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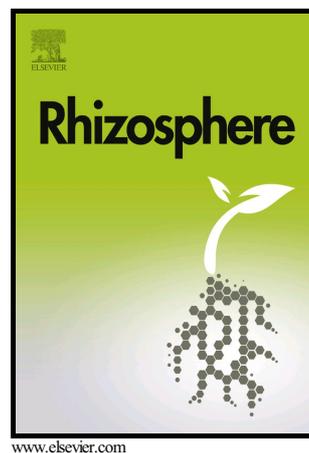
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Linking the depletion of rhizosphere phosphorus to the heterologous expression of a fungal phytase in *Nicotiana tabacum* as revealed by enzyme-labile P and solution ^{31}P NMR spectroscopy

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Short Title: Effect of phytase exudation on the composition of phosphorus in the rhizosphere

ABSTRACT

Root exudation of phytase could improve the ability of plants to access organic forms of soil phosphorus (P), thereby minimizing fertilizer requirements and improving P use efficiency in agroecosystems. After 75 days growth in a high available P soil, shoot biomass and P accumulation, soil pH, and rhizosphere P depletion were investigated in *Nicotiana tabacum* wild-type and transgenic plant-lines expressing and exuding *Aspergillus niger* phytase (*ex::phyA*), or a null-vector control. Solution ^{31}P NMR analysis revealed a 7% to 11% increase in orthophosphate and a comparable depletion of undefined monoester P compounds (-13 to -18%) in the rhizosphere of tobacco plants relative to the unplanted soil control. Wild-type plants had the greatest impact on the composition of rhizosphere P based on the depletion of other monoester P, polyphosphate, and phosphonate species. The depletion of phytase-labile P by *ex::phyA* plants was associated with decreased proportions of other monoester P, rather than *myo*-InsP6 as expected. Rhizosphere pH increased from 6.0 to 6.5-6.7 in transgenic plant soils, beyond the pH optimum for *A. niger phyA* activity (pH=5), and may explain the limited specificity of *ex::phyA* plants for phytate in this soil. The efficacy of single exudation traits (e.g., phytase) therefore appear to be limited in P-replete soil conditions and may be improved where soil pH matches the functional requirements of the enzyme or trait of interest.

KEYWORDS phosphorus, phytate, phytase, tobacco, ^{31}P NMR spectroscopy, pH

INTRODUCTION

Organic phosphorus (P_o) represents a large proportion of the total P in agricultural soils, which if made more accessible to plants, could promote the sustainable use of inorganic fertilizers and global crop productivity (Stutter et al. 2012). In order for plants to utilize P_o, it must first be hydrolysed to orthophosphate. Phosphatase enzymes of plant or microbial origin catalyse the hydrolysis of P_o in soils (Richardson et al. 2011). Phytate (*myo*-inositol hexakisphosphate, *myo*-InsP6) is among the most abundant and well-studied forms of P_o in soils and is specifically hydrolysed by phytases (EC 3.1.3.8, EC 3.1.3.26) (Greiner 2007). However, phytate is poorly hydrolysed in soil, which may explain its high abundance in most soils. One approach to overcoming the limited availability of phytate in soils has been to develop plants that overexpress fungal phytase genes (i.e., *Aspergillus niger PhyA* in: *Nicotiana tabacum* (George et al. 2005b); *Arabidopsis thaliana* (Richardson et al. 2001); *Trifolium subterraneum* (George et al. 2005a)) and release extracellular enzymes in the rhizosphere of plants to 'mine' soil P_o (Stutter et al. 2012). Whilst these and other studies have demonstrated gains in growth and P acquisition by phytase-exuding plants supplied with phytate in controlled laboratory studies, such gains have not translated widely to plants grown in soils (George et al. 2005a; George et al. 2005b). Therefore, further study is needed to understand the limits of manipulating single traits (i.e., phytase) with regard to plant P acquisition and P availability in the rhizosphere.

Abiotic (e.g., sorption, precipitation) and biotic (e.g., incorporation into microbial biomass) factors in soil influence the interaction of P_o substrates and phytase and ultimately, the release of plant available orthophosphate. Due to its abundance in most soils and strong association with the soil solid phase, phytate has been used widely to investigate mechanisms of P_o hydrolysis in soils and in particular for understanding limitations to mineralisation. Transgenic tobacco serves as a model for the potential improvements that could be gained through the manipulation of single exudation traits. Tobacco plant lines expressing the *A. thaliana Frd3* gene showed increased citrate efflux and utilization of phosphate sorbed to synthetic goethite (Giles et al. 2012), and tobacco that expressed the *A. niger PhyA* gene (*ex::phyA*), which accessed a greater amount of P from calcium-phytate in comparison to wild-type plants (Giles et al. 2014). However, when provided with phytate sorbed to goethite, the growth advantage of the *ex::phyA* plant lines was only observed when the amount of phytate in the growth media exceeded the sorption capacity of the goethite (Giles et al. 2014). In soil-based experiments, plant lines that exuded phytase accumulated more P than control plants, but only when substrate availability was improved by the addition of lime or when soils were directly amended with phytate (George et al. 2005b). Collectively, these studies indicate that the benefit of phytase exudation in plants is controlled by various abiotic factors including pH and soil mineralogy,

which may impact substrate availability and the efficacy of substrate-enzyme interactions (George et al. 2005b; Giles et al. 2014).

Implicit to the study of phytase-exuding plants is the hypothesis that an increased presence of phytase in the rhizosphere will lead to the greater mineralization of soil P_o and generation of plant-available orthophosphate. However, if phytase is similarly limited by immobilization to the solid phase, as has been demonstrated widely for phytate (George et al. 2007a), then the conditions necessary for mineralization will be severely limited. Phytase acts non-specifically on a wide range of orthophosphate monoester compounds with hydrolysis requiring that both the substrate and enzyme be in soluble forms (Giaveno et al. 2010; Tang et al. 2006). In the presence of different minerals (e.g., goethite, montmorillonite), however, the sorption of phytate and partitioning of phytase activity to the solid phase may restrict mineralization (Celi and Barberis 2007; Giaveno et al. 2010). Furthermore, the optimal phytase activity and isoelectric point of specific proteins occur at specific pH values, which may further influence the efficacy of enzyme-substrate interactions in soil (George et al. 2007a; George et al. 2007b). *A. niger phyA* has a maximum activity and isoelectric point at pH 5.0 (George et al. 2007b; Menezes-Blackburn et al. 2015). Soil conditions below or above pH 5 could therefore limit the potential gains in soil phytate hydrolysis afforded by the phytase exudation trait.

George et al. (2005b) reported expression of *PhyA* in transgenic tobacco lines, which allowed plants to accumulate 3.7-fold more P from soluble phytate when grown on agar. Whilst direct amendment of phytate to low P soils improved dry weight and P accumulation of phytase-exuding plants (George et al. 2005b), similar studies in high available P soils have not been conducted. Arable soils receiving continuous phosphate application may be so replete with available P that belowground strategies for soil P recovery may not work (Stutter et al. 2012). Therefore, the evaluation of genotypic and phenotypic traits expected to improve soil P scavenging should be conducted in soils of differing P_i and P_o contents (George et al. 2005a). Specifically, it will be necessary to determine the effect of the phytase exudation trait on plant growth and rhizosphere characteristics including pH and P species composition under P sufficiency (George et al. 2006).

We hypothesize that when grown under non-limiting P conditions, tobacco lines with *phyA* expression will differentially influence the composition and depletion of P in the rhizosphere when compared to wild type plants, and that compositional differences in the acquisition of soil P may occur irrespective of changes in plant growth or total P uptake. To test these hypotheses, a plant growth study was conducted in an arable soil with high 'plant-available P' to (1) assess biomass production and P acquisition by wild-type tobacco plants and transgenic lines that express the fungal

phyA gene or contain only the null-vector control, (2) analyse rhizosphere soils for pH, extractable inorganic and organic P and phytase-labile P and (3) determine the P composition of the rhizosphere soils using solution ^{31}P NMR spectroscopy.

EXPERIMENTAL

Plant Lines. *Nicotiana tabacum* wild-type (var. Wisconsin-38), and transgenic plant lines (*ex::phyA-1, ex::phyA-2, ex::phyA-3*) and a null-vector control (vector) line were obtained from CSIRO Agriculture (Canberra ACT). The transformation of tobacco plants with the *A. niger* phytase gene (*phyA*) was carried out as described previously (George et al. 2005b). Briefly, *phyA* was fused 5' with the carrot extension gene extracellular targeting sequence (*ex*) and expressed under the control of a 35S promoter from cauliflower mosaic virus and *ocs* terminator (Richardson et al. 2001). The pPLEX502 vector (Schünmann et al. 2003) was used for all transformed plant-lines. Phytase activity is negligible in root exudate solutions from *N. tabacum* wild-type and vector control plants, whereas *ex::phyA-1*, *ex::phyA-2,* and *ex::phyA-3* produce 66.8, 66.7, and 24.2 nKat g^{-1} root dry weight d^{-1}, respectively (George et al. 2005b).

Soil. Topsoil (0-10 cm depth) used for the plant growth experiments was collected from a field site near the James Hutton Institute (Dundee, Scotland; 56°42'33.03"N -2°88'75.16"W). The 'Tayport' soil is a freely drained Cambisol (FAO 2014) and is typically under cultivation with winter barley. Stutter et al. (2015) reported the chemical properties of the soil to be slightly acidic (pH 5.95 in CaCl$_2$) with a degree of P saturation of 50%. The soil contains 1475 mg kg^{-1} total P (determined by NaOH fusion) and relatively high Olsen extractable P (84.5 mg kg^{-1}). Solution ^{31}P NMR analysis of the Tayport soil showed 765 mg kg^{-1} total inorganic P and 410 mg kg^{-1} orthophosphate monoester P (including degradation products of diester P)(Stutter et al. 2015). Soil was air-dried and sieved to 4 mm prior to use in plant growth experiments.

Plant Growth and Phosphorus Uptake. Plants were grown in controlled glasshouse conditions at the James Hutton Institute for 75 d (22°C/14°C day/night, 16 h light, 200 W m^{-2}). Tobacco seeds were pre-germinated on 0.1% distilled water agar and transferred to growth pots containing 60 g of field moist soil. Soil moisture was maintained at ~80% water holding capacity (30 g water per 100 g dry soil) with distilled water. Five mL of P-free nutrient solution was added to each pot weekly (3 mM NH$_4$Cl, 4 mM Ca(NO$_3$)$_2$, 4 mM KNO$_3$, 3 mM MgSO$_4$, 0.1 mM Fe-EDTA, micronutrients: 6 μM MnCl$_2$, 23 μM H$_3$BO$_3$, 0.6 μM ZnCl$_2$, 1.6 μM CuSO$_4$, 1.0 μM Na$_2$MoO$_4$, 1.0 μM CoCl$_2$; pH 5.5). Five replicate pots were prepared for each plant line (wild-type, vector, *ex::phyA-1, ex::phyA-2, ex::phyA-3*) and a plant-free soil control (no-plant). Shoot and root materials were harvested at the end of the growth period

for the determination of dry weights and shoot P content. Soil adhering to roots was removed with gentle shaking and either stored at 4°C for pH measurements or dried (30°C, 2 weeks) and sieved (2 mm) for soil analyses. Roots were rinsed thoroughly and roots and shoots were dried at 70°C for 3 d. Shoot P was determined on approximately 50 mg of dried and milled material by sulphuric acid-peroxide digestion (Heffernan 1985) followed by malachite green (MG) colorimetry (Irving and McLaughlin 1990) as described previously (George et al. 2011). For rhizosphere pH, 2 g fresh sieved (2 mm) soil was mixed with 6 mL distilled water plus 0.85 mL 0.1M $CaCl_2$ and allowed to settle prior to measurement of the supernatant fluid with a glass pH electrode (Mettler Toledo, Ltd., Leicester UK).

Extractable- and Phytase-labile Phosphorus. Extractions were carried out by shaking (100 rpm) 2 g air-dried soils from the rhizosphere and unplanted soil treatments with distilled water (1:4 w/v) or 50 mM citrate (pH 5.0, 1:2 w/v) for 1 h (Stutter et al. 2015). Extracts were centrifuged (4,000g, 15 min), filtered (0.45 μm polyethersulfone), and stored at 4°C prior to analysis. Water and citrate-extractable inorganic P (WEP$_i$, CEP$_i$) concentrations were based on reactive P concentrations determined by MG colorimetry, whereas total water- and citrate-extractable P (WEP$_{tot}$, CEP$_{tot}$) were determined by Inductively-Coupled-Plasma-Optical Emission-Spectroscopy (ICP-OES). Unreactive fractions (WEP$_o$, CEP$_o$) were determined by the difference between total and reactive phosphate concentrations. In this study, unreactive and reactive P are considered analogous to organic and inorganic fractions in soil, respectively.

Phytase-labile P was determined in citrate extracts as described previously (Giles et al. 2016). Briefly, Natuphos (3-Phytase, EC 3.1.3.8; BASF SE, Ludwigshafen Germany) was added to citrate extracts (100 μL) at an excess final activity of 10 nkat mL^{-1} and combined with 30 μL of MES buffer (150 mM MES, 10 mM EDTA, pH 5.5) to a final incubation volume of 300 μL (37°C, 48 h). Reactions were stopped with an equal volume of cold (4°C) trichloroacetic acid (10% w/v) and phosphate release was measured by MG colorimetry (Irving and McLaughlin 1990).

Solution ^{31}P Nuclear Magnetic Resonance Spectroscopy. The initial soil, no-plant control soil (receiving nutrient additions for 75 d), and rhizosphere soils collected from the wild-type, vector, and *ex::phyA-1* plant lines were analysed using solution ^{31}P NMR spectroscopy. Rhizosphere soil from the *ex::phyA-1* plant line (as opposed to *ex::phyA-2* and *ex::phyA-3*) was selected for NMR analysis based on its similarity to the vector control in terms of shoot growth and soil pH (see results). Soils were bulked across replicates (~1 g per pot) and extracted in 0.25 M NaOH + 0.05 M EDTA (1:20 w/v, 16 h, 200 rpm, 20°C) followed by centrifugation (3500g, 40 min) and filtration (Whatman No. 42 paper; Whatman Filters, GE Healthcare Life Sciences, Buckinghamshire, UK) as described previously

(Stutter et al. 2015). A 10 mL aliquot of extract was collected for the determination of total P, Fe, and Mn by ICP-OES and the remaining extract (~80 mL) freeze-dried for ^{31}P NMR analysis. Extraction efficiencies were calculated based on total P in NaOH-EDTA extracts relative to total soil P determined by aqua regia digestion (Yara International, Analytical Services, Pocklington, York, UK).

Immediately prior to NMR analysis, 150 mg of freeze-dried extract was dissolved in 1.5 mL of 1 M NaOH containing 10% D$_2$O (v/v) and 0.2 mM methylene diphosphonic acid (MDPA; M9508, Sigma-Aldrich, Inc., Dorset UK) as an internal standard (17.3 ppm). NMR analysis was carried out using a Bruker Avance 500 II instrument (Bruker, Germany) operating at 202.458 MHz with decoupling, with a 5 mm probe, 90° pulse angle, and internal temperature maintained at 25°C. The T1 value for the orthophosphate peak (1.154 s) was determined by inversion-recovery experiments. Cade-Menun and Liu (2014) recommended using delay times of 5xT1 to achieve 99% signal equilibrium for experiments operating with 90° pulse angle. We used a pulse time of 4 μs with a 6 s pulse delay (Actual 5xT1 = 5.772 s), an acquisition time of 0.41 s, 5 Hz line-broadening, and 5000 scans (8.9 h).

Spiking experiments were carried out by adding 0.1 mL of known P$_o$ compound solutions (50 mg each in 5 mL distilled water) to blank extract samples, prepared in the same manner as all other soil extracts. Compounds from Sigma-Aldrich included phytic acid dodecasodium salt hydrate (*myo*-InsP6; P3168), D-glucose-6-phosphate sodium salt (G6P; G7879), DL-α-glycerol phosphate magnesium salt hydrate (α -glyc; 17766), and β-glycerophosphate disodium salt hydrate (β-glyc; G5422). Chemical shift (ppm) assignments were determined based on values reported in the literature (Cade-Menun and Liu 2014; Cade-Menun 2015; Stutter et al. 2015; Turner et al. 2003b) and those confirmed by spiking experiments: inorganic orthophosphate (6.0), polyphosphate (-4.23), phosphonates (10-20), total orthophosphate monoester (3.5-7.0), G6P (5.00), α -glyc (5.28), β-glyc (4.71), *myo*-InsP6 (5.76, 4.86, 4.49, 4.35), *scyllo*-InsP6 (4.02), other monoesters (3.5-5.9), orthophosphate diesters (2.5 − -3.0). Alkaline conditions in the NaOH-EDTA extract are known to promote phospholipid degradation resulting in the accumulation of α-glyc and β-glyc in ^{31}P NMR samples (Schneider et al. 2016). We assumed complete degradation of phospholipids would occur during the nearly 9 h NMR experiment. The full proportions of α-glyc and β-glyc are therefore added to the estimates of total diester P.

Theory and Calculation

Data were analysed in JMP Pro 11.2.0 (SAS 2013). One-way analysis of variance was used to determine significant differences between plant and soil treatments (Tukey's Least Squares Difference; $p<0.05$). Variables were checked for normality using the Shapiro-Wilk test and log-

transformed as required. Pearson correlations for extractable soil P fractions and plant characteristics were evaluated at the 95% confidence interval.

^{31}P NMR spectra were aligned by setting the orthophosphate peak to 6 ppm (Cade-Menun and Liu 2014) followed by manual peak picking and Global Spectral Deconvolution in MestReNova (MestReNova 2015). To confirm the quantitative detection of P, calibration curves were generated using the peak area and total MDPA P in each sample. The calibration used to calculate the total NaOH-EDTA P in each NMR sample was then checked against the actual NaOH-EDTA P concentration determined by ICP-OES. NaOH-EDTA P concentrations calculated within 5% of the actual value were deemed acceptable for analysis. The concentration of individual P species was calculated using the NMR compositional data and total NaOH-EDTA P in extracts. For comparisons of ^{31}P NMR species composition across plant treatments, data was transformed using the isometric log ratio procedure (*ilr*)(Filzmoser and Hron 2009) and sequential binary partition (SBP) as described previously (Abdi et al. 2015). Aitchison distances for the P species compositions of the rhizosphere and no-plant soils were determined relative the initial soil (Abdi et al. 2015; Egozcue and Pawlowsky-Glahn 2006).

RESULTS

Plant Growth and Phosphorus Uptake. Shoot and root dry weights of the tobacco plant lines were compared after 75 d growth in the Tayport soil which, based on Olsen extractable P (84.5 mg kg^{-1}), would be expected to contain sufficient P to support maximum plant growth. Shoot dry weights of phytase-expressing plant lines (*ex::phyA-1, -2, -3*) were not significantly different from either the vector control or wild-type plants (Table 1). Root dry weights of vector control plants (0.18 g) were however significantly larger than the wild-type, *ex::phyA-1*, and *ex::phyA-2* plants (p<0.05; Table 1). As a result, shoot:root ratios differed significantly across plant lines with the widest ratio in the wild-type (7.6) followed by *ex::phyA-1* (4.7), *ex::phyA-2* (4.1), *ex::phyA-2* (3.5) and vector (3.5) plants. Across all plant lines, this indicates differences in growth patterns and partitioning of resources in plants with expression of phytase. Shoot P concentrations were up to 29% greater in *ex::phyA-1* and *ex::phyA-2* as compared to the vector control and wild type plants (p<0.05; Table 1). However, there were no significant differences in total shoot P accumulation across any of the plant lines (Table 1).

Changes in readily extractable soil P and soil pH. The initial soil contained 5 mg kg^{-1} WEP$_{TOT}$ of which 75% was inorganic (Table 2). Total WEP and WEP$_i$ content did not change over the period of plant growth in the no-plant control soil (4.8 mg P kg^{-1}). In contrast, soils obtained from the rhizosphere of all plant lines contained significantly larger concentrations of WEP$_{TOT}$ at the end of the growth period, which was predominantly WEP$_o$ (54-65%; Table 2). Total WEP, WEP$_i$, and WEP$_o$

concentrations were similar in rhizosphere soils collected from the vector control and wild-type plants and the no-plant control soil (4.8 to 7.2 mg P kg^{-1}). Concentrations of WEP were also similar in rhizosphere soils from the three $ex::phyA$ plant lines, which contained 1.8 to 2-fold more WEP_{TOT} and approximately 2.9-fold more WEP_o in comparison to the no-plant soil (p<.05; Table 2). As a result, rhizosphere soil from $ex::phyA$ plants contained the largest percentage of WEP_o (60-65%) in comparison to other soil treatments (Table 2).

Citrate (50 mM, pH 5.5) extracted 2.4-fold more P from the planted and no-plant control soils in comparison to extractions by water. Total CEP in the initial soil (64 mg P kg^{-1}) consisted of 54% CEP_i and decreased significantly during the growth period in both the no-plant control soil and in the rhizosphere of all plant lines (Table 2). In comparison to the initial soil, CEP_{TOT} in the no-plant soil declined approximately 3-fold with the remaining 20.5 mg P kg^{-1} being 80% CEP_i (Table 2). Growth of all plant lines resulted in a further ~2 mg P kg^{-1} reduction of CEP_{TOT} concentrations in comparison to no-plant control soils and this was associated with the preferential depletion of CEP_i by plants (Table 2). Soils from $ex::phyA$ plants contained 25-30% less CEP_i than soil from the no-plant control. In contrast, CEP_i concentrations in wild-type soils were 2-fold less than in the no-plant control soil, and no significant difference was found between CEP_i in no-plant and vector soils (Table 2). Only in soil from the $ex::phyA-1$ plant line were CEP_i concentrations significantly less than the no-plant, wild-type, and vector control soils (Table 2). Soil from the $ex::phyA-1$ plant line also contained the largest concentration (6.8 mg P kg^{-1}) and proportion (37%) of CEP_o in comparison to the other soil and plant treatments (Table 2).

Phytase-labile P in citrate extracts represented 38% of CEP_{TOT} in the initial soil (24.1 mg P kg^{-1}) and was 10-fold less in the no-plant soil at the end of the growth period (p<0.05; Table 2). Soils collected from wild-type, vector control, and $ex::phyA-1$ plants contained similar concentrations and proportions of phytase-labile P (1.1-1.6 mg P kg^{-1}; Table 2). Wild-type and $ex::phyA-1$ soils contained 1.4 to 2-fold less phytase-labile P relative to the no-plant soil (Table 2). In contrast, phytase-labile P in soil from $ex::phyA-2$ and $ex::phyA-3$ plants was approximately 2-fold greater than the no-plant soil and represented 23-25% of the CEP_{TOT}.

The pH of the no-plant soil, which received nutrient solution in the glasshouse for 75 d, increased by 0.53 from an initial pH value of 5.95 to 6.48 (Table 2). On average, pH was significantly higher (+0.05-0.17 units) in rhizosphere soils compared to the no-plant control soil. Soil pH of the $ex::phyA-1$ (6.63) and the vector control plant lines (6.66) were significantly more alkaline than the wild-type, $ex::phyA-2$, and no-plant control soils (Table 2).

Depletion of total and NaOH-EDTA extractable phosphorus in soils. The concentration of total P ranged from 642 mg kg^{-1} in the wild-type soil to 1072 mg kg^{-1} in the initial soil (Table 3). Sodium-hydroxide-EDTA extractable P concentrations ranged from 418 mg kg^{-1} in the vector control soil to 672 mg kg^{-1} in the initial soil (Table 3). Across all soils NaOH-EDTA extracted 63% to 97% of the total P. The assessment of P forms by ^{31}P NMR in this study is therefore considered indicative of the total P species composition, though incomplete and selective extraction could have influenced P compositions, particularly in soils with low extraction efficiency (i.e., initial, vector control).

Soil from the no-plant control contained 37% less total P in comparison to the initial soil. Relative to the no-plant control, planted soils contained a further 8% (wild-type, vector control) to 13% (*ex::phyA-1*; Table 3) less total soil P. Shoot P accumulation accounted for 19 to 38% of the total P loss measured in the planted soils (Table 1). Differences in the recovery of P into shoots by these plant lines may be due to the accumulation of P in root materials with contrasting biomass, or through greater mobilization and leaching of P from the planted soils.

Phosphorus species composition of soils determined by ^{31}P NMR. Figure 1 shows the solution ^{31}P NMR chemical shift spectra and peaks identified in the monoester region relative to the MDPA internal standard for the different soils. The concentration and proportion of P_i was greatest in the initial soil (477 mg kg^{-1}; 71% of total NaOH-EDTA P) and was substantially less in soils following the growth period either without plants (217 mg kg^{-1}; 33%) or in the planted soils (182-252 mg kg^{-1}; 40-44%; Table 3). Orthophosphate was the primary form of P_i in all soils (>99%). Polyphosphate accounted for less than 1% of total P (<1.4 mg P kg^{-1}) in the NaOH-EDTA soil extracts (Table 3). Organic P in the initial soil (194 mg P kg^{-1}, 29%) was primarily monoester P, of which *myo*-InsP6 was the most abundant, and G6P the least abundant (7.8 mg kg^{-1}, 1%; Table 3). Total diester P in the initial soil (15.3 mg kg^{-1}) was entirely composed of diester degradation products (α-glyc, β-glyc) and accounted for 2% of P in the NaOH-EDTA extract (Table 3). Relative to planted and no-plant soils, the initial soil contained the smallest proportion of other monoester P (10%; Table 3).

Soil from the no-plant control contained the largest concentration and proportion of total P_o (434 mg P kg^{-1}, 67% of the total NaOH-EDTA P), which was primarily composed of monoester P (Table 3). The no-plant soil contained 70 mg kg^{-1} *myo*-InsP6 (11%) and 19 mg kg^{-1} *scyllo*-InsP6 (3%; Table 3). Other monoester P compounds represented 50% (328 mg kg^{-1}) of the P in extracts from the no-plant control soil. Glucose-6-phosphate (1%) and total diester P (1%) were the least abundance P_o forms in the no-plant control soil. The large concentration of P_o in the no-plant soil (relative to the initial soil) indicates that a considerable transformation of P into P_o occurred in the soils during the plant growth period and that this was primarily driven by an increase in other monoester P.

Proportions of P_i in the planted soils (182-252 mg kg^{-1}; 40-44%) were greater than in the no-plant control soil (217 mg kg^{-1}; 33%; Table 3). The proportion of P_i in planted soils at the end of the growth period was greater for wild-type and vector control plants (44%) compared to soils from *ex::phyA-1* (40%). Absolute concentrations of P in these soils show a decrease in P_i in vector control and *ex::phyA-1* soils relative to the no-plant soil (Table 3). This is primarily due to small NaOH-EDTA-P concentrations in the vector and *ex::phyA-1* soils (418-464 mg kg^{-1}) relative to the other soil treatments (568-572 mg kg^{-1}; Table 3). Total P was also diminished in the vector and *ex::phyA-1* soils relative to the no-plant soil and may therefore represent a real decrease in the concentration of monoester P during the growth period.

The concentrations and proportions of total P_o were smaller in soils from the wild-type, vector control, and *ex::phyA-1* plants (236-317 mg kg^{-1}; 56-60%) in comparison to the no-plant control soil (434mg kg^{-1}, 67%; Table 3). *Myo*-InsP6 was greater in the planted soils (15-16%) relative to the no-plant soil (11%). In contrast, other monoester P was depleted in the planted soils (132-187 mg kg^{-1}; 32-37%) relative to the no-plant soil (328 mg kg^{-1}, 50%; Table 3). The proportion of total diester P was greater in the planted soils (2-4%) relative to the no-plant soil and consisted primarily of phospholipid degradation products (α–glyc, β-glyc; Table 3).

Rhizosphere soil from wild-type plants contained the greatest concentration of *myo*-InsP6 (86 mg kg^{-1}, 15%) among the plant treatments. Relative to the transgenic soils, wild-type soils contained similar proportions of *myo*-InsP6 (15%), *scyllo*-InsP6 (4%), and other monoester P (33%), with differences in absolute concentration being driven by total and NaOH-EDTA extractable P (Table 3). In terms of concentration, the only monoester pools to be depleted by wild-type plants relative to the no-plant control were other monoester P (-141 mg kg^{-1}) and G6P (-3 mg kg^{-1}; Table 3). Relative to the no-plant control, wild-type soils contained +16 mg kg^{-1} *myo*-InsP6, +3 mg kg^{-1} *scyllo*-InsP6, and +9 mg kg^{-1} total diester P (Table 3). Phosphonate concentration and proportion was smallest in the wild-type soils (0%, 0.1 mg kg^{-1}) as compared to no-plant (0.1%), vector (0.3%) and *ex::phyA-1* (0.2%) soils (Table 3).

Rhizosphere soils from transgenic plants contained 7-11% less P_o relative to the no-plant control soil with the depletion of P_o being greatest in the vector control soil compared to the *ex::phyA-1* soil. In both the vector control and *ex::phyA-1* soils, changes in P_o were primarily driven by the depletion of other monoester P (132 mg kg^{-1}, 32%; Table 3). Otherwise, the proportions of P_o species (*myo*-InsP6, *scyllo*-InsP6, G6P) in the transgenic soils were found to be greater than or similar to the no-plant soil (Table 3). Total diester P was an exception to this due to its elevated proportions in vector (4%; Table 3) and *ex::phyA-1* soils (2%; Table 3) compared to the no-plant soil.

Major differences in the P composition of soils from the wild-type and transgenic plant lines were associated with orthophosphate, other monoester P, polyphosphate, and phosphonates. Although the latter two P forms accounted for less than 1% of the P forms identified, these were consistently found to be absent or in the lowest abundance in the wild-type soil. Orthophosphate was most abundant in the wild-type soil (44%) and other monoester P also occurred in relatively low abundance in this soil (33%) compared to the transgenic plants (Table 3). Major differences between the transgenic soils include the relatively greater abundance of orthophosphate in the vector soil (43%) and lower abundance of other monoester P (32%) relative to the *ex::phyA-1* soil (Table 3). Collectively, the contrasting compositions of P in wild-type and transgenic rhizosphere soils suggest different mechanisms of P turnover in the rhizosphere.

Aitchison distances were calculated as described by Abdi et al. (2015) to assess differences in P composition among the initial soil and planted soil treatments relative to the no-plant control soil. The sequential binary partition matrix and *ilr* values for n minus one P species are provided in the appendices (Table A1, Table A2). This analysis provides an unbiased comparison of the P species distribution in the soils relative to a soil reference (no-plant). The initial soil (1.85) was most similar to the no-plant soil, followed by soils from *ex::phyA-1* (1.99), vector control (2.14), and wild-type plants (3.73; Table 3). Although the lack of replication in the ^{31}P NMR analysis limits our ability to determine the statistical significance of these values, Aitchison distances that differ from zero indicate a global change to the composition of all treatments relative to the reference condition (Egozcue and Pawlowsky-Glahn 2006). Furthermore, small differences in Aitchison distance between treatments indicate the similarity of soil P compositions, whereas larger differences are more dissimilar (Abdi et al. 2015). Aitchison distances indicate that the wild-type tobacco had the largest impact on the composition of soil P relative to the no-plant soil. Aitchison distances of the transgenic plant soils differed by 0.15 and were therefore more similar to one another than to the wild-type soil (Table 3).

DISCUSSION

Phosphorus transformations in unplanted soil. We observed large differences in the concentration and composition of P between the initial soil and the unplanted (no-plant) soil, which was watered, received plant nutrients, and was maintained alongside the other plant treatments for the duration of the growth experiment. A conversion of orthophosphate to other monoester P during the 75 d growth period occurred (Table 2, Table 3). In addition to the incubation of soils, soil rewetting can lead to a release of P from the microbial biomass and an increase in concentrations of available P (Blackwell et al. 2009; Bünemann et al. 2013; Butterly et al. 2011). Blackwell et al. (2009) found the

majority of P leached from rewetted soil columns to be organic. However, Butterly et al. (2011) found that the 'pulse' of P resulting from soil rewetting had stabilized after 49 h in incubation experiments, despite evidence of continued soil respiration. Differences in the P composition of soils in the current study may not be solely due to differences in rewetting between the initial and incubated soils (no-plant and planted), but a combination of P released by the initial rewetting and, as shown in other studies, using ^{32}P isotopic dilution (George et al. 2006), the continual microbial cycling and stabilisation of P in moist soils during the growth period.

The generation of inositol phosphates in incubated soils was first observed by Caldwell and Black (1958) and Cosgrove (1964). More recently, the similarity of microbial P compositions (polyphosphate, phospholipids, nucleic acids) to that of soil has been demonstrated using ^{31}P NMR (Makarov et al. 2002; Makarov et al. 2005). In the current study, these changes were primarily reflected as larger proportions of other monoester P in the nutrient-amended soils (Table 3). Inositol hexaphosphate stereoisomers other than *myo-* and *scyllo*-InsP6 were not identified in the current study, however *neo-* and *chiro*-InsP6 are likely to occur among the peaks identified as other monoester as are inositols with fewer than six phosphate moieties (Cade-Menun 2015). In contrast, CEP_i, CEP_o, and CEP_{phy} concentrations were depleted in the no-plant soil relative to the initial soil. In the absence of plant uptake, the reduction of CEP concentrations coupled with increased concentrations of NaOH-EDTA extractable P species indicates the possible partitioning of P between these fractions during the incubation of the no-plant soils.

Plant-induced changes to pH and readily extractable phosphorus in the rhizosphere. Water- and citrate-extractable P were assessed to monitor plant-induced changes to immediately bioavailable and readily extractable P. These pools are expected to change rapidly in response to microbial activity, rhizodeposition, and plant uptake processes. Whilst extraction with water essentially targets soil solution P, citrate may solubilize adsorbed and precipitated forms of P through competitive displacement (ligand exchange) or the chelation of metal cations (e.g., Ca^{2+}, Al^{3+}, Fe^{3+})(Martin et al. 2004; Ström et al. 2005). The increased concentration of WEP_i and depletion of CEP_i beyond that observed in the no-plant control soil (Table 2), therefore indicates a plant-induced partitioning of soil P into readily available pools and possible preferential uptake of P_i previously sorbed or in complex with soil minerals and organic matter. This is also reflected in the negative relationship of shoot dry weight with CEP_i (r=-0.491; p=.0159) and CEP_{TOT} (r=-0.507, p=.010; Appendix Table A3).

The depletion of phytase-labile P concentrations in planted soils indicates the transformation or utilization of phytate-like compounds (e.g., inositol phosphates, other monoester P) by the wild-type and *ex::phyA-1* plants (Table 2, Table 3). Based on the NMR analysis, the pool of CEP_o targeted by the

Natuphos phytase was likely P forms characterized as other monoesters, which were more abundant than *myo*-InsP6 and depleted in the planted soils (Table 3). Phytase-labile P was 2-fold greater in rhizosphere soils from *ex::phyA-2/-3* plants in comparison to the no-plant soil (Table 2). The inability of these plant lines to deplete the phytase-labile P pool may be related to the relatively low rate of phytase exudation by *ex::phyA-3* (24.2 nKat g^{-1} root dry weight d^{-1})(George et al. 2005b) or the small root dry weight of *ex::phyA-2* plants, though this could not be confirmed. However, *ex::phyA-1* had the greatest rate of phytase exudation (66.8 nKat g^{-1} root dry weight d^{-1})(George et al. 2005b), a greater root dry weight, and, in most cases, the greatest effect on extractable P and rhizosphere pH relative to vector control and wild-type plants (Table 2). The relationship between rhizosphere pH and phytase-labile P concentrations among the plant lines tested indicates that, holding shoot P accumulation constant, small plant-induced increases in pH (<0.2 units) could significantly affect the amount and bioavailability of P in the tobacco rhizosphere (Table A3).

Phosphorus species transformations in the rhizosphere of tobacco. Solution ^{31}P NMR analysis revealed the transformation of P_i to P_o by tobacco plants grown in a high available P soil. Relative to the no-plant soil, there was 7% to 11% more P_i in the soil of wild-type and transgenic plants and a proportional decrease in P_o, primarily as other monoester P. Depletion of other monoester P relative to the no-plant soil followed the order: Vector (-18%), wild-type (-17%), ex::phyA-1 (13%). The pattern of P species depletion by the wild-type and transgenic plant lines was contrary to our original hypothesis. Based on previous work with tobacco (George et al. 2005b; Giles et al. 2014; Giles et al. 2012) and other phytase-exuding plants (George et al. 2008; George et al. 2006; Hayes et al. 1999; Richardson et al. 2001), it could be expected that *ex::phyA-1* would not only deplete a larger proportion of the total soil P_o, but would specifically access more *myo*-InsP6 than vector control or wild-type plants.

Depletion of *myo*-InsP6 was not apparent in the planted soils. Rather, *myo*-InsP6 increased relative to the no-plant soil and it was other monoester P that was the most depleted. The depletion of other monoester P was consistent with changes to the phytase-labile CEP pools as noted above. George et al. (2005a) reported the greatest depletion of NaOH-extractable P forms, particularly monoester P, by subterranean clover (*Trifolium subterraneum* L.) expressing *phyA* in comparison to wild-type clover; however, *myo*-InsP6 was not specifically quantified in that study. Our results show that rhizosphere soils from wild-type tobacco contained the smallest proportions and concentrations of other monoester P and phytase-labile CEP, and that *myo*-InsP6 was elevated in all planted soils. Therefore, undefined forms of P_o in the monoester region were the largest and most dynamic forms

of P accessible to both the transgenic and wild-type plants and no specificity for the utilisation of *myo*-InsP6 by the phytase-exuding *ex::phyA-1* plant line was observed.

The exudation of a native purple-acid phosphatase (PAP) by tobacco could have contributed to the utilisation and transformation of P_o in the pH 6-6.5 soils (Lung et al. 2008). Purple-acid-phosphatases isolated from tobacco (Lung et al. 2008), sweet potato (Olczak et al. 2003), soybean (Hegeman and Grabau 2001), and *Arabidopsis* (Kuang et al. 2009) have been shown to act non-specifically on phytate and other orthophosphate monoester compounds. Wild-type tobacco produces low levels of phytase activity in root exudates (0.2 nKat plant^{-1} d^{-1}) and could acquire 3-10% of P from soluble or insoluble sources of phytate *in vitro* (Giles et al. 2014; Giles et al. 2012). Activity profiles of the *N. tabacum* PAP indicate a slightly higher catalytic efficiency and pH optimum (pH 5.5) than the *A. niger* phyA, which may be impaired above pH 6.5 (Lung et al. 2008). In contrast to the fungal *phyA* trait, which is constitutively expressed in *ex::phyA* plants, the production of native PAP will be expressed based on growth stage, P availability and potentially other environmental cues (Lung et al. 2008). However, at the pH of wild-type and transgenic soils (pH 6.5-6.7), even a low level of PAP activity could explain the decrease in monoester P by plants lacking expression of the *A. niger phyA*. (Lung et al. 2008)

The interaction and regulation of phosphatase activity by soil microorganisms in the rhizosphere of phosphatase-exuding plants may also have a considerable effect on P_o utilization by plants (Nannipieri et al. 2008). Spohn and Kuzyakov (2013) showed that P fertilization diminished the production of plant phosphatases by white lupin (*Lupinus albus* L.), but did not affect the abundance or distribution of acid and alkaline phosphatase activity by soil microorganisms. Thus in addition to the exudation of native PAP, the production of microbial phosphatases may have contributed to the differential depletion of soil phytate by the wild-type and transgenic plant lines. The measurement of soil phosphatase activity in future studies would help to identify the contribution of secondary plant phosphatases or responses of the microbial community to single plant exudation traits.

Factors controlling the utilisation of soil organic phosphorus by tobacco. *Nicotiana tabacum* was used as a model plant to investigate the effects of phytase exudation on the composition and depletion of P_o in soils. Our results indicate however that although a wider range and concentration of P_o forms were depleted by the vector control and plants expressing a heterologous phytase from *A. niger* (*ex::phyA*), wild-type plants had the largest effect on the composition of soil P when grown in a high available P soil. Our analysis highlights two factors, rhizosphere pH and root biomass, which may explain the differing effects of the wild-type and transgenic plants on the soil P_o pool.

Rhizosphere pH was similar between vector control (6.7) and *ex::phyA-1* plant lines (6.6) and greater than in the wild-type, initial (6.0), and no-plant soils (6.5; Table 2). The isoelectric point of *phyA* occurs at pH 5 (*pI* = 5). At this pH, the enzyme is adsorbed to the soil surface, but with minimal inhibition to phytase activity associated with the solid phase (George et al. 2007b). At pH 7.5, repulsive forces between the negatively charged protein and soil surface lead to greater amounts and activities of the enzyme in solution (George et al. 2007b), meaning that *phyA* should be soluble and available in the transgenic rhizosphere at pH 6.5. However, the pH optima of *A. niger phyA* activity in solution occurs at approximately pH 2.5 and 5.0 (George et al. 2007b; Sariyska et al. 2005) and can decrease by more than 60% above pH 6 (Mezeli et al. 2015; Naves et al. 2012). Naves et al. (2012) reported *A. niger* phytase activity (y; FTU g^{-1}) to depend on pH (x) following the cubic relationship: $y = -501.23x^3 + 4994.6x^2 - 13296x + 10733$ (R^2 = 0.9684). Based on this analysis, phytase activity decreases by 18% from pH 5.0 to 5.5, by 53% from pH 5.5 to 6.0, and by an additional 97% from 6.0 to 6.3, where solution activity is expected to go to zero. Whilst a greater amount of the enzyme would be expected in the soil solution at the pH of vector and *ex::phyA-1* soils, these pH conditions could have also limited or even abolished *phyA* activity beyond pH 6.5.

Although the composition of P in soils from the vector control and *ex::phyA* plants were the most similar based on Aitchison distances, the abundance and concentration of specific P forms was least in soil from the vector control plants. This is largely due to smaller NaOH-EDTA extractable P concentrations in vector soils, but is reflected in ^{31}P NMR proportional data as well (Table 3). Physiological differences between the vector control and *ex::phyA-1* may explain these differences. For example, root biomass was 38% larger in the vector control than *ex::phyA-1* plant line. Larger roots provide plants with greater access to the entire soil volume, which can facilitate root-induced changes to soil abiotic (e.g., pH, P solubilisation) and biotic conditions (e.g., plant-microbe interactions; Badri and Vivanco 2009). As summarized by Brown et al. (2013), plants respond to P deficiency through conservation or active acquisition strategies. Root elongation and branching (Hammond and White 2008; Lynch 2011), root hair growth (Bates and Lynch 1996), exudation of organic anions (Hoffland et al. 1992), and modification of pH (Hinsinger et al. 2003) pertain to active strategies, which promote P availability, whereas altered metabolism and diminished root growth are examples of conservation responses (Johnson et al. 1994; Peret et al. 2011). Furthermore, as reported for the exudation of other organic anions and enzymes (Badri and Vivanco 2009; Lopez-Bucio et al. 2000), the metabolic cost of *phyA* production and transport may have contributed to reduced root growth in the *ex::phyA* plants, though this could not be confirmed in the current study. Thus, differences in the root biomass of the vector control and *ex::phyA-1* plants may be related to (1) a root growth response by the vector control plants to low P availability, (2) root growth

inhibition in *ex::phyA-1* plants due to the cost of *phyA* production and transport, or (3) the efficient acquisition of soil P by phytase-exuding plants leading to reduced root growth by *ex::phyA-1* plants.

In conclusion, the complex multi-genic and multi-trait interactions that occur between plants and P limitation in soil may mean that a single gene or 'root solution' (Stutter et al. 2012) to improving plant P acquisition are difficult to achieve and, at best, unpredictable. Future studies aimed at achieving the full potential of single traits such as *phyA* should consider (1) the nutritional and metabolic trade-offs associated with soil P acquisition in wild-type and transgenic plant systems and (2) the appropriate selection or modification of rhizosphere chemical conditions (e.g., pH, organic anion efflux) to match the functional requirements of the enzyme. Trait-based solutions such as these may be best suited to P limited systems where plant P requirements are not already satisfied by legacy available P, particularly in non-GM plant systems where trait expression depends on nutrient availability and other edaphic factors.

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Figure 1. Solution ^{31}P nuclear magnetic resonance spectra of rhizosphere soils collected after 75 d growth of *N. tabacum* wild-type, vector control and *ex::phyA-1* plant lines along with a no-plant control soil. Insert shows enlargement of the 8 to 2 ppm monoester region; MDPA, methylene diphosphonic acid internal standard; o, orthophosphate; m, *myo*-InsP6; G6P, glucose-6-phosphate; α, α–glycerol phosphate; β, β-glyerophosphate; * Other monoester P.

Table 1 Shoot and root dry weight, shoot phosphorus (P) concentration and total shoot P content in *N. tabacum* plant-lines grown for 75 days in the Tayport soil.

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Plant treatment	Shoot dry wt. (g)		Root dry wt. (g)		S:R		Total dry wt. (g)		Shoot P concentration (μg mg^{-1})		Shoot P accumulation (μg)	
ex::phyA-1	0.60	a	0.13	b	4.7	ab	0.73	a	1.02	b	619	a
ex::phyA-2	0.41	a	0.10	b	4.1	ab	0.51	b	1.10	a	489	a
ex::phyA-3	0.48	a	0.14	ab	3.5	b	0.62	ab	0.90	bc	437	a
Vector control	0.63	a	0.18	a	3.5	b	0.81	ab	0.87	c	540	a
Wild type	0.84	a	0.11	b	7.6	a	0.95	ab	0.85	c	731	a

Plant treatments: Transgenic *N. tabacum* plants expressing *Aspergillus niger* phytase (*ex::phyA-1, -2, -3*), vector control and wild-type plants. Data are presented as the mean ± standard error of 5 replicates and compared using Least Significant Difference, whereby within each column different letters represent significantly different means (*p<0.05*).

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Table 2. Total, inorganic, organic, and phytase-labile concentrations of water (WEP) and citrate-extractable (CEP) phosphorus and pH of initial soil and soils after 75 days with and without (no-plant) the growth of *N. tabacum* plant lines.

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	pH (CaCl$_2$)	Water-extractable P (mg kg^{-1})			Citrate-extractable P (mg kg^{-1})			
		WEP$_{TOT}$	WEP$_i$	WEP$_o$	CEP$_{TOT}$	CEP$_i$	CEP$_o$	Phy-labile
Initial soil	5.95 d	5.0 b	3.4 abc	1.2 c	64.0 a	34.5 a	29.5 a	24.1 a
No plant	6.48 c	4.8 b	2.8 c	2.0 bc	20.5 b	16.3 b	4.2 c	2.2 c
ex::phyA-1	6.63 a	9.5 a	3.7 ab	5.8 a	18.2 c	11.4 d	6.8 b	1.2 d
ex::phyA-2	6.55 bc	9.7 a	3.9 a	5.8 a	18.5 c	12.1 cd	6.4 b	4.6 b
ex::phyA-3	6.60 ab	9.0 a	3.1 bc	5.9 a	18.2 c	12.1 cd	6.1 bc	4.1 b
Vector control	6.66 a	6.9 ab	3.2 bc	3.7 abc	18.4 c	13.4 c	5.0 bc	1.6 cd
Wild type	6.53 bc	7.2 ab	2.8 c	4.3 ab	18.5 c	13.5 c	5.0 bc	1.1 d

Plant treatments: Transgenic *N. tabacum* plants expressing *Aspergillus niger* phytase (*ex::phyA-1, -2, -3*), vector control and wild-type plants. Means of 5 replicates are compared using Least Significant Difference, whereby within each column different letters represent significant differences between plant treatments (*p<0.05*).

Table 3. Total phosphorus, NaOH-EDTA extractable P, monoester organic P content of soils as determined by solution ^{31}P nuclear magnetic resonance analysis of initial Tayport soil and soil collected after 75 days with or without (no-plant) growth of *N. tabacum* plant lines, and Aitchison distance comparisons for plant and soil treatments.

Compound Class	Initial soil	Plant treatments			
		No Plant	W38	Vector	*ex::phyA-1*
		-----------mg P/kg (%)-----------			
Total P	1072	674	642	643	621
NaOH-EDTA-P	672	651	568	418	464
Total Inorganic	477.2 (71)	216.8 (33)	251.6 (44)	182.0 (44)	185.7 (40)
Orthophosphate	476.8 (71)	216.6 (33)	250.9 (44)	180.5 (43)	184.4 (40)
Polyphosphate	0.5 (0.1)	0.2 (0)	0.7 (0.1)	1.5 (0.4)	1.4 (0.3)
Total Organic	194.4 (29)	433.8 (67)	316.8 (56)	235.6 (56)	278.5 (60)
Phosphonate	0.8 (0.1)	0.6 (0.1)	0.1 (0)	1.4 (0.3)	0.7 (0.2)
Total Monoester	178.3 (27)	423.9 (65)	298.6 (53)	219.3 (53)	268.3 (58)
Glucose-6-phosphate	7.8 (1)	6 (1)	3.2 (1)	6.5 (2)	3.9 (1)
myo-InsP6	83.7 (12)	70.3 (11)	85.8 (2)	63.8 (15)	73.7 (16)
scyllo-InsP6	20.8 (3)	19.4 (3)	22.6 (15)	16.8 (15)	19.2 (16)
Other Monoester	66.0 (10)	328.1 (50)	187.1 (33)	132.1 (32)	171.5 (37)
Total Diester	15.3 (2)	9.3 (1)	18.1 (3)	14.9 (4)	9.4 (2)
α-glycerol phosphate	5.3 (1)	3.2 (0)	7.4 (1)	5.7 (1)	3.0 (1)
β-glycerophosphate	10.0 (1)	6.1 (1)	10.7 (2)	9.2 (2)	6.4 (1)
Aitchison Distance	0.95	0.00	3.24	2.52	1.69

Chemical shift assignments (ppm) of P species and compound classes determined by solution ^{31}P NMR spectroscopy: Orthophosphate (6.0), Polyphosphate (-4.2), Phosphonate (10-20), Monoester (Total orthophosphate monoesters, 3.5-7.0), Glucose-6-phosphate (5.0), Inositol hexaphosphates (*myo*-InsP6: 5.76, 4.86, 4.49, 4.35; *scyllo*-InsP6: 4.0), Other Monoester P (3.5-5.9), α-glycerol phosphate (5.3), β-glycerophosphate (4.7). Aitchison Distances are based on ^{31}P

NMR species proportions and concentrations determined for the initial soil and *N. tabacum* rhizosphere soils, including wild-type, vector control, and a transgenic plant line expressing the *A. niger* phytase (*ex::phyA-1*) and were calculated relative to the composition of the no-plant soil.

Phosphorus species in soils identified by solution 31P NMR spectroscopy

Isometric log ratio, i	OrthoP	Poly P	Phon	*myo* IHP	*scyllo* IHP	G6P	Oth Mono	α-glyc	β-glyc	r	s	Coefficient
ilr1	1	1	-1	-1	-1	-1	-1	-1	-1	2	8	1.265
ilr2	1	-1	0	0	0	0	0	0	0	1	1	0.707
ilr3	0	0	1	1	1	1	1	-1	-1	5	2	1.195
ilr4	0	0	1	-1	-1	-1	-1	0	0	1	4	0.894
ilr5	0	0	0	1	1	-1	-1	0	0	2	2	1.000
ilr6	0	0	0	1	-1	0	0	0	0	1	1	0.707
ilr7	0	0	0	0	0	1	-1	0	0	1	1	0.707
ilr8	0	0	0	0	0	0	0	1	-1	1	1	0.707

Table A1. Sequential binary partition matrix for phosphorus species proportions identified by solution ^{31}P nuclear magnetic resonance analysis of soils and used to calculate isometric log ratio values as described by Filzmoser and Hron (2009) and Abdi et al. (2015).

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Table A2. Isometric log ratio values and differences based on Table S1 SBP matrix and used to calculate the Aitchison distance, $A(x,y)$ (Abdi et al. 2015; Egozcue 2006).

Soil condition	Isometric log ratio values							
	ilr1	ilr2	ilr3	ilr4	ilr5	ilr6	ilr7	ilr8
Initial Soil	0.29	4.88	-2.71	-2.89	0.61	0.98	-1.51	-0.46
No plant	-0.66	4.86	-2.45	-3.15	-0.18	0.91	-2.83	-0.44
Wild-type	0.41	4.13	-5.41	-4.83	0.59	0.94	-2.89	-0.26
Vector	0.30	3.37	-1.98	-2.35	0.11	0.94	-2.12	-0.33
ex::phyA-1	0.55	3.46	-2.20	-2.82	0.37	0.95	-2.67	-0.54

	Isometric log ratio differences from No plant soil								
	ilr1	ilr2	ilr3	ilr4	ilr5	ilr6	ilr7	ilr8	A(x,y)
Initial Soil	0.952	0.018	-0.262	0.264	0.795	0.074	1.314	-0.015	1.847
No plant	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Wild-type	1.068	-0.735	-2.967	-1.676	0.777	0.034	-0.059	0.176	3.733
Vector	0.955	-1.487	0.469	0.804	0.292	0.033	0.703	0.106	2.140
ex::phyA-1	1.207	-1.402	0.247	0.334	0.553	0.043	0.158	-0.103	1.985

Table A3. Pair-wise correlations and significant probabilities (*, p<.05) of the measured plant and soil properties. Highlighted cells represent significant correlations or correlations referred to in the text.

	CEPi	CEPo	CEPtot	CEPphy	WEPi	WEPo	WEPtot	pH(CaCl2)	Shoot dry wt.	Root dry wt.	Tot. Biomass	S:R	Log Shoot P (µg/mg)	Shoot P Accum.	NaOH-EDTA-Pi	NaOH-EDTA-Fe	Log NaOH-EDTA-Mn	Log NaOH-EDTA-Al
CEPi(mg/kg)	1	0.911(<.0001)*	0.870(0.0242)*	0.870(0.0242)*	0.954(<.0001)*	0.611(<.0001)*	0.904(<.0001)*	-0.947(<.0001)*	-0.408(0.0429)*	-0.103(0.6234)	-0.382(0.0593)	-0.163(0.4364)	-0.336(0.515)	0.015(0.9451)	0.531(0.2196)	-0.434(0.3301)	0.893(0.0165)*	0.873(0.0104)*
CEPo(mg/kg)		1	0.981(<.0001)*	0.929(0.0073)*	0.994(<.0001)*	0.818(<.0001)*	0.986(<.0001)*	-0.917(<.0001)*	0.174(0.4047)	-0.163(0.4353)	0.123(0.559)	0.298(0.1484)	0.229(0.6614)	0.011(0.9626)	0.436(0.3284)	-0.338(0.459)	0.9575(0.0027)*	0.766(0.0444)*
CEPtot(mg/kg)			1	0.925(0.0082)*	0.993(<.0001)*	0.743(<.0001)*	0.972(<.0001)*	-0.952(<.0001)*	-0.507(0.0098)*	-0.356(0.0812)	-0.519(0.0078)*	0.129(0.5688)	-0.0671(0.8995)	-0.278(0.1792)	0.512(0.2402)	-0.417(0.3525)	0.9497(0.0037)*	0.848(0.0159)*
CEPphy(mg/kg)				1	0.183(0.7284)	-0.39(0.4347)	-0.29(0.5726)	-0.91(0.01)*	0.198(0.7061)	0.044(0.9385)	0.184(0.7268)	-0.04(0.8329)	0.4766(0.3392)	0.217(0.6792)	0.441(0.3811)	-0.35(0.4836)	0.9589(0.0025)*	0.831(0.04)*
WEPi(mg/kg)					1	0.798(<.0001)*	0.99(<.0001)*	-0.944(<.0001)*	0.209(0.3156)	-0.122(0.5609)	0.162(0.4399)	0.25(0.2279)	0.5532(0.2549)	0.281(0.1732)	0.463(0.2952)	0.357(0.4323)	0.1152(0.828)	0.828(0.0215)*
WEPo(mg/kg)						1	0.874(<.0001)*	-0.698(<.0001)*	0.27(0.1926)	-0.243(0.242)	0.192(0.3587)	0.482(0.0146)*	0.2986(0.5654)	0.245(0.2378)	0.245(0.5963)	-0.053(0.9108)	-0.5215(0.2886)	0.132(0.7787)
WEPtot(mg/kg)							1	-0.922(<.0001)*	0.281(0.1732)	-0.235(0.2584)	0.204(0.3289)	0.472(0.0173)*	0.3909(0.4435)	0.273(0.1863)	0.459(0.3003)	-0.325(0.4766)	-0.4667(0.3507)	0.747(0.0536)
pH(CaCl2)								1	-0.025(0.9074)	0.193(0.3545)	0.016(0.9392)	-0.152(0.4682)	-0.2268(0.6657)	-0.308(0.1337)	-0.625(0.1332)	0.65(0.1143)	-0.859(0.0284)*	-0.958(0.0007)*
Shoot dry wt.(g)									1	0.501(0.0107)*	0.986(<.0001)*	0.184(0.3783)	-0.2049(0.6969)	0.369(0.0697)	0.15(0.8099)	-0.353(0.5601)	-0.0573(0.9141)	-0.031(0.9608)
Root dry wt.(g)										1	0.641(0.0006)*	0.986(<.0001)*	-0.2163(0.6806)	0.054(0.7995)	-0.396(0.5091)	0.388(0.1483)	0.6664(0.1483)	0.186(0.7648)
Tot. Biomass(g)											1	0.031(0.884)	-0.2242(0.6693)	0.338(0.0988)	0.092(0.8832)	-0.282(0.6459)	0.0269(0.9597)	-0.006(0.992)
S:R												1	0.038(0.9431)	0.186(0.3731)	0.605(0.2795)	-0.783(0.1177)	-0.7523(0.0844)	-0.131(0.8333)
Log Shoot P (µg P/mg)													1	0.0028(0.9957)	0.0055(0.9918)	-0.0588(0.9118)	0.9241(0.0084)*	0.8534(0.0307)*
Shoot P Accum.(mg)														1	-0.541(0.3468)	0.433(0.4659)	0.9004(0.0144)*	0.602(0.283)
NaOH-EDTA-Pi(mg/kg)															1	-0.889(0.0075)*	0.341(0.5083)	0.625(0.1335)
NaOH-EDTA-Fe(mg/kg)																1	-0.250(0.6325)	-0.631(0.1286)
Log NaOH-EDTA-Mn(mg/kg)																	1	0.8326(0.0397)*
Log NaOH-EDTA-Al(mg/kg)																		1

CEPi, citrate-extractable inorganic P; CEPo, citrate-extractable organic P; CEPtot, citrate-extractable total P; CEPphy, Phytase-labile CEP; WEPi, water-extractable inorganic P; WEPo, water-extractable organic P; WEPtot, water-extractable total P; S:R, shoot to root biomass ratio. Data were checked for normality by Shapiro-Wilk test and log-transformed accordingly.

Fig. 1

OrthoP

myo-InsP6

InsP6
stereo.

G6P

scyllo-InsP6

MDPA
17.3ppm

α

β

7   6   5   4   3

Wild-type

Vector control

ex::phyA-1

No plant

20   10   0   −10   −20   −30

Chemical Shift (ppm)

Rhizosphere phosphorus cycling

Nicotiana tabacum

Wild-type   Null-vector   ex::phyA

Phytase
Exudation

Plant available
Inorganic
Orthophosphate monoesters
Orthophosphate diesters
Phosphonates (C-P)
organic