Title

Engineered Root Bacteria Release Plant-Available Phosphate from Phytate

Journal

Applied and Environmental Microbiology, 85(18)

ISSN

0099-2240

Authors

Shulse, Christine N

Chovatia, Mansi

Agosto, Carolyn

et al.

Publication Date

2019-09-15

DOI

10.1128/aem.01210-19

Peer reviewed

Engineered root bacteria release plant-available phosphate from phytate

Christine N. Shulse[a], Mansi Chovatia[a], Carolyn Agosto[a], Gaoyan Wang[a], Matthew Hamilton[a], Samuel Deutsch[a], Yasuo Yoshikuni[a], Matthew J Blow[a*]

Running title: Engineered phytate-hydrolyzing root bacteria

[a]Department of Energy Joint Genome Institute, Walnut Creek, CA, USA.

***Correspondence**: Dr. Matthew J. Blow, mjblow@lbl.gov

Abstract

Microorganisms that release plant-available phosphate from natural soil phosphate stores may serve as biological alternatives to costly and environmentally damaging phosphate fertilizers. To explore this possibility, we engineered a collection of root bacteria to release plant available orthophosphate from phytate, an abundant phosphate source in many soils. We identified 82 phylogenetically diverse phytase genes, refactored their sequences for optimal expression in *Proteobacteria,* then synthesized and engineered them into the genomes of three root-colonizing bacteria. Liquid culture assays revealed 41 engineered strains with high levels of phytate hydrolysis. Among these, we identified 12 strains across three bacterial hosts that confer a growth advantage on the model plant *Arabidopsis thaliana* when phytate is the sole phosphate source. These data demonstrate that DNA synthesis approaches can be used to generate plant-associated strains with novel phosphate solubilizing capabilities.

Importance

Phosphate fertilizers are essential for high yield agriculture, yet are costly and environmentally damaging. Microbes that release soluble phosphate from naturally occurring sources in the soil are appealing as they may reduce the need for such fertilizers. In this study, we used

synthetic biology approaches to create a collection of engineered root-associated microbes with the ability to release phosphate from phytate. We demonstrate that these strains improve plant growth under phosphorus-limited conditions. This represents a first step in the development of phosphate-mining bacteria for future use in crop systems.

Introduction

Phosphorus is an essential and limiting nutrient for plant growth and is obtained by uptake of orthophosphate (Pi) through the roots. Phosphate fertilizers sustain high yield agriculture but are non-renewable, from politically sensitive regions of the world, and pollute aquatic environments (1). Alternatives to phosphate-based fertilizers are needed to alleviate these problems.

One potential source of phosphate is the abundant but recalcitrant forms of phosphate already present in the soil (2). Soil inorganic phosphates include iron, calcium and aluminum precipitates of phosphate and phosphate adsorbed to the surface of soil minerals. Organic phosphates, derived from soil biomass (plant, microbial, and metazoan), are chemically diverse but dominated by phytic acid, primarily in the salt form, referred to as ‘phytate’ (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, IP6; (3)). Organic phosphates such as phytate can be further bound by soil particles through adsorption-desorption reactions (4, 5). It has been estimated that accumulated phosphorus in agricultural soils may be sufficient to sustain high yield agriculture for many decades (6). However, only ~0.1% is in a form available to plants (7). The remainder must first be converted to soluble phosphate.

Plants and soil microorganisms have evolved diverse mechanisms to obtain phosphate from existing sources in the soil. These include expression of phosphatase and phytase enzymes to release Pi from organic phosphates (8), and exudation of organic acids and siderophores to solubilize inorganic phosphate (9-11). However, most natural plant and microbial communities are unable to produce high yields in the absence of added phosphate fertilizers. One approach is engineering plants to metabolize alternative forms of phosphate (12, 13). However each crop species must be engineered individually and must be labeled as a genetically modified organism. An alternative is to develop phosphate solubilizing soil microorganisms that grow in the vicinity of plant roots, and release plant-available phosphate (14, 15). Bacteria are more amenable to engineering at scale, and potentially applicable to a broad range of plant species and environments. Furthermore, different bacteria would be expected to be differently successful at colonizing distinct crops, and distinct soil environments. Therefore it may be useful to develop several engineered bacterial strains, which could be used in a customized manner, depending on crop and environment.

Previous studies exploring the use of phosphate solubilizing microbes have largely focused on natural phosphate-solubilizing plant-growth-

promoting bacteria (PGPB) (e.g. (16-19)). Other studies have introduced P-solubilization genes into plant-associated hosts (20-22), but these typically rely on plasmids which are genetically unstable (23). Genomic integrants have been created for a single phosphate-solubilization pathway (24) but the resulting strains were not tested for activity in plants.

Here, we used a combinatorial synthetic biology-based approach to generate a collection of plant-associated bacteria capable of efficient phytate hydrolysis. We engineered 82 biochemically diverse phytase enzymes directly into the genomes of three bacterial hosts, and demonstrated that the resulting strains are highly efficient hydrolyzers of phytate in liquid culture. Inoculation of *Arabidopsis thaliana* with several of these strains results in significantly improved plant growth under soilless conditions.

Results

Generation of transgenic bacterial strains overexpressing a diversity of phytases

We aimed to create a collection of plant-associated Pi-releasing bacteria by engineering diverse Pi-liberating enzymes into the genomes of multiple host bacteria (**Fig. 1**). Combining multiple hosts with diverse enzymes should result in a collection of strains with potential activity across a broader range of environmental conditions than any single host-gene combinations.

For bacterial hosts, we chose the proteobacteria *Pseudomonas simiae* WCS417r*, Ralstonia* sp. UNC404CL2/Col and *Pseudomonas putida* KT2440. These bacteria are known to be stably associated with plant roots (25, 26) and each has previously been engineered to contain a 'landing pad' sequence that facilitates permanent cloning into the genome versus unstable cloning into plasmids (27), creating strains *P. simiae* WCS417r:pw17, *Ralstonia* sp. UNC404CL2/Col:SB352_2, and *P. putida* KT2440:SB98_8.1. Furthermore all three strains were shown to release Pi from tricalcium phosphate after 1 to 3 days of growth (**Fig. S1**), presumably through the production of organic acids. This is important as organic acids increase phosphate mobilization in soils by completing with phosphate for positively charged binding sites on soil colloids (28).

A broad suite of genes are potentially involved in Pi mineralization in soils (**Table S1).** We selected phytases as these catalyze the hydrolysis of phytate, one of the most abundant phosphorus-containing molecules in soil (3). There are three major classes of enzyme with demonstrated phytase activity in microorganisms: 1) histidine acid phosphatase (HAP), 2) β-propeller phytase (BPP), and 3) cysteine phosphatase (CP) (29). A search for phytase genes among all available microbial genomes and environmental metagenomes in the Integrated Microbial Genomes database (37,985 genomes and 6674 metagenomes as of July 1st 2015 (30, 31)), revealed a total of 2,518 potential phytases across these three classes. Using a phylogenetic sampling approach (32), we selected 82 genes with the maximum degree of sequence diversity. This set of enzymes included 29 HAPs, 24 BPPs and 29 CPs, with representative sequences from across eight bacterial phyla and 7 sequences of unknown origin from metagenomic datasets (**Table S2**). Of the three bacterial hosts, *P. simiae* WCS417r, *Ralstonia* sp. UNC404CL2, and *P. putida* KT2440, only *P. simiae* WCS417r contains a putative phytase gene (a BPP; **Note S1**).

The selected phytase gene sequences were refactored *in silico* for optimal expression in *Proteobacteria* (33). Briefly, DNA sequences were redesigned to reflect the nucleotide sequence composition and

regulatory sequences of the *Pseudomonas* hosts, while maintaining the original amino acid sequences. We then synthesized these genes, cloned them into high-expression, IPTG-inducible expression cassettes, and sequence verified them using an established DNA synthesis pipeline (34). Finally, we transferred all 82 sequences into the genomes of *P. simiae* and *Ralstonia* sp. using conjugation and reconfirmed the sequences of the final engineered strains. We additionally transferred 21 sequences into *Pseudomonas putida*. In total, we generated 185 phytase-containing strains (**Table S3**).

Engineered strains hydrolyze phytate in liquid culture

We next determined the ability of engineered strains to solubilize phytate in liquid culture. Strains engineered with a landing pad sequence, but no phytase gene released no or negligible Pi from phytate at 10 days (*P. simiae* WCS417r:pw17 = 8 µM; *P. putida* KT2440:SB98_8.1 = 0 µM; *Ralstonia* sp. UNC404CL2/Col:SB352_2 = 84 µM; **Fig. 2**). In contrast, 17 / 82 *P. simiae* phytase-encoding strains, 16 / 82 *Ralstonia* sp. phytase-encoding strains and 8 / 21 tested *P. putida* phytase-encoding strains released high levels of Pi (>500 µM) after 10 days (**Fig. 2** and **Table S3**). These include 26 strains releasing over 10,000 µM Pi, representing conversion of over 22% of theoretically available Pi (**Note S2**). These high levels of Pi hydrolysis were reproducible across replicates (**Fig. S2**).

Overall, we found that 9 / 29 (31%) HAP and 9 / 29 (31%) CP enzymes were active in at least one host. In general, activity of these enzymes was the same across the three bacterial hosts. If a phytase was active in *P. simiae*, it was likely to be active in *Ralstonia* ($r = 0.933$; $p < 0.01$). None of the BPPs showed activity in any strain. The reasons for this are unclear, but may reflect a requirement for other cofactors or proteins (**Note S1**).

Among HAPs and CPs, sequences with extremes of length (either short (HAPs: 103 – 387 amino acids; CPs: 162 - 291 amino acids) or long (HAPs: 549 – 736 amino acids; CPs 332 – 822 amino acids)) were more likely to be inactive (**Fig. S3**). Active HAPs fell within the range of 397 – 544 amino acids while active CPs were restricted to between 293 – 331 amino acids. We considered the possibility that phytase genes deriving from bacterial lineages more closely related to our host bacterial strains were more likely to be active *in situ*. HAPs and CPs from *Proteobacteria* were slightly more likely to be active (10 / 22, 45%) than phytases originating from other bacterial phyla or metagenomes (8 / 36, 22%) (**Fig. S4**), however, this difference was not significant (Fisher's exact test, $P = 0.08$).

Growth capacity of phytase-engineered strains

When grown under Pi-limited conditions, with phytate as the only available Pi source, 86% of engineered *P. simiae* strains shown to have phosphate-solubilizing activity in the liquid medium grew to higher final cell densities than corresponding landing pad strains (**Table S4**). Under Pi-replete conditions, most engineered *P. simiae* strains had an extended lag phase when induced but ultimately had a similar maximum population size as the wild type and landing pad strains (**Fig. S5**). These results suggest that carrying an engineered phytase does not impose an excessive burden on the cell, and may offer a growth advantage in Pi-limited conditions.

Localization and pH range of phytase activity

To further characterize the active phytate-hydrolyzing strains, we isolated cell-associated and extracellular protein fractions from four of the constructs (three HAPs and one CP), and tested phytase activity across a range of pH. While all were active at acidic pHs they have different optima (from 2 to 6) (**Fig. 3**). The CP analyzed had higher levels of phytase activity in the cell-associated fraction than in the extracellular fraction (**Fig. 3D**). Results from the four HAPs were mixed – two (H07 and H19) had higher levels of phytase activity in the extracellular fraction, while one had similar levels in of activity in both fractions (H11). These results suggest that phytases are exuded into

the media and exhibit different biochemical properties. This is important in generating consortia of bacteria with potentially diverse capabilities such that they complement one another under different environmental conditions.

Plants treated with phytase-expressing bacterial strains exhibit improved growth under Pi-limited conditions

Our next aim was to determine whether these engineered strains would result in improved plant growth on phytate. The engineered strains used here include those used in the previous activity localization studies (**Fig. 3**). They represent the best performing strains from the initial determination of Pi mobilization (**Fig. 2**). We used an experimental system where *Arabidopsis* is grown on agar plates containing either 1 mM Pi or 0.8 mM phytate. Plates were then amended with engineered bacteria, control bacteria (no phytase), or no bacteria ('mock'). For each condition, we used at least 25 plants grown across at least 5 replicate plates. At the end of three weeks, we compared plants grown under different conditions and determined whether the engineered bacterial strains provide a growth advantage to the plants (**Methods)**. In total 14 different host / gene combinations were tested in assays on *Arabidopsis* with phytate as the sole

phosphate source. These included representatives of each host, and both functional classes of phytase (HAPs and CPs (**Fig. 4**)).

The growth of *Arabidopsis* grown on phytate as the only phosphate source (Pi-limited), and no added bacteria is consistently less than under Pi-replete conditions (1 mM Pi added) (**Fig. S6 – S8**). Plants exhibit limited growth and accumulation of anthocyanin resulting in a darker leaf color, consistent with the inability of *Arabidopsis* to access sufficient Pi from phytate (**Fig. 4A and 4D**).

Arabidopsis grown on phytate and treated with the control strains did not show increased growth benefit when compared with the mock treatment (**Fig 4B and 4E and Fig. S6 – S8**). These plants still have a P-starvation phenotype suggesting that the control bacteria are not releasing sufficient Pi from phytate to improve plant growth.

In contrast, *Arabidopsis* treated with 12 / 14 of the engineered strains had higher fresh weight, dry weight, and/or rosette size compared to the controls (**Fig. 4C, 4F and 4G**), with plant growth only slightly less than under Pi-replete conditions **(Fig. S6 – S8**). We consistently recovered an average of 2×10^8 – 2×10^9 colony-forming units (CFUs) per gram of root from the bacteria-treated plants tested at the conclusion of the assay, while no CFUs were recovered from the mock

controls (**Fig. S9**). These results suggest that engineered bacteria are releasing Pi from phytate, and some of this is available to the plants.

We observed improved growth of *Arabidopsis* in the presence of all 10 strains that were engineered to contain HAPs (four *Ralstonia* strains, four *P. simiae* strains, and two *P. putida* strains; containing either H07, H11, H13, or H19 – **Fig. 4G**). The CPs tested (C10 and C24) only improved plant growth when expressed in the pseudomonads. This is consistent with the relatively low activity of C10 and C24 *Ralstonia* strains versus pseudomonad strains in liquid culture assays (**Fig. 2**), and highlights the value of testing multiple different strains. The impact on plant growth as assessed by weight was generally correlated with absolute Pi mobilization (dry weight, r = 0.57 (p value = 0.03); rosette size, r = 0.48 (p value = 0.08); fresh weight, r = 0.60 (p value = 0.02)).

Discussion

Phosphate solubilizing microorganisms have potential as biological complements to phosphate fertilizers (9, 15). Here, we used a combinatorial synthetic biology approach to engineer phylogenetically diverse phytases into the genomes of three root-associated bacteria (**Fig. 1**). Through liquid culture assays, we identified 41 strains, with diverse pH optima, capable of hydrolyzing Pi from phytate (**Fig. 2 and 3**). The majority (12 / 14) of tested phytate-solubilizing strains improved the growth of *Arabidopsis* in agar plate assays with phytate as the only phosphate source (**Fig. 4**). Overall plant growth was generally correlated with absolute Pi mobilization by the strain applied. Strains releasing lower amounts of Pi in liquid cultures had no significant impact on plant growth (C10 and C24 expressed in *Ralstonia*), while those that released high amounts of Pi in liquid culture positively impacted plant growth (e.g. H11 expressed in *Ralstonia* or *P. simiae*, **Fig. 4**).

The combinatorial approach described here offers several benefits to the development of biological complements to phosphate fertilizers. Firstly, it provides control over the bacterial host. While environmental isolates with high levels of phytase activity have been identified (35), it is often not known if these strains are also capable of colonizing plant roots. In contrast, our approach starts with known root-colonizing

bacteria as hosts, and introduced high levels of desired phytase activity. Secondly, synthetic approaches enable the generation of consortia of bacteria with complementary properties. For example, in this study we used three bacterial host species in combination with phytases that have peak activity across a range of pH, such that one strain may always be active in diverse conditions. In the future, this approach may be expanded to harness other phosphate-solubilizing activities (for example different enzymes or pathways which produce metabolites capable of inorganic phosphate solubilzation) or other growth promoting capabilities such as N_2 fixation or hormone production. Finally, by directly cloning new enzymes into the genome of root-associated strains, versus utilizing plasmids as has been done previously, we can circumvent plasmid loss under non-selective conditions (23).

The bacteria engineered in this study may be expected to colonize and persist in environments similar to those from which they were isolated, namely the rhizosphere and soils. Specifically, *P. simiae* WCS417 was originally isolated from the rhizosphere of wheat(36) and subsequently shown to colonize crops such as lettuce, tomato, cucumber, and potato, where it outcompeted the indigenous microbial community(25). It additionally was successful as a biocontrol against the plant pathogen *Gaeumannomyces graminis var*. *tritici* (causative agent of

wheat take-all disease) when applied as a seed treatment prior to planting in marine loam soil(36). This is especially relevant as one of the failures of biocontrol of plant pathogens, and hence possibly PGPB in general, relates to the inability of the desired strains to compete with the many different microbes native to those environments (37). *P. simiae* WCS4174 has already proven itself successful in this regard. Likewise *P. putida* KT2440 has been found to establish itself at high levels when applied as a seed coating in the rhizosphere of broad bean plants and corn, as well as in the surrounding bulk soils, in field trials (26) in Granada, Spain.

Bacteria that naturally hydrolyze phytate have been isolated from a number of sources, including soil (38), poultry feces (39), and river sediment (35). For example, Richardson and Hadobas (1997) isolated two fluorescent pseudomonads with identical 16S rRNA gene sequences from composted garden soil. They found that these strains hydrolyzed up to 81% of phosphate contained within added phytate. The best-performing strains in this study hydrolyzed 60% of phosphate contained within added phytate. An advantage of the engineered strains is that as the bacteria are root-associated, the root will likely capture a higher proportion of liberated phosphate than it could from the naturally phytate-hydrolzying bacteria potentially found in the bulk soil.

While the approach described here represents a potential avenue to improve Pi availability in the rhizosphere, other challenges remain. In particular, improving the accessibility of phytate and other organophosphates sequestered on soil colloids remains an important area of research (4, 5). One approach may be to expand the engineering of root microbiota to include additional genes that may aid mobilization, such as siderophores for the solubilization of iron-phosphate complexes (40). In summary, our data provide proof of principle that DNA synthesis approaches can be used to generate plant-associated strains with novel capabilities benefitting plant growth.

Methods

Identification of phylogenetically diverse phytases

A comprehensive set of 2,518 putative phytase enzyme nucleotide and amino acid sequences was obtained by downloading all sequences annotated with pfam domains PF00328 (HAPs), PF02333 (BPPs), or PF14566 (CPs) in the Integrated Microbial Genomes & Microbiomes database (IMG/M, accessed May 2015; img.jgi.doe.gov). Amino acid sequences were aligned using MAFFT (41) and a phylogenetic tree was constructed using FastTree (42). To extract a highly informative set of representatives that cover maximal phylogenetic distance, we used the MaxPD algorithm as described in (32). This yielded a set of 96 phytase representatives for synthesis. Due to failure at various subsequent steps this was reduced to a final set of 82 phytases for evaluation.

Phytase gene synthesis and cloning

Amino acid sequences were codon optimized for expression in *P. simiae* using BOOST (33). Assembled products were cloned into pW26 vector by In-Fusion cloning (Clontech) in TransforMax EC100D *pir*$^{+}$ cells (Epicentre). Each plasmid was then transformed into *E. coli* WM3064, a diaminopimelic acid (DAP) auxotroph, in preparation for conjugation.

Transformation of root-associated bacteria

The plant root-associated strains *P. simiae* WCS417r:pw17, Ralstonia sp. UNC404CL2/Col:SB352_2, and *P. putida* KT2440:SB98.8_1 were selected as hosts for phytase solubilizing genes. Each of these strains has previously been engineered with a genomic insertion of a *lox* targeting cassette (a 'landing pad') to facilitate easier genomic integration of other genes and pathways (27). To create the engineered phytase-containing strains, each host strain was conjugated with *E. coli* WM3064::pw26-phytase. After 6 – 8 hours growth on LB + DAP, conjugations were resuspended in 2 mL LB, and 100 µl of a 10^{-3} dilution of this LB was plated on LB-agar plates with apramycin to isolate single colonies. After 36 hr growth at 28°C, individual colonies were streaked out on LB-kanamycin and LB-apramycin to identify apramycin-resistant, kanamycin-sensitive colonies. These colonies were additionally verified for proper integration by colony PCR using primers that hit within the landing pad close to the site of gene insertion (primer pair: 5'-TCCCGCGAAATTAATACGAC-3' and 5'-CAGCCAACTCAGCTTCCTTT-3').

Bacterial strains, growth, and inoculation

All bacterial strains were routinely cultured in LB Broth (Lennox) (L7658, Sigma-Aldrich, St. Louis, MO) or LB Miller Broth (MBLE-7030, GrowCells, Irvine, CA) supplemented with the appropriate antibiotic at 28°C in a shaking incubator at 250 rpm. For plant assays, cultures

were grown for approximately 4 - 7 hours depending on the strain until the culture reached mid- to late-log phase (OD_{600} = 0.4 – 0.7). Media were supplemented with apramycin (SB352_2: 1000 µg/ml, pw17: 50 µg/ml, SB98_8.1: 50 µg/ml) or kanamycin (500 µg/ml) as required. Cells were then harvested by centrifugation (4000 rpm for 5 min) and washed 2 times by resuspending in 10 mL of ¼ strength Ringer's solution (Sigma-Aldrich). After washing, the cells were resuspended in 10 mL of ¼ strength Ringer's solution and the OD of the resuspension was calculated by spectrophotometer (using a 1:10 dilution).

Experiments to study the growth parameters of *P. simiae* WCS417r, *P. simiae*:pw17, and the engineered phytase-containing derivates were carried out in LB broth containing the appropriate antibiotic and 1 mM IPTG in 96 well plates. Optical density was determined at 600 nm on a Tecan infinite 200Pro. Data were analyzed using the R package growthcurver (v0.2.0).

Liquid culture assays for phytase activity and tricalcium phosphate solubilization

To assess phytase activity, cells of each engineered strain were grown in Phytase Specific Medium (PSM) (1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4x7H_20$, 0.01% NaCl, 0.01% $CaCl_2x2H_20$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 and 0.5% sodium phytate (39))

and the supernate was collected at various time-points. To assess tricalcium phosphate solubilization as a proxy for organic acid production, cells of each landing pad strain were grown in National Botanical Research Institute's phosphate growth medium (NBRIP) with a minor modification (1% glucose, 0.5% $MgCl_2x6H_20$, 0.025% $MgSO_4x7H_20$, 0.02% KCl, 0.01% $(NH_4)_2SO_4$, 0.4% $Ca_3(PO_4)_{2\ (43)}$) and the supernate was collected after 1 to 3 days of growth. The release of Pi from phytate or NBRIP was monitored using the QuantiChrom phosphate assay kit (Bioassay Systems, Hayward, CA) across 3 (phytate) or 2 (NBRIP) technical replicates in 96 well plates at 600 nm on a Tecan infinite 200Pro.

Phytase enzyme assays

All assays were conducted using freshly prepared cultures. Starter cultures of pw17::phytase were grown overnight and used to inoculate a flask (250 µl of culture into 25 ml of LB medium) containing 50 µg/ml apramycin and 1 mM IPTG. After 46 h of growth, crude supernatant and cells were separated by centrifugation (4000 × g for 15 min at 4°C). Supernatant and cell pellet were then separately assayed for phytase activity.

Fifteen ml of the supernatant was transferred to an Amicon® Ultra-15 Centrifugal Filter Unit (MilliporeSigma) and subject to centrifugation at

4554000 × g for 30 min. The flow through was discarded; the remaining 456supernatant was applied to the filter unit and again subject to 457centrifugation. The flow through was discarded and the concentrated 458proteins were resuspended with 14 ml of 1 M Tris-HCl (pH 7) and 459subsequently subjected to a third centrifugation. The concentrated 460proteins were then transferred to a new tube, the volume was brought 461up to 2 ml with 1 M Tris-HCl (pH 7), and protein concentration was 462measured using the Quick Start Bradford Protein Assay Kit (Bio-Rad 463Laboratories).

464

465The culture cell pellet was suspended in lysis buffer (50 mM HEPES, 466300 mM NaCl, 10 mM imidazole, pH 8), 0.8 mg ml^{-1} lysozyme and 72.5 467U Benzonase® nuclease were added and the mixture was incubated 468for 15 – 30 min on ice. The lysate was then subject to centrifugation at 46912,000 × g for 15 – 30 min at 4°C. The resulting supernatant was 470decanted into an Amicon® Ultra-4 Centrifugal Filter Unit 471(MilliporeSigma) and centrifuged at 4000 × g for 30 min. The flow 472through was discarded and concentrated proteins were resuspended 473within the filter unit in 2 ml Tris-HCl (pH 7) and subsequently subject to 474a second centrifugation. The concentrated proteins were then 475transferred to a new tube, the volume was brought up to 2 ml with 1 M 476Tris-HCl (pH 7), and protein concentration was measured using the 477Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories).

All enzymes were assayed using sodium phytate (5 mM) as a substrate at 37°C against a control without the enzyme source in the reaction mixture. Phytase pH optima were determined using the buffers as in (44) at 0.1 M with slight modifications: glycine-HCl (pH 2 – 3), sodium acetate-HCl (pH 4 and 5), sodium acetate (pH 4.5), MES-Tris-HCl (pH 5.5 – 6), Tris-HCl (pH 7 – 8), glycine-NaOH (pH 9). Briefly, 70 µl of buffer/substrate mixture was added to 4 µg of enzyme preparation (in a 30 µl volume) in a 96 well plate. After 30 min of incubation, the reaction was stopped with 100 µl of 15% trichloroacetic acid and the concentration of liberated Pi was determined at 600 nm on a Tecan infinite 200Pro.

Plant growth assays

All plants used in this study were *Arabidopsis thaliana* ecotype Columbia (Col-0). Prior to growth, seeds were surface-sterilized by immersion in 70% ethanol for 5 minutes, followed by 10 minutes in 50% bleach + 0.1% Triton X-100 and several rinses in sterile water. Seeds were germinated in 24-well plates containing ½ strength Murashige and Skoog (MS) media with no phosphate and 0.5% sucrose. Seven to 10 days after planting, 5 seedlings were transplanted to each square petri dish containing 0.5X MS media with no sucrose, either 0.8 mM phytate or 1 mM Pi (KH_2PO_4) as the sole phosphate source, 10 mM

5013-(*N*-morpholino)propanesulfonic acid (MOPs), an IPTG overlay (48 µl of
502100 mM IPTG spread over the 10 cm^2 Petri dish), 1% Noble agar, and
503adjusted to pH 7.0, and inoculated with 100 µl of either ¼ strength
504Ringer's solution or bacteria diluted to A_{600} = 0.125 in ¼ strength
505Ringer's solution using sterile glass beads. Each treatment, including
506controls, was replicated on 5 plates, for a total of 25 plants per
507treatment. Seventeen to 21 days after inoculation plants were imaged
508and then removed from plates and fresh and dry weight was
509determined as described in (45), with some modifications. Briefly, roots
510were placed onto tared aluminum weight boats and weighed on a
511laboratory scale (Mettler Toledo MS105, Columbus, OH) to determine
512fresh weight. Samples were then dried in a gravity convection oven at
51375±2°C for 16 h and dry weight was determined on the same
514laboratory scale. Rosette sizes were calculated using the software
515ImageJ (v1.50i; http://imagej.nih.gov/ij). Statistical analyses of rosette
516size, fresh weight, and dry weight were performed using ANOVA and
517Tukey's Honest Significance Difference method within the R software
518package (http://www.r-project.org/; (46)). Statistical analysis for
519supplementary figures 5 – 7 was performed using a *t*-test within Excel.
520Plant images used for rosette size estimation are available at the Open
521Science Framework database: https://osf.io/m6gde/. These files can be
522accessed directly by downloading them from the OSF server. Roots
523were washed six times to removed bacteria not attached to the root

524and then bead beat and the resulting lysate was serially diluted to 525determine CFUs.

526

Acknowledgements

This research was supported by funding from Lawrence Berkeley Lab's Lab Directed Research and Development Program. We thank B. Cole for advice and assistance with developing plant assays. We also thank two anonymous reviewers for their helpful comments, which greatly improved the manuscript.

Author Contributions

C.N.S., M.C., and C.A. performed experiments. C.N.S. and M.C. carried out data analysis. G.W. and Y.Y. created landing pad strains. M.H. and S.D. refactored and provided synthesized phytase genes. M.J.B. obtained funding for and oversaw all aspects of the study. C.N.S. and M.J.B. prepared the manuscript with input from M.C. All authors approved the submitted version.

Competing Interests

The authors declare no competing interests.

References

Obersteiner M, Penuelas J, Ciais P, van der Velde M, Janssens IA. 2013. The phosphorus trilemma. Nat Geosci 6:897-898.

Menezes-Blackburn D, Giles C, Darch T, George TS, Blackwell M, Stutter M, Shand C, Lumsdon D, Cooper P, Wendler R, Brown L, Almeida DS, Wearing C, Zhang H, Haygarth PM. 2017. Opportunities for mobilizing recalcitrant phosphorus from agricultural soils: a review. Plant Soil 427:5.

Turner BL, Paphazy MJ, Haygarth PM, McKelvie ID. 2002. Inositol phosphates in the environment. Philos Trans R Soc 357:449-469.

Anderson G, Williams EG, Moir JO. 1974. A comparison of the sorption of inorganic orthophosphate and inositol hexaphosphate by six acid soils. J Soil Sci 25:51-62.

Tinker PB, Nye PH. 2000. Solute Movement in the Rhizosphere. Oxford University Press, New York.

George TS, Hinsinger P, Turner BL. 2016. Phosphorus in soils and plants - facing phosphorus scarcity. Plant Soil 401:1-6.

Zou XM, Binkley D, Doxtader KG. 1992. A new method for estimating gross phosphorus mineralization and immobilization rates in soil. Plant Soil 147:243-250.

Rodriguez H, Rossolini GM, Gonzalez T, Li JP, Glick BR. 2000. Isolation of a gene from *Burkholderia cepacia* IS-16 encoding a protein that facilitates phosphatase activity. Curr Microbiol 40:362-366.

Richardson AE, Simpson RJ. 2011. Soil microorganisms mediating phosphorus availability. Plant Physiol 156:989-996.

Rodriguez H, Fraga R, Gonzalez T, Bashan Y. 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil 287:15-21.

Rodriguez H, Gonzalez T, Goire I, Bashan Y. 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften 91:552-555.

Lopez-Arredondo DL, Herrera-Estrella L. 2012. Engineering phosphorus metabolism in plants to produce a dual fertilization and weed control system. Nat Biotechnol 30:889-U123.

Belgaroui N, Berthomieu P, Rouached H, Hanin M. 2016. The secretion of the bacterial phytase PHY-US417 by *Arabidopsis* roots reveals its potential for increasing phosphate acquisition and biomass production during co-growth. Plant Biotechnol J 14:1914-1924.

Rodriguez H, Fraga R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319-339.

58715. Richardson AE. 2001. Prospects for using soil microorganisms to
588 improve the acquisition of phosphorus by plants. Aust J Plant
589 Physiol 28:897-906.
59016. Srinivasan R, Alagawadi AR, Yandigeri MS, Meena KK, Saxena AK.
591 2012. Characterization of phosphate-solubilizing microorganisms
592 from salt-affected soils of India and their effect on growth of
593 sorghum plants [*Sorghum bicolor* (L.) Moench]. Ann Microbiol
594 62:93-105.
59517. Baig KS, Arshad M, Shaharoona B, Khalid A, Ahmed I. 2012.
596 Comparative effectiveness of *Bacillus* spp. possessing either dual
597 or single growth-promoting traits for improving phosphorus
598 uptake, growth and yield of wheat (*Triticum aestivum* L.). Ann
599 Microbiol 62:1109-1119.
60018. Unno Y, Okubo K, Wasaki J, Shinano T, Osaki M. 2005. Plant
601 growth promotion abilities and microscale bacterial dynamics in
602 the rhizosphere of Lupin analysed by phytate utilization ability.
603 Environ Microbiol 7:396-404.
60419. Giles CD, Hsu PC, Richardson AE, Hurst MRH, Hill JE. 2014. Plant
605 assimilation of phosphorus from an insoluble organic form is
606 improved by addition of an organic anion producing
607 Pseudomonas sp. Soil Biol Biochem 68:263-269.
60820. Patel KJ, Vig S, Nareshkumar G, Archana G. 2010. Effect of
609 transgenic rhizobacteria overexpressing *Citrobacter braakii appA*
610 on phytate-P availability to mung bean plants. J Microbiol
611 Biotechnol 20:1491-1499.
61221. Liu LL, Du WY, Luo WY, Su Y, Hui JJ, Ma SW. 2015. Development
613 of an engineered soil bacterium enabling to convert both
614 insoluble inorganic and organic phosphate into plant available
615 phosphate and its use as a biofertilizer. Mol Biotechnol 57:419-
616 429.
61722. Wagh J, Shah S, Bhandari P, Archana G, Kumar GN. 2014.
618 Heterologous expression of pyrroloquinoline quinone (*pqq*) gene
619 cluster confers mineral phosphate solubilization ability to
620 *Herbaspirillum seropedicae* Z67. Appl Microbiol Biotechnol
621 98:5117-5129.
62223. Rodriguez H, Gonzalez T, Selman G. 2000. Expression of a
623 mineral phosphate solubilizing gene from *Erwinia herbicola* in
624 two rhizobacterial strains. J Biotechnol 84:155-161.
62524. Adhikary H, Sanghavi PB, Macwan SR, Archana G, Kumar GN.
626 2014. Artificial citrate operon confers mineral phosphate
627 solubilization ability to diverse fluorescent pseudomonads. PLoS
628 ONE 9.
62925. van Peer R, Schippers B. 1989. Plant growth responses to
630 bacterization with selected *Pseudomonas* spp. strains and
631 rhizosphere microbial development in hydroponic cultures. Can J
632 Microbiol 35:456-463.

63326. Molina L, Ramos C, Duque E, Ronchel MC, Garcia JM, Wyke L,
634 Ramos JL. 2000. Survival of *Pseudomonas putida* KT2440 in soil
635 and in the rhizosphere of plants under greenhouse and
636 environmental conditions. Soil Biol Biochem 32:315-321.
63727. Yoshikuni Y, Wang G, Zhao Z, Coates RC, Cheng J-F, Robinson D.
638 2019. Pathway integration and expression in host cells. US
639 patent application publication 2019/0048354 A1.
64028. Kruse J, Abraham M, Amelung W, Baum C, Bol R, Kuhn O,
641 Lewandowski H, Niederberger J, Oelmann Y, Ruger C, Santner J,
642 Siebers M, Siebers N, Spohn M, Vestergren J, Vogts A, Leinweber
643 P. 2015. Innovative methods in soil phosphorus research: A
644 review. J Plant Nutr Soil Sci 178:43-88.
64529. Mullaney EJ, Ullah AHJ. 2007. Phytases: attributes, catalytic
646 mechanisms and applications. *In* Turner BL, Richardson AE,
647 Mullaney EJ (ed), Inositol Phosphates: Linking Agriculture and the
648 Environment. CAB International, Oxfordshire, UK.
64930. Chen IMA, Markowitz VM, Chu K, Palaniappan K, Szeto E, Pillay M,
650 Ratner A, Huang JH, Andersen E, Huntemann M, Varghese N,
651 Hadjithomas M, Tennessen K, Nielsen T, Ivanova NN, Kyrpides
652 NC. 2017. IMG/M: integrated genome and metagenome
653 comparative data analysis system. Nucleic Acids Res 45:D507-
654 D516.
65531. Markowitz VM, Chen IMA, Chu K, Pati A, Ivanova NN, Kyrpides NC.
656 2015. Ten years of maintaining and expanding a microbial
657 genome and metagenome analysis system. Trends Microbiol
658 23:730-741.
65932. Wu D, Hugenholtz P, Mavromatis K, Pukall R, Dalin E, Ivanova NN,
660 Kunin V, Goodwin L, Wu M, Tindall BJ, Hooper SD, Pati A, Lykidis
661 A, Spring S, Anderson IJ, D'Haeseleer P, Zemla A, Singer M,
662 Lapidus A, Nolan M, Copeland A, Han C, Chen F, Cheng J-F, Lucas
663 S, Kerfeld C, Lang E, Gronow S, Chain P, Bruce D, Rubin EM,
664 Kyrpides NC, Klenk H-P, Eisen JA. 2009. A phylogeny-driven
665 genomic encyclopaedia of Bacteria and Archaea. Nature
666 462:1056-1060.
66733. Oberortner E, Cheng JF, Hillson NJ, Deutsch S. 2017. Streamlining
668 the design-to-build transition with build-optimization software
669 tools. ACS Synth Biol 6:485-496.
67034. Heins RA, Cheng XL, Nath S, Deng K, Bowen BP, Chivian DC,
671 Datta S, Friedland GD, D'Haeseleer P, Wu DY, Tran-Gyamfi M,
672 Scullin CS, Singh S, Shi WB, Hamilton MG, Bendall ML, Sczyrba A,
673 Thompson J, Feldman T, Guenther JM, Gladden JM, Cheng JF,
674 Adams PD, Rubin EM, Simmons BA, Sale KL, Northen TR, Deutsch
675 S. 2014. Phylogenomically guided identification of industrially
676 relevant GH1 beta-glucosidases through DNA synthesis and
677 nanostructure-initiator mass spectrometry. ACS Chem Biol
678 9:2082-2091.

67935. Hill JE, Kysela D, Elimelech M. 2007. Isolation and assessment of
680 phytate-hydrolysing bacteria from the DelMarVa Peninsula.
681 Environ Microbiol 9:3100-3107.
68236. Lamers JG, Schippers B, Geels FP. 1988. Soil-borne diseases of
683 wheat in the Netherlands and results of seed bacterization with
684 pseudomonads against *Gaeumannomyces graminis* var. *tritici*, p
685 134 - 139. *In* Jorna ML, Slootmaker LAJ (ed), Cereal breeding
686 related to integrated cereal production, Pudoc, Wageningen.
68737. Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The
688 rhizosphere microbiome and plant health. Trends Plant Sci
689 17:478-486.
69038. Richardson AE, Hadobas PA. 1997. Soil isolates of *Pseudomonas*
691 spp. that utilize inositol phosphates. Can J Microbiol 43:509-516.
69239. Hosseinkhani B, Emtiazi G, Nahvi I. 2009. Analysis of phytase
693 producing bacteria (*Pseudomonas* sp.) from poultry faeces and
694 optimization of this enzyme production. Afr J Biotechnol 8:4229-
695 4232.
69640. Sharma SB, Sayyed RZ, Trivedi MH, Gobi TA. 2013. Phosphate
697 solubilizing microbes: sustainable approach for managing
698 phosphorus deficiency in agricultural soils. Springerplus 2.
69941. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment
700 software version 7: improvements in performance and usability.
701 Mol Biol Evol 30:772-780.
70242. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2-approximately
703 maximum-likelihood trees for large alignments. PLoS ONE
704 5:e9490.
70543. Nautiyal CS. 1999. An efficient microbiological growth medium
706 for screening phosphate solubilizing microorganisms. FEMS
707 Microbiol Lett 170:265-270.
70844. Golovan S, Wang GR, Zhang J, Forsberg CW. 2000.
709 Characterization and overproduction of the *Escherichia coli appA*
710 encoded bifunctional enzyme that exhibits both phytase and acid
711 phosphatase activities. Can J Microbiol 46:59-71.
71245. Bashan Y, de-Bashan LE. 2005. Fresh-weight measurements of
713 roots provide inaccurate estimates of the effects of plant growth-
714 promoting bacteria on root growth: a critical examination. Soil
715 Biol Biochem 37:1795-1804.
71646. R Core Team. 2015. R: A language and environment for
717 statistical computing, R Foundation for Statistical Computing,
718 Vienna, Austria. https://www.R-project.org/.
719

Figures

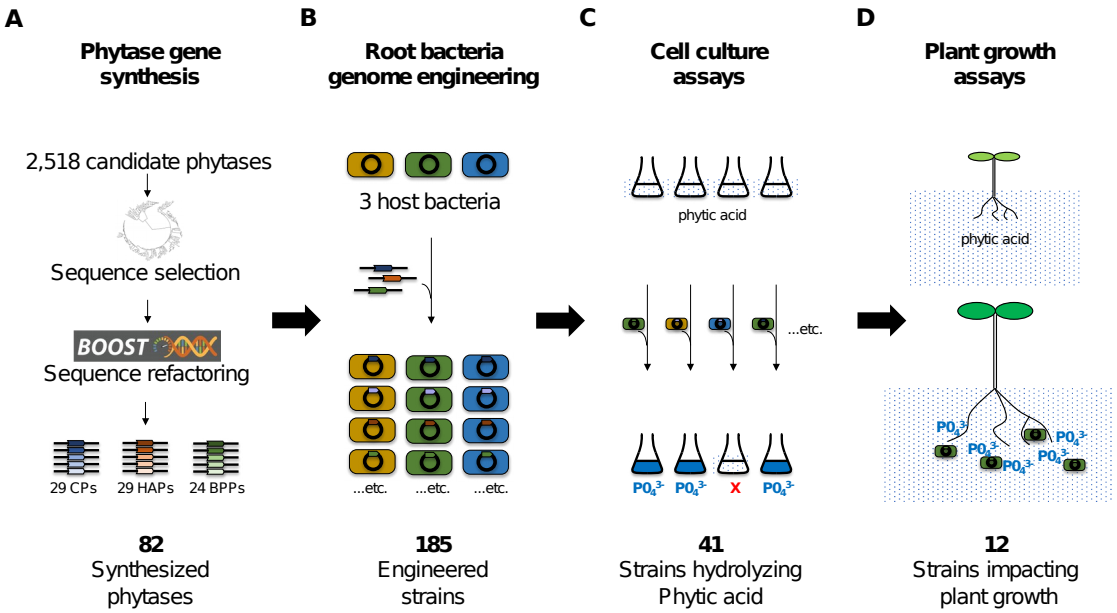
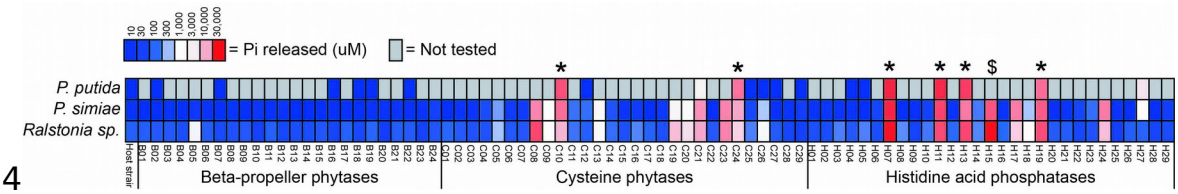


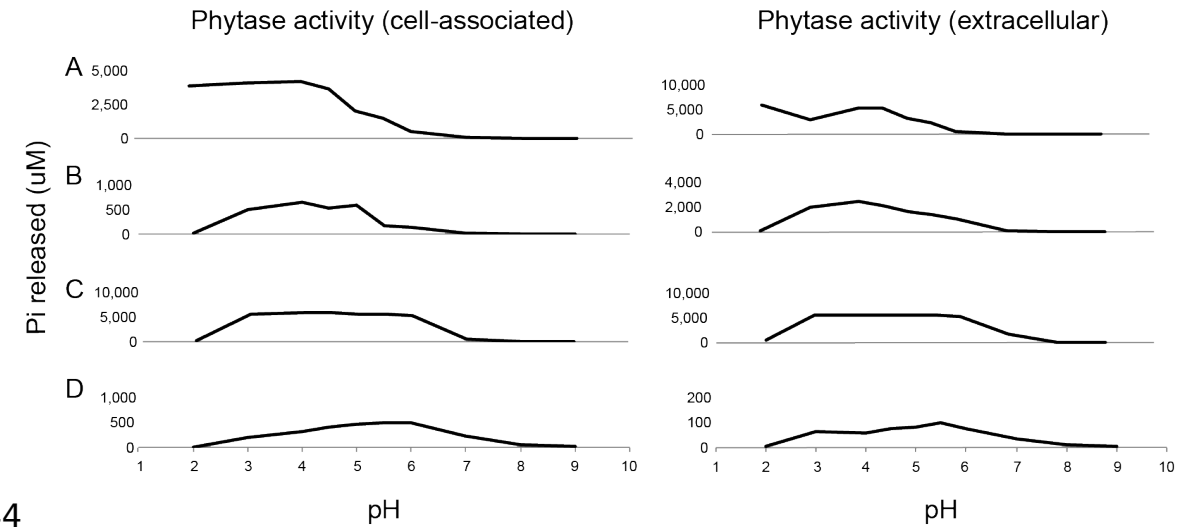
Figure 1. Study overview. (A) Diverse phytases were selected following a search of the Joint Genome Institute's Integrated Microbial Genomes & Microbiomes (IMG/M) database. Figure includes the three classes of phytase enzymes in microbes: Cysteine Phytases (CPs), Histidine Acid Phytase (HAPs), and Beta Propeller Phytases (BPPs). (B) Genes were optimized for expression in *Proteobacteria*, synthesized, and engineered into three bacterial hosts. (C) Engineered strains were evaluated *in vitro* for their ability to hydrolyze phytate and release Pi. (D) *Arabidopsis* were inoculated with the best performing strains and monitored for improvements in fresh and dry weight and rosette size.



734

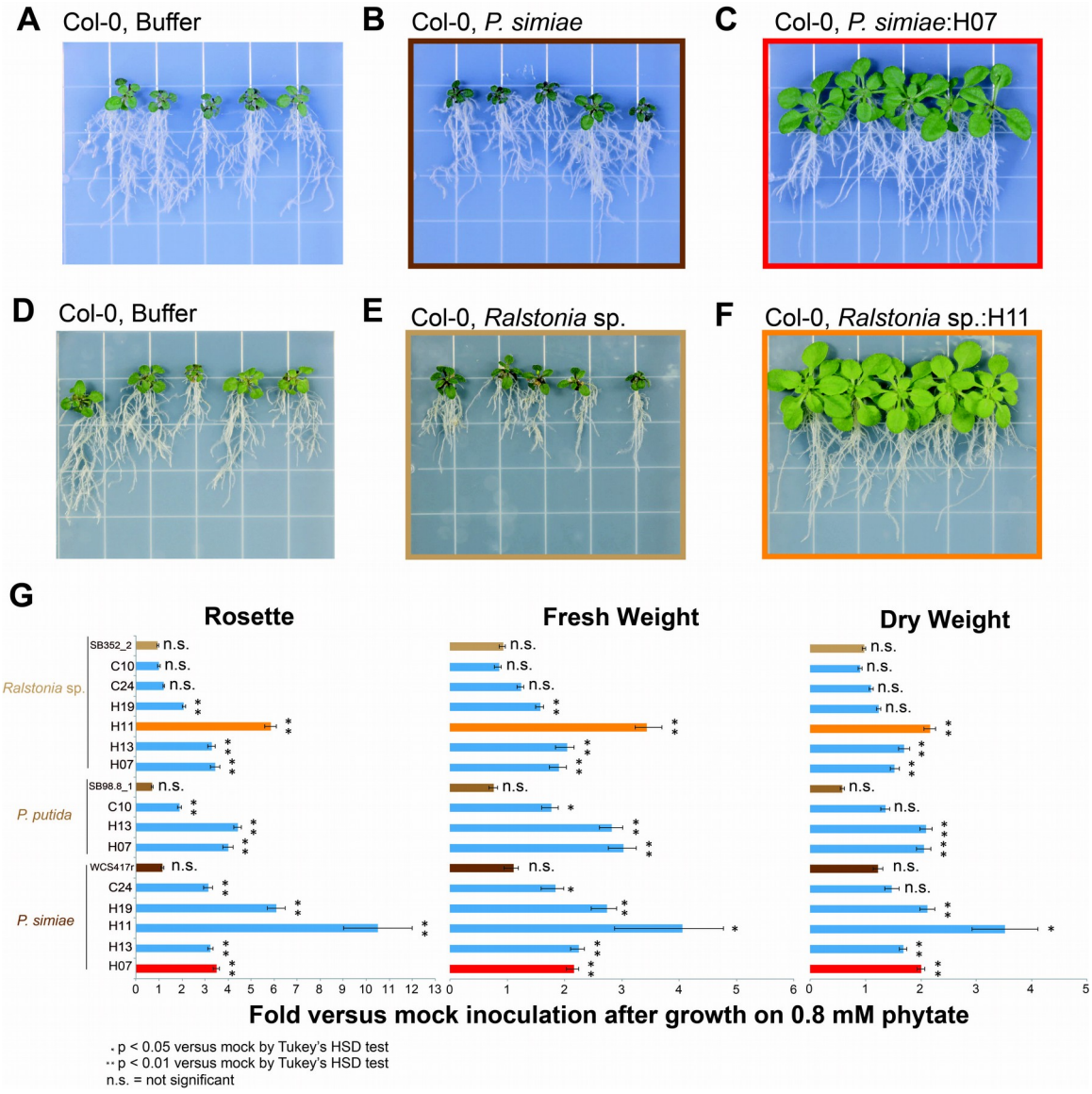
735**Figure 2. Phytase activity of engineered strains in liquid culture.** Pi levels in
736culture supernatant where phytate is the only phosphate source at 10 days for each
737construct expressed in *P. putida* KT2440, *P. simiae* WCS417r, and *Ralstonia* sp**.** PGPB
738with engineered phytases are screened for the ability to release Pi from phytate
739using the malachite green-based QuantiChrom Phosphate Assay Kit (BioAssay
740Systems). Each category of enzyme is ordered by amino acid length, from shortest to
741longest. Gray space indicates no measurement taken. *Strains used in plant assays.
742$*E. coli* AppA included as positive control.

743



744

745**Figure 3. Effect of pH on phytase activity**. Phytase activity in the extracellular
746medium for (A) H07 (B) H19 (C) H11 and (D) C24. The values are the averages of
747three experiments. The tested phytases have optimal activities across a range of
748pHs.



749

750**Figure 4. Engineered strains improve plant growth under Pi-limited**
751**conditions.** *Arabidopsis* was grown on ½ strength Murashige & Skoog agar,
752supplemented with 0.8 mM sodium phytate and specific bacterial treatments.
753Representative images 17 days post inoculation with (A) ¼ strength Ringer's
754solution, (B) *P. simiae* WCS417r, or (C) *P. simiae*:H07 or 19 days post inoculation with
755(D) ¼ strength Ringer's solution, (E) *Ralstonia* sp. 352_2, or (F) *Ralstonia* sp:H11 (G)
756Fold change in rosette size, fresh weight, and dry weight upon engineered and
757unengineered strain treatments versus mock inoculation for all strains tested. Brown

758bars indicate control strains without engineered phytases. Individual plant photos in
759B, C, E, and F are colored to correspond with data in G.