinguished these possibilities in the context of a live plant and thereby, confirmed that Pi export is the physiologically relevant role for PHT4;2.

CONCLUSION

This series of cpFLIPPi sensors provides the necessary spatial and temporal resolution to follow critical aspects of Pi metabolism in live plants. One of these, cpFLIPPi-6.4m, was particularly useful because it detected reduced cytosolic Pi concentrations in response to varied external supply and also detected a mutation-induced increase in Pi accumulation within an organelle. These sensor proteins are also likely to be suitable for studies in microbial and animal cell systems, and the approaches described here for their optimization may be broadly applicable to other genetically encoded sensors. Future efforts to target cpFLIPPi sensors to additional subcellular compartments and additional engineering to enhance signal responses in acidic environments will provide valuable insight into the mechanisms underlying Pi metabolism as well as the sensing and signaling of Pi status.
MATERIALS AND METHODS

Plasmids

We obtained FLIPPi plasmid clones pRSET FLIPPi-200u and pRSET FLIPPi-30m (Gu et al., 2006) from Addgene (www.Addgene.com). To replace the eYFP coding region in pRSET FLIPPi-200u with a cpVenus, we first amplified the cp173 coding region from the YC3.60 Ca2+ sensor (Nagai et al., 2004). We then used PCR and the In-Fusion Cloning Kit (Clontech) to introduce this sequence into the same region occupied by the eYFP coding region in the pRSET FLIPPi-200u plasmid to generate pRSET cpFLIPPi-200u.

For cpFLIPPi sensor expression in plants, a 2,455-bp HindIII-BamHI segment of the desired pRSET cpFLIPPi clone was ligated into pCN-UBQ10, a modified pCB1A3300 plasmid. The modifications included insertion of the Arabidopsis (Arabidopsis thaliana) UBQ10 promoter (Norris et al., 1993) to direct expression of the sensor gene and replacing the 35S promoter located upstream of the phosphinothricin resistance gene with the nopaline synthase promoter of pBI121. A plastid-targeted variant of each clone was constructed by inserting a 237-bp fragment encoding the 79-amino acid RbcS chloroplast transit sequence into the same region occupied by the eYFP coding sequence in the Arabidopsis plasmid to generate pRSET cpFLIPPi-200u.

Construction of a cpFLIPPi Affinity Mutant Library

We used SCA (Lockless and Ranganathan, 1999; Socolich et al., 2005) as a guide to generate a sensor mutant library biased for altered affinity. Sequences were collected from the nr Database (as of May 5, 2010) using PSI-BLAST (Altschul et al., 1997). The Escherichia coli PiBP (Gene Index: 157829674) was used as the query for five iterations of PSI-BLAST. Sequences with an e-value < 1e-10 were aligned using MUSCLE (Edgar, 2004). Identical sequences were eliminated, and the alignment of the remaining 1,020 sequences was used for SCA. SCA was performed as previously described (Lockless and Ranganathan, 1999; Socolich et al., 2005) to identify the set of positions that coevolves with one another. Two rounds of hierarchical clustering were performed to yield a self-consistent set of positions that statistically covaries with each other. PiBPs have one self-consistent cluster consisting of 43 amino acids (13% of protein), which was mapped onto the structure of the E. coli PiBP using PyMol (Supplemental Fig. S3).

A stretch of six amino acids (positions 18–23) within the 43-amino acid cluster was identified as a high-value mutational target based on the following observations: (1) Four of the positions (18, 19, 21, and 23) coevolve with one another. (2) One position (20) is highly conserved. (3) They are located near the Pi binding site. (4) The positions could be simultaneously mutated with one set of oligonucleotides. The mutagenesis oligonucleotides were designed such that each position was mutated to the three most frequently observed amino acids in the alignment that are found in at least 9% of sequences. In practice, this means that more than the desired amino acids are possible at some positions because the nucleotides required to encoded the target amino acids can also encode other amino acids. For example, Ala (GCC codon), Asp (GAT codon), and Ser (TCT codon) are needed at position 19, but the nucleotides required in the oligonucleotide at each position can also encode for Tyr (TAT codon).

The pRSET cpFLIPPi-200u plasmid was used as a template for PCR mutagenesis. The mutagenesis oligonucleotides 5'-ccacacgtggaatttccbbcttggctgkcggc-mdm-cdyggggaattttgtcgttcgacaggtg and 5'-ggtgctagaaagggcgcggcagcggdggcgcggcagcggc-gutttygcgaatttgggtgcttcgtt introduce approximately 1,728 possible amino acid combinations for the six-amino acid (positions 18–23) target region. The amplicon population was restriction digested and then ligated into pRSET cpFLIPPi-200u, replacing the original sequence. A portion of this mixed ligation product was introduced into E. coli DH5α cells, and >10,000 colonies were pooled for bulk plasmid isolation. The isolated plasmids collectively represent the affinity mutant library.

cpFLIPPi Affinity Mutant Screen

Portions (0.1 ng) of the sensor affinity mutant plasmid library were introduced into E. coli BL21 (DE3) cells. The pRSET cpFLIPPi-200u plasmid served as a positive control, and pUC19 served as a negative control for each transformation and subsequent screen. Individual colonies were transferred to a 96-well plate containing 45 μL of Luria-Bertani medium in each well and mixed thoroughly. Aliquots of 20 μL were transferred to duplicate 96-well deep-well plates containing 1.5 mL of Luria-Bertani medium and then covered with a gas-permeable seal. Plates were shaken at 300 rpm for 16 h at 25°C. Glyceraldehyde 3-phosphate dehydrogenase was prepared from 0.1-mL aliquots of each culture in a separate 96-well plate and stored at -80°C. The growth plates were centrifuged for 30 min to pellet cells; then, the supernatants were removed by vacuum aspiration. Cell pellets were washed two times with 20 μL Tris-HCl, pH 7.5, and then frozen at -20°C. To lyse cells, 0.35 mL of lysis solution (1× BugBuster [Novagen], 20 μL Tris-HCl, pH 7.5, 150 μM K-gluconate, and 3 μL of Lysonase per milliliter) was added to each well, mixed thoroughly, and then incubated at room temperature for 20 min with mixing every 5 min. Plates were then centrifuged for 10 min at 2,800 rpm and 20°C to remove cell debris.

Fluorescence assays were conducted with lysates in 96-well black plates. We transferred 20-μL aliquots of each lysate to eight wells containing 200 μL of pseudocytosol medium (100 mM K-gluconate, 30 mM NaCl, 25 mM MES, 25 mM HEPES, 40% [w/v] Suc, 1 mg mL-1 bovine serum albumin, pH 7.5; Messieli and Robinson, 1998; Feijó et al., 1999) and varied amounts of Pi (0–30 mM). Fluorescence was measured using a microplate reader (Synergy HT) with excitation at 420/27 nm and emission at 485/20 and 540/25 nm. Fluorescence from direct excitation of cpVenus (500/20 nm) was also monitored with emission at 540/25 nm. The KD value for each sensor was estimated by fitting data to a single-binding isotherm as described (Gu et al., 2006).

In Vitro Characterization of cpFLIPPi Proteins

Selected cpFLIPPi mutant clones were reintroduced into E. coli BL21 (DE3) cells, and single colonies were cultured in 100 mL of medium, harvested, and lysed essentially as described above for the mutant screen. The expressed sensor proteins were purified from cell lysates using His-affinity chromatography with elution in 20 mM Tris-HCl, pH 7.5, 150 mM K-gluconate, and 0.1 M imidazole. The eluent was dialyzed overnight at 4°C against 20 mM Tris-HCl, pH 7.5, and then transferred to a new tube, and stored at 4°C. The purified sensors could be stored for at least 1 month without measurable change in fluorescence or binding properties. KD values were determined as described above for the mutant screen, but 12 Pi concentrations were used to generate binding curves. Assays for binding affinity, specificity, and pH sensitivity were all conducted with at least three independent protein preparations.

Generation of Transgenic Arabidopsis plants

Sensor transgenes were introduced into Arabidopsis plants by floral dip transformation (Clough and Bent, 1998).

Subcellular Localization of Sensor Proteins

Protoplasts were prepared as described (Yoo et al., 2007) from leaves of 5-week-old transgenic plants expressing cpFLIPPi sensors engineered with and without an N-terminal RbcS chloroplast transit peptide. Protoplasts were imaged with a BioRad ES confocal laser-scanning microscope equipped with a 63× (numerical aperture of 1.2) water-immersion objective (515-nm excitation and 540-nm emission) for direct excitation of the cpVenus sensor component and 488-nm excitation and 680-nm emission for chlorophyll autofluorescence.

Live Imaging of Sensors in Plants

Transgenic plants were grown in individual wells of 96-well plates with 0.3 mL of one-half-strength Murashige and Skoog medium containing 1% (w/v) Suc and, unless indicated otherwise, 0.5 mM Pi. The plates were incubated in a growth chamber (60% relative humidity, 21°C, and 90 μmol m-2 s-1 light intensity for a 16-h photoperiod). After 5 d, the seedlings were either imaged or transferred to fresh medium as indicated. For imaging, seedlings were placed in a custom-made chamber (http://microscopy.tamu.edu/lab-protocols/light-microscopy-protocols.html) containing the same growth medium. Seedlings were covered with a small square of nylon mesh and then gently immobilized with a glass weight. A similar procedure was described recently (Kruger et al., 2013). Seedlings were analyzed for FRET using an inverted Olympus IX81 laser spinning disc confocal microscope equipped with an Andor Opti splitter to allow simultaneous dual emission. Root epidermal cells were viewed with a 40× (numerical aperture of 1.3) oil immersion objective (445-nm excitation for eCFP and 485-/32- and 542-/27-nm emission wavelengths for eCFP and FRET-derived cpVenus, respectively). Laser intensity and exposure settings were kept constant. For acceptor photobleaching experiments, a 515-nm excitation laser was used with maximum laser intensity. Untransformed seedlings and transgenic seedlings expressing eCFP alone and cpVenus separately were imaged each day to allow correction for background fluorescence, spectral bleed through, and cross excitation. All image analyses were done using ImageJ software. Mean intensity values for untransformed seedlings were used to establish background fluorescence.
values, and these were subtracted from all sensor images before conducting additional analyses. For analysis of cytosol-localized sensors, mean intensity values for eCFP and cpVenus were measured for selected regions of interest, which typically included three to four cells for each field of view. For analysis of plastid-localized sensors, a uniform threshold was applied to the eCFP and FRET cpVenus images, and mean intensity values above the threshold were used to calculate sensitized FRET ratios. FRET efficiencies (e values) were calculated from partial acceptor photobleaching images as described (Rozsik et al., 2008). Briefly, mean intensity values for eCFP (donor) and directly excited cpVenus (acceptor) for each sample were recorded before photobleach and again for at least four photobleach time points. These values were corrected for spectral bleed through and cross excitation and then used to calculate mean FRET efficiencies. An ImageJ plugin, AccPFRET, is available to automate much of this analysis (Rozsik et al., 2008).

Colorimetric Pi Assay

Plants were grown exactly as for live imaging with and without a final 24 h of Pi deprivation. Roots were harvested and pooled in groups of 15. Pi contents were determined using a phosphomolybdate colorimetric assay as described previously (Ames, 1966; Versaw and Harrison, 2002).

Secreted Acid Phosphatase Assay

Plants were grown exactly as for live imaging with and without a final 24 h of Pi deprivation. Individual seedlings were then transferred to 0.3 mL of fresh media of Pi deprivation. Roots were harvested and pooled in groups of 15. Pi concentrations were determined using a phosphomolybdate colorimetric assay as described previously (Ames, 1966; Versaw and Harrison, 2002).

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