

# Phosphorus and Nitrogen Regulate Arbuscular Mycorrhizal Symbiosis in *Petunia hybrida*

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## Abstract

Phosphorus and nitrogen are essential nutrient elements that are needed by plants in large amounts. The arbuscular mycorrhizal symbiosis between plants and soil fungi improves phosphorus and nitrogen acquisition under limiting conditions. On the other hand, these nutrients influence root colonization by mycorrhizal fungi and symbiotic functioning. This represents a feedback mechanism that allows plants to control the fungal symbiont depending on nutrient requirements and supply. Elevated phosphorus supply has previously been shown to exert strong inhibition of arbuscular mycorrhizal development. Here, we address to what extent inhibition by phosphorus is influenced by other nutritional pathways in the interaction between *Petunia hybrida* and *R. irregularis*. We show that phosphorus and nitrogen are the major nutritional determinants of the interaction. Interestingly, the symbiosis-promoting effect of nitrogen starvation dominantly overruled the suppressive effect of high phosphorus nutrition onto arbuscular mycorrhiza, suggesting that plants promote the symbiosis as long as they are limited by one of the two major nutrients. Our results also show that in a given pair of symbiotic partners (*Petunia hybrida* and *R. irregularis*), the entire range from mutually symbiotic to parasitic can be observed depending on the nutritional conditions. Taken together, these results reveal complex nutritional feedback mechanisms in the control of root colonization by arbuscular mycorrhizal fungi.

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## Introduction

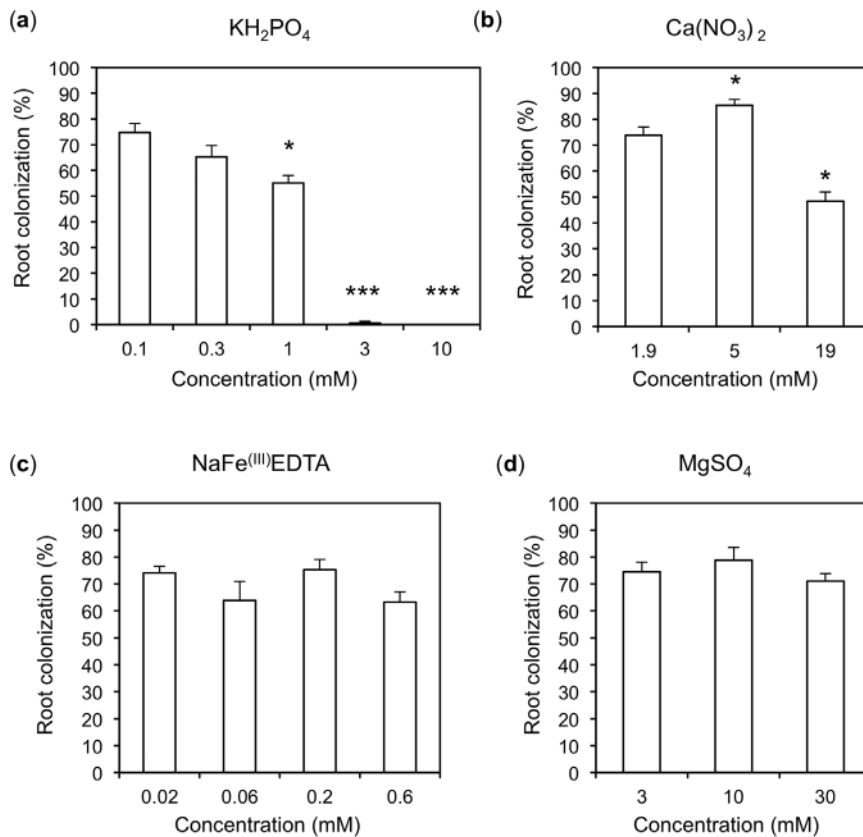
### Plant nutrition and AM symbiosis

In many environments, plant growth is limited by inadequate nutrient supply [1,2]. This condition is alleviated by a mutualistic association with soil fungi of the order *Glomeromycota* which provide the plant host with diverse mineral nutrients in exchange for assimilates [3]. This symbiosis, referred to as arbuscular mycorrhiza (AM), emerged approximately 450 Ma ago, and is thought to have facilitated the colonization of land by early vascular plants [4]. The fungal mycelium emanating from the root system reaches far beyond the rhizosphere and therefore can acquire nutrients from soil volumes to which roots have no access [5]. Furthermore, fungal hyphae are much thinner than roots [6], allowing them to explore small cracks in the micrometer range that are inaccessible to roots. Besides better nutrient supply, mycorrhizal associations provide other benefits to plants, such as increased drought tolerance and disease resistance [7–9].

The predominant nutrient element acquired through AM is phosphorus (P), which is delivered to the plant in its inorganic oxidized form phosphate (P<sub>i</sub>) [10]. Furthermore, nitrogen (N) can be transferred to mycorrhizal plants through the fungal symbiont [11–14], involving a complex pathway that has been elucidated in considerable detail in recent years [15–17]. Apart from these central macronutrients, sulfur (S) [18,19] and several micronutrients can be delivered to plants through AM fungi [20,21].

Mycorrhizal plants have two routes of nutrient acquisition, the direct pathway (DP) through the root epidermis and its root hairs, (representing the only uptake route of non-mycorrhizal plants), and the mycorrhizal pathway (MP) through the fungal mycelium that delivers nutrients to the root cortex through the arbuscules. Surprisingly, P<sub>i</sub> supply through the two pathways is not necessarily additive [22]. The contribution of the DP can be reduced in mycorrhizal plants, via the repression of constitutive phosphate transporter (PT) genes, to the extent that the plant becomes almost entirely dependent on the MP [23,24]. Hence, total P nutrition of mycorrhizal plants is not necessarily higher than in non-mycorrhizal plants. This effect is one possible reason for the observation that mycorrhizal colonization does not always result in a net gain of plant growth, i.e. in a positive mycorrhizal growth response (MGR). Indeed, even negative MGRs occur quite frequently depending on the pairing of host and fungal symbiont [25,26], raising the question why such interactions are tolerated by the plant. A plausible explanation for neutral or negative growth effects could be that AM confer a benefit other than growth promotion, e.g. a qualitative benefit, or that the benefit is not evident under the respective experimental conditions.

Modern agricultural practice has in many instances resulted in progressively reduced AM fungal diversity and frequency, an effect that is believed to be related to tillage methods [27] and to the use of mineral fertilizers [28,29]. In particular, P<sub>i</sub> has long been known to negatively impact on AM [30–38]. Inhibition of AM



**Figure 1. Exogenous phosphate and nitrate inhibit root colonization by *Rhizophagus irregularis*.** Plants inoculated with *Rhizophagus irregularis* were watered with the basic nutrient solution, additionally supplemented with the indicated nutrient concentrations. The first column to the left in each graph corresponds to the concentration in the basic nutrient solution, except for KH<sub>2</sub>PO<sub>4</sub> (see Table S2); the other columns represent elevated nutrient levels as indicated. Columns represent the average of four replicate plants with standard deviations. doi:10.1371/journal.pone.0090841.g001

development by P<sub>i</sub> is a systemic effect that depends on the nutritional status of the shoot. Since AM fungi can consume a considerable fraction of the assimilates of their hosts [26,39], the suppression of AM by high P<sub>i</sub> levels can be regarded as an energy-saving negative feedback mechanism under conditions under which the plant is optimally supplied with nutrients without the fungal symbiont. However, inhibition of AM development by P<sub>i</sub> can potentially lead to starvation for other nutrients for which the DP is not sufficiently active, hence the P<sub>i</sub> effect may reduce plant fitness depending on the supply with other nutrients. This raises the question whether the inhibition of AM development by P<sub>i</sub> may become attenuated when plants are limited for other nutrients. Indeed, such nutritional interactions have been observed under natural conditions [40], however, these findings need to be substantiated under controlled conditions.

The aim of this study was to evaluate which nutrients, besides P, influence AM development, and with which nutritional pathway the P-pathway interacts. First, the major nutrient elements P, N, K, S, Mg, Ca, and Fe were tested for inhibitory effects on mycorrhizal colonization of *Petunia hybrida* by *Rhizophagus irregularis*. These experiments, together with previous findings [34], showed that strong nutritional repression of AM is specific to P. Secondly, to evaluate how the starvation for other nutrients influences the P-effect, combinatorial treatments of plants were performed with nutrient mixtures containing high P<sub>i</sub> levels, but lacking several other nutrients alone or in combination. Our results show that the P- and N-related pathways interact, and that the AM-promoting

effect of low N supply is dominant over the AM-suppressive effect of high P<sub>i</sub> supply. These observations can serve as a basis to approach the underlying regulatory mechanisms with genetic means. Our results also show that in a given AM system (in this case *P. hybrida* and *R. irregularis*), nutrient supply has a strong influence not only on plant growth and nutrient content, but also on AM colonization and MGR. Depending on the nutritional context, AM can result in strong promotion of plant growth or in a reduction in qualitative and/or quantitative traits. Hence, the outcome of AM symbiosis is highly context-dependent.

## Materials and Methods

### Plant material, fungal inoculum and growth conditions

Two *Petunia hybrida* lines with indistinguishable interaction with arbuscular mycorrhizal fungi were used: Mitchell diploid (line W115) and line W138 (experiment 2; see below). Seeds were germinated on seedling substrate (Klasmann, <http://www.klasmann-deilmann.com>). After four weeks, plantlets were transferred to small pots (volume: 20 ml) of a sterilized mixture of 70% sand with 30% unfertilized soil (v/v; further referred to as “substrate”) for another two weeks. The substrate was sterilized by heating to 90°C for 3 h with vapor. Subsequently, plantlets were transferred to larger pots (volume: 150 ml) with the same substrate and inoculated with 1 teaspoon (*ca.* 10 g) of mycorrhizal inoculum per plant directly to the root system. Inoculum of *R. irregularis* (MUCL 43204) was produced in leek

pot cultures. The inoculum consisted of a mixture of soil and roots, and was tested for the presence of spores before use. In general, the plants were cultured in growth chambers with a day:night cycle (16 h day at 25°C; 8 h night at 20°C) with a photosynthetic photon flux density (PPFD) of 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Sylvania 36 W Luxline-Plus). Nutrient composition of the substrate (Table S1) was determined by extraction of 10 g substrate in 20 ml 0.5 M  $\text{NaHCO}_3$  (pH = 8.5) for 30 min under shaking. For the determination of sodium content, 10 g of substrate was extracted with 20 ml distilled water. Nutrient levels in the resulting soil extracts were determined as described below.

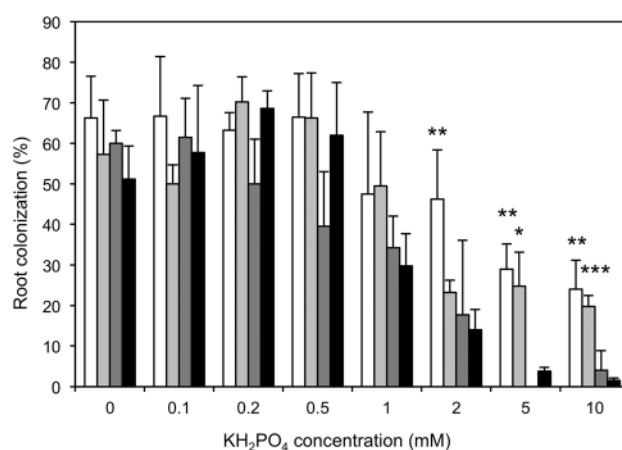
### Experimental design and nutrient treatments

Two experiments were carried out to assess the influence of nutrient supply on the level of AM colonization (Figures 1, 2). A third experiment was designed to assess the effect of nutrient supply and mycorrhizal colonization on plant growth, nutrient content, and gene expression (Figures 3–6, Table 1). A fourth experiment was carried out to confirm gene expression results from microarray analysis by qPCR (Figure 7,8). And a final experiment tested the effect of nutrient starvation on mycorrhizal colonization (Figure 9). All experiments were carried out with the experimental system described above. After the onset of the experiments, the plants were treated once per week for five weeks with the indicated solutions (experiments 1,2,4,5). Hence, each of these plants received a total of 250 ml of the indicated solutions over the five weeks of the experiment. In experiment 3, the plants were grown in a time course for 12 d (2 treatments), 22 d (4 treatments), 29 d (5 treatments), 36 d (6 treatments), or 48 d (7 treatments), with each treatment involving 50 ml of the respective nutrient solution. In general, the watering resulted in minimal leaching from the soil, thus the plants received the entire amount of nutrients applied to the pot. If necessary, in particular at later time points when plants had grown larger, they were watered a second time per week with only tap water to avoid water stress. Composition of the basic nutrient solution, of the modified solutions with elevated  $\text{P}_i$  concentration, and of the solutions lacking individual nutrients, is listed in Table S2. In the latter case, the omitted nutrients were replaced by other nutrient salts to maintain similar osmotic conditions. The effect of increasing concentrations of individual nutrients was tested with the indicated concentrations of  $\text{MgSO}_4$ ,  $\text{Fe}^{\text{III}}\text{-EDTA}$ ,  $\text{Ca}(\text{NO}_3)_2$ , and  $\text{KH}_2\text{PO}_4$ , respectively, in combination with basic nutrient solution.

### Determination of plant weight and AM colonization

Plants were harvested at the indicated time points to determine the fresh weight of the shoot and the root. Subsequently, root samples were taken for determination of total intraradical root colonization and, in case of experiments 3 and 4, the remaining root system was frozen in liquid  $\text{N}_2$  for RNA extraction. The shoots were dried (overnight at 120°C) for the determination of leaf nutrient content. The shoot:root ratio was calculated by dividing the fresh weight of the shoot by the fresh weight of the root. The mycorrhizal growth response (MGR) was calculated as the ratio of the shoot fresh weights of mycorrhizal and non-mycorrhizal plants, respectively. Data shown in Figures 3 to 6 and Table 1 come from the same experiment.

Root staining and quantification of mycorrhizal colonization were carried out as described [41]. Briefly, roots were harvested, washed and stored overnight in 10% KOH (w/v) in glass tubes. Then, they were cleared for 30 min at 95°C, washed twice with water, stained for 10 min with Trypan Blue (TB) staining solution at 95°C, and rinsed twice with 30% lactic acid (v/v). TB staining



**Figure 2. Inhibition of AM colonization by exogenous phosphate depends on the supply with other nutrients.**  $\text{KH}_2\text{PO}_4$  was applied to inoculated plants at the indicated concentrations either with basic nutrient solution (black columns) or alone (white columns). Additional treatments involved the application of  $\text{KH}_2\text{PO}_4$  with only micronutrients (light grey) or only macronutrients (dark grey), respectively. Columns represent the average of four replicate plants with standard deviations. Asterisks indicate significant differences between phosphate alone (white bars) and phosphate with micronutrients (light grey bars), respectively, vs. the treatment with a combination of  $\text{P}_i$  and basic nutrient solution (black bars). doi:10.1371/journal.pone.0090841.g002

solution consisted of 20% glycerol (w/v), 30% lactic acid (v/v) and 0.01% Trypan Blue (w/v). Microscopic inspection and quantification of root colonization was carried out as described with a modified grid intersection method [41].

In general, the columns represent the mean of the values from the indicated number of biological replicates (see figure legends). Error bars represent the standard deviations. Tests for significance of differences between treatments were carried out by pairwise t-test. Significance is classified as follows: \*  $0.05 > p > 0.01$ , \*\*  $0.01 > p > 0.001$ , \*\*\*  $p > 0.001$ .

### Determination of metal mineral nutrients

For the quantification of K, Ca, Mg, Cu, Mn, Fe, Zn and Ni by atomic absorption spectrometry, dry leaves from time point 36 d in experiment 3 were weighed, dry ashed at 550°C for 8 h and then further processed as described [42]. Briefly, the residue was dissolved in 0.2 ml 10 M HCl and 7.8 ml water. The solutions were then mixed and diluted with 1.267 g  $\text{L}^{-1}$  CsCl suprapur in 0.1 M HCl (for potassium), with 13.37 g  $\text{L}^{-1}$   $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$  in 0.1 M HCl (for calcium and magnesium) and with 0.1 N HCl (for iron, manganese, copper, zinc, and nickel) prior to measuring the elements by atomic absorption spectrometry.

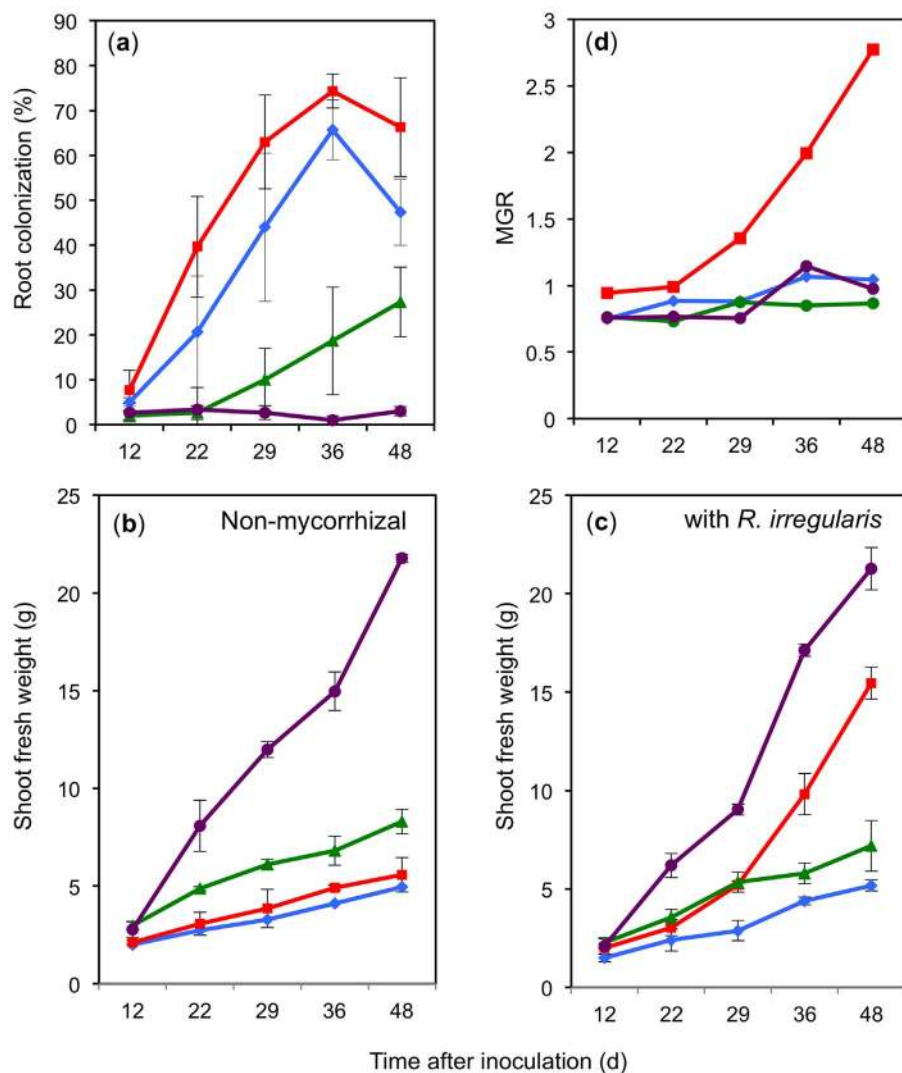
### Determination of reduced nitrogen in leaves

Determination of total reduced nitrogen followed a simplified version of the micro-Kjeldahl procedure reported by [43]. Leaf material (30 mg dry matter) was homogenized in 1 ml 0.01 M KOH and 200  $\mu\text{l}$  of this homogenate was transferred to micro-Kjeldahl tubes, mixed with 100  $\mu\text{l}$  combustion acid (4.2 M  $\text{H}_2\text{SO}_4$  containing 5.4 g/l  $\text{HgCl}_2$ ) and incubated in a sand bath first at 160°C for 40 min and then for 2 h at 300°C. The samples were allowed to cool to room temperature, then 50  $\mu\text{l}$   $\text{H}_2\text{O}_2$  (30%) were added, followed by further incubation at 160°C during 1 h and at 300°C for 2 h. After a second addition of 50  $\mu\text{l}$   $\text{H}_2\text{O}_2$  (30%) and further incubation at 160°C for

**Table 1.** Microarray analysis of N-related transporter genes in relation to the nutritional status.

	Fertilized plants			Non fertilized plants (Starved)			
	1	2	3	4	5	6	7
	AM	AM+Pi	Pi	water	AM	AM+Pi	Pi
N/P ratio (relative to fertilized control)	0.541	0.212	0.266	0.653	0.234	0.126	0.127
Cn8666 (PhNRT2)	1.44	18.08	21.27	18.2	11.2	16.33	37.53
CL1918 (PhNitr1)	7.63	13.64	10.59	6.15	26.57	27.23	23.75
cn5943 (PhNitr1)	6.42	19.23	15.71	6.23	41.61	41.41	17.95
cn8665	1.07	10.26	11.86	2.87	5.13	6.23	6.97
cn5272 (PhNRT1)	0.61	2.86	3.35	3.63	5.28	7.32	5.73
CL5245	3.95	2.69	4.05	2.51	2.98	2.58	4.58
SG_SGN-U210769	1.21	1.73	1.12	1.83	4.71	10.41	3.72
cn7864	1.32	3.01	2.96	2.83	2.69	2.85	3.51

Microarray analysis was carried out with root RNA from the plants shown in Figures 3–6 in the presence or absence of *R. irregularis* 36 d after inoculation. N/P ratio and gene expression ratios are expressed relative to reference plants supplemented with basic nutrient solution (fertilized). Relative gene induction is shown for the indicated treatments including inoculation with *R. irregularis* (AM), treatment with basic nutrient solution (fertilized plants) or water (non-fertilized plants), and treatment with 5 mM KH<sub>2</sub>PO<sub>4</sub> (Pi). The list represents nitrogen-related genes among the top ten in Table S4. Values represent induction ratios between expression values from the indicated treatments divided by the expression value from the reference treatment (only nutrient solution). The individual expression values and the corresponding variation coefficients are listed in Table S3. doi:10.1371/journal.pone.0090841.t001



**Figure 3. Dynamics of fungal root colonization and plant growth under different nutritional conditions.** Plants inoculated or not with *R. irregularis* were supplied with water (blue), basic nutrient solution (red), 5 mM KH<sub>2</sub>PO<sub>4</sub> (green), or with a combination of 5 mM KH<sub>2</sub>PO<sub>4</sub> and basic nutrient solution (purple). Samples were harvested at the indicated time points to determine root colonization (a), shoot fresh weight (b,c), and mycorrhizal growth response (MGR; (d)). MGR is defined as the ratio of the shoot weight of mycorrhizal versus non-mycorrhizal control plants. Values are the mean of three biological replicates. Error bars represent standard deviations. doi:10.1371/journal.pone.0090841.g003

15 min, 1 ml water was added followed by colorimetric determination of the reduced nitrogen (ammonium) by mixing 50  $\mu$ l of this sample solution with 100  $\mu$ l 2.5% K-Na-tartrate (w/v) and 100  $\mu$ l dilute Nessler reagent (1 part of Nessler Reagent Merck Nr 109028 and two parts 2.5 M NaOH). Absorbance was measured at 450 nm.

#### Determination of leaf phosphorus content

Dry leaf material (20 mg) was ashed overnight at 550°C in heat-resistant glass tubes. The residue was dissolved in 2 ml H<sub>2</sub>O and 100  $\mu$ l HCl (32%). 1 ml solution was then transferred to Eppendorf tubes and centrifuged in a table centrifuge (12'000 rpm for 10 min). From the supernatant, 150  $\mu$ l were transferred to microtiter plates, mixed with 40  $\mu$ l phosphate reagent, and absorbance was measured at 405 nm. Phosphate reagent (100 ml) was prepared by mixing 20 ml ammonium heptamolybdate (5% w/v (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> in water), 20 ml

ammonium metavanadate (2.5% w/v NH<sub>4</sub>VO<sub>3</sub> in water), 13.8 ml concentrated nitric acid, and 46.2 ml water.

#### Microarray analysis

Microarray analysis was performed as described [34]. Briefly, total root RNA from plants harvested at time point 36 d after inoculation (experiment 3) was extracted with the hot phenol procedure [44], pooled per treatment (equal amounts; n = 3), and sent to Nimblegen on dry ice. Array design of a four-plex microarray with 72'000 features was carried out using the Array-Scribe software from NimbleGen (<http://www.nimblegen.com>) to generate three optimized independent probes per gene, with an average length of 36 base pairs per probe. For shorter sequences, two probes per sequence were designed. Array design, probe synthesis, synthesis of labeled cDNA, hybridization, and data acquisition was carried out by Nimblegen as described [45]. Average expression values and a coefficient of variation for the gene expression values (Table S3) were derived by across-array

quantile normalization using the Robust Multichip Average (RMA) algorithm [46]. Comparative analysis of expression data sets was carried out with Fire2.2 [47]. Table S3 compiles the entire data set resulting from microarray analysis. Gene expression ratios in Table 1 and Tables S4 and S5 represent the ratios of the expression values of the respective treatment divided by the expression values of the standard treatment (fertilized plants at low  $P_i$  levels).

### Quantitative real time RT-PCR (qPCR)

For the independent assessment of gene expression by quantitative real-time polymerase chain reaction coupled to reverse transcription (qPCR), a new experiment was carried out under identical conditions as the one represented in Table 1 and Tables S4 and S5, but in this case with five biological replicates per treatment. Total RNA was extracted using the hot phenol procedure [44]. First-strand cDNA synthesis was performed with the Omniscript RT kit according to the manufacturer's guidelines. qRT-PCR was carried out with the ABsolute qPCR SYBR Green mastermix (Thermo Scientific, <http://www.thermo.com>) in a Rotorgene thermocycler (Corbett Life Science, <http://www.corbettlifescience.com>) with the primers listed in Table S6. The PCR conditions included an initial denaturation cycle for 15 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 20 s at 60°C and extension for 20 s at 72°C, followed by a final extension for 5 min at 72°C. Primer design was performed with the online primer design tool primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) on the entire predicted cDNA sequence derived from the genomic sequence, which had been identified using the EST sequences. For this reason, the qPCR results (Figure 8) relate to gene names (as in the case of the phylogenetic analysis on the predicted proteins; Figure 7), and not to the array IDs. (as in Tables S4 and S5). Table 1 and Figure 7 show which array ID corresponds to which gene name.

Each treatment is represented by five biological replicate plants that were analyzed with two technical replicates each. First, the relative expression value for each gene was obtained with the delta-delta-CT method [48] using GAPDH as constitutive reference gene for normalization. Then the relative expression ratios per treatment were derived by averaging the five biological replicates  $\pm$  standard deviations. Subsequently, induction ratios were calculated between the indicated treatments relative to the reference treatment (non-mycorrhizal plants with fertilizer; see Figure 8). T-test was performed for each treatment relative to the reference treatment. Standard deviations in Figure 8 were derived using the law of propagation of the error, based on the standard deviations resulting from the five biological replicates according to the following formula (for a pair of treatments a,b):  $StDev = \sqrt{(StDev_a/Ave_a)^2 + (StDev_b/Ave_b)^2} * (Ave_b/Ave_a)$ . Significance is classified as follows: \*  $0.05 > p > 0.01$ , \*\*  $0.01 > p > 0.001$ , \*\*\*  $p > 0.001$ .

### Phylogenetic sequence analysis

Predicted protein sequences were either retrieved from the public databases (*Arabidopsis thaliana*), or derived from genomic petunia sequences with the prediction tool Augustus (<http://bioinf.uni-greifswald.de/augustus>). The predicted amino acid sequences listed in File S1 were used for phylogenetic analysis (<http://www.phylogeny.fr/version2>).

## Results

### Phosphate and nitrate influence AM colonization

In order to test whether AM development is sensitive to other nutrients than phosphate ( $P_i$ ), the macronutrients nitrate, sulfate, calcium, magnesium, and the micronutrient iron were applied to petunia plants inoculated with *Rhizophagus irregularis* at elevated levels in the form of  $MgSO_4$ ,  $Ca(NO_3)_2$ , and  $Fe^{III}EDTA$ , respectively (Figure 1). The latter was used only at sub-millimolar concentrations because of its potential toxicity [49]. Apart from  $P_i$  (Figure 1a), the only nutrient salt that significantly affected AM development was  $Ca(NO_3)_2$  which caused a modest reduction of colonization from approximately 80% to 50% when applied at a concentration of 19 mM, whereas at 5 mM, a small enhancement of AM colonization was observed (Figure 1b). A control experiment with  $CaCl_2$  had no effect on AM (data not shown), suggesting that the effect of  $Ca(NO_3)_2$  is caused by the nitrate anion rather than by the calcium cation.  $Fe^{III}EDTA$  did not affect AM colonization (Figure 1c), and likewise,  $MgSO_4$  as a control for osmotic effects did not interfere with AM colonization (Figure 1d; compare also with [34]).

### Suppression of AM colonization by $P_i$ depends on supply with other nutrients

To test whether the inhibition of AM development by high  $P_i$  depends on the nutritional context, inoculated plants were supplied with 5 mM  $KH_2PO_4$  either alone or in combination with basic nutrient solution. In two additional treatments,  $P_i$  was supplied together with only micro- or macronutrients, respectively. While  $P_i$  caused a marked decrease of AM colonization in the presence of basic nutrient solution, or with only the macronutrients, inhibition reached only about 40% relative to fertilized plants (from ca. 65% colonization to ca. 25% colonization), when  $P_i$  was applied alone or with only micronutrients (Figure 2). Hence, the starvation for some macronutrient(s) generates an AM-promoting signal that counteracts the inhibitory effect of high  $P_i$ .

### Dynamics of plant growth and root colonization in dependence of nutrient supply

To address the dynamics of AM colonization and plant growth in dependence of nutrient supply, a time course experiment was performed with plants treated with 5 mM  $KH_2PO_4$  and nutrient solution in different combinations and in the presence and absence of *R. irregularis*. Shoot and root weight, as well as AM colonization, were determined 12, 22, 29, 36, and 48 days after inoculation. AM colonization increased most rapidly in plants treated with nutrient solution, reaching over 40% already after 22 d, whereas treatment with water resulted in generally lower colonization levels (Figure 3a). The treatment with 5 mM  $KH_2PO_4$  in combination with nutrient solution strongly inhibited AM colonization, however, the inhibition was only partial in treatments with  $P_i$  alone.

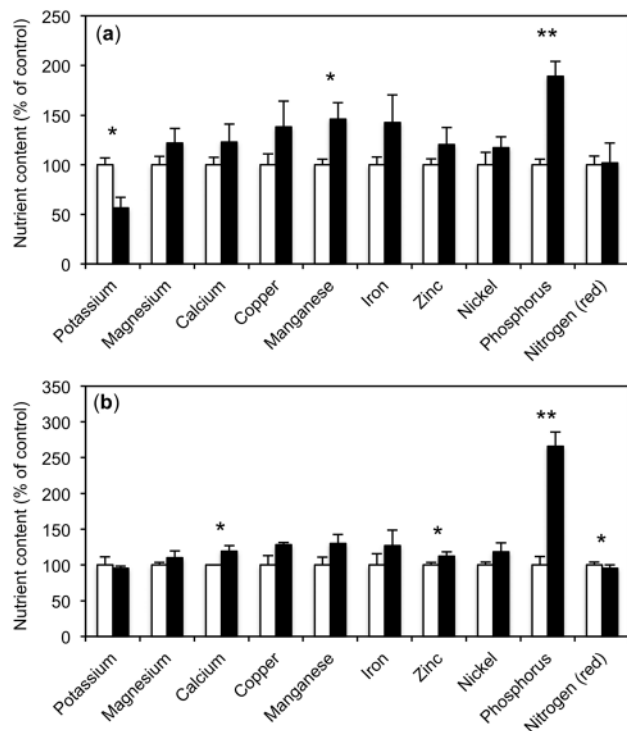
$P_i$  in combination with nutrient solution caused strong growth promotion, whereas  $P_i$  alone did only moderately stimulate growth (Figure 3b,c). This was the case irrespective of the presence of *R. irregularis*, indicating that under these conditions, fungal colonization did not provide a significant benefit. These results confirm that starvation for other nutrients reduces the inhibitory effect of  $P_i$  on AM fungal colonization (compare with Figure 2). Interestingly, a positive mycorrhizal growth response (MGR) was observed only in the presence of nutrient solution (Figure 3b-d), showing firstly that the plants could not profit from nutrient supply without the fungus, and secondly, that fungal efficiency has its limits, since plants that were not supplied with nutrient solution (and therefore

relied entirely on the low nutrient concentrations in the substrate) did not show a significant MGR despite considerable colonization levels (Figure 3a,d).

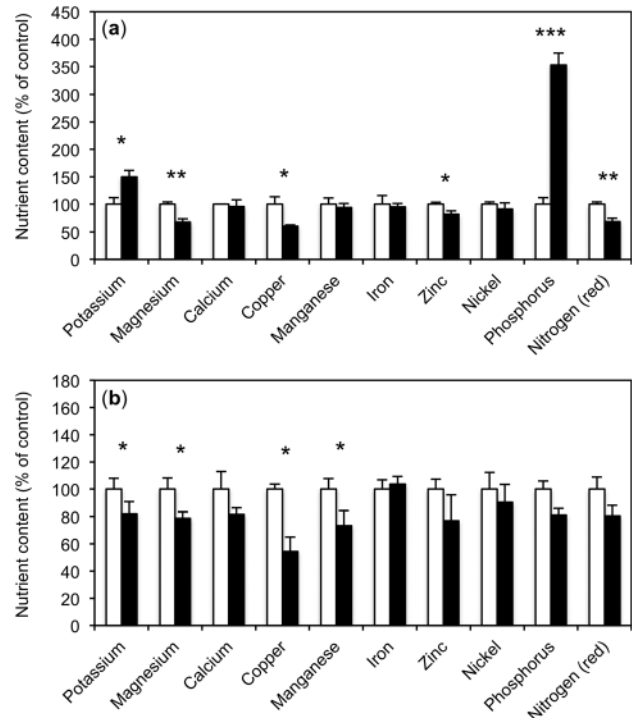
#### *R. irregularis* improves plant nutrition in petunia

The marked differences in AM colonization and MGR between the nutritional treatments (Figure 3) prompted us to evaluate the nutrient status of these plants as an indicator for plant fitness and qualitative mycorrhizal benefits (Figure S1). For easier comparison, the nutrient levels of the different treatments were normalized to the respective controls that were set at 100% (Figures 4, 5). First, the effect of AM on the levels of various metal ions, phosphate and nitrate in fertilized and non-fertilized plants were determined. Fertilized plants showed an AM-related increase in several mineral nutrient levels, however, only manganese and phosphate reached the significance threshold. In contrast, potassium levels were reduced in mycorrhizal roots relative to the non-mycorrhizal controls (Figure 4a). Hence, in addition to the quantitative benefit in growth (Figure 3), mycorrhizal plants profited from a qualitative improvement in mineral nutrition (in particular of phosphorus and manganese).

Similarly, plants treated only with water exhibited increased levels of calcium, zinc, and phosphorus, (Figure 4b). These results show that mycorrhizal plants profit, in addition to the mycorrhizal



**Figure 4. *R. irregularis* increases nutrient content of plants supplied with water or with nutrient solution.** Nutrient levels in leaves were determined 36 days after inoculation in the plants shown in Figure 3. Values are the mean of three biological replicates. Error bars represent standard deviations. Asterisks indicate significant differences between mycorrhizal roots (black columns) and non-mycorrhizal controls (white columns). (a) Plants were fertilized with basic nutrient solution. Values are expressed relative to the non-mycorrhizal fertilized controls that were set to 100% for each nutrient. (b) As in (a), but without nutrient solution. Values are expressed relative to the non-colonized water-treated controls that were set to 100% for each nutrient. doi:10.1371/journal.pone.0090841.g004



**Figure 5. Treatment with  $\text{KH}_2\text{PO}_4$  alone causes depletion of other nutrients and interferes with nutrient acquisition in mycorrhizal roots.** Nutrient levels in leaves were determined 36 days after inoculation in the plants shown in Figure 3. Values are the mean of three replicates. Error bars represent standard deviations. Asterisks indicate significant differences between  $\text{P}_i$  and water treatment (a) or between mycorrhizal and non-mycorrhizal roots (b). (a) Treatment of non-inoculated plants with 5 mM  $\text{KH}_2\text{PO}_4$  alone (black columns) compared to controls with water alone (white columns). Values are expressed relative to the water-treated controls that were set to 100% for each nutrient. (b) Nutrient content of mycorrhizal plants treated with 5 mM  $\text{KH}_2\text{PO}_4$  (black columns) compared to non-inoculated controls treated with 5 mM  $\text{KH}_2\text{PO}_4$  (white columns; corresponding to black columns in (a)). Values are expressed relative to the non-colonized  $\text{P}_i$ -treated controls that were set to 100% for each nutrient. doi:10.1371/journal.pone.0090841.g005

growth effect, from a qualitative benefit in mineral nutrition. This qualitative benefit was also observed in the absence of a positive growth response as observed in the water-treated plants.

#### Exogenous $\text{P}_i$ supply interferes with plant nutrition

To determine how  $\text{P}_i$  supply in the absence of other nutrients impacts on general plant nutrition, the nutrient levels in plants treated only with  $\text{P}_i$  were determined relative to plants treated with water (Figure 5a; Figure S1). As expected, supply with  $\text{P}_i$  alone resulted in a strong increase of P in the shoot, however, the levels of other nutrients, in particular Mg, Cu, Zn and nitrate, were reduced. This may reflect the dilution of these nutrients in the leaves as a result of the moderately stimulated growth in response to elevated  $\text{P}_i$ .

Examination of the effect of *R. irregularis* on mineral nutrition in plants treated with  $\text{P}_i$  alone (without fertilization) revealed a surprising trend. The levels of several nutrients were further reduced in these mycorrhizal plants relative to the respective non-mycorrhizal controls treated with only  $\text{P}_i$  (Figure 5b, compare with 4a,b). This indicates that the AM fungus did not provide a nutritional benefit, but rather acted like a parasite by consuming carbohydrates, and at the same time holding back mineral

nutrients. In addition, the DP for phosphate, represented by *PhPT1* and *PhPT2*, was repressed under these conditions (Tables S4, S5).

### Shoot/root ratio and N/P ratio indicate that elevated $P_i$ levels cause N-starvation

Several lines of evidence indicated that  $P_i$ -treated plants were starved for other nutrients, in particular N (see above). Therefore, three parameters that are indicative of nutritional status in plants were compared, namely shoot weight, shoot/root (S/R) ratio, and N/P ratio. Shoot growth was significantly induced by AM in fertilized plants, and by high  $P_i$  in combination with nutrient solution, indicating that these plants experienced favorable nutrient supply (Figure 6a). The S/R ratios (Figure 6b) showed a similar trend, in particular, the supply with high  $P_i$  in combination with nutrient solution caused the S/R ratio to double compared to water and  $P_i$  alone. Similarly, the strong growth effect of mycorrhizal plants with nutrient solution (Figure 3d) translated into a pronounced increase of S/R ratio (Figure 6b). In contrast, the treatments of water or  $P_i$  alone resulted in low S/R ratio even in mycorrhizal plants, indicating that these plants were nutrient-limited despite their mycorrhizal status.

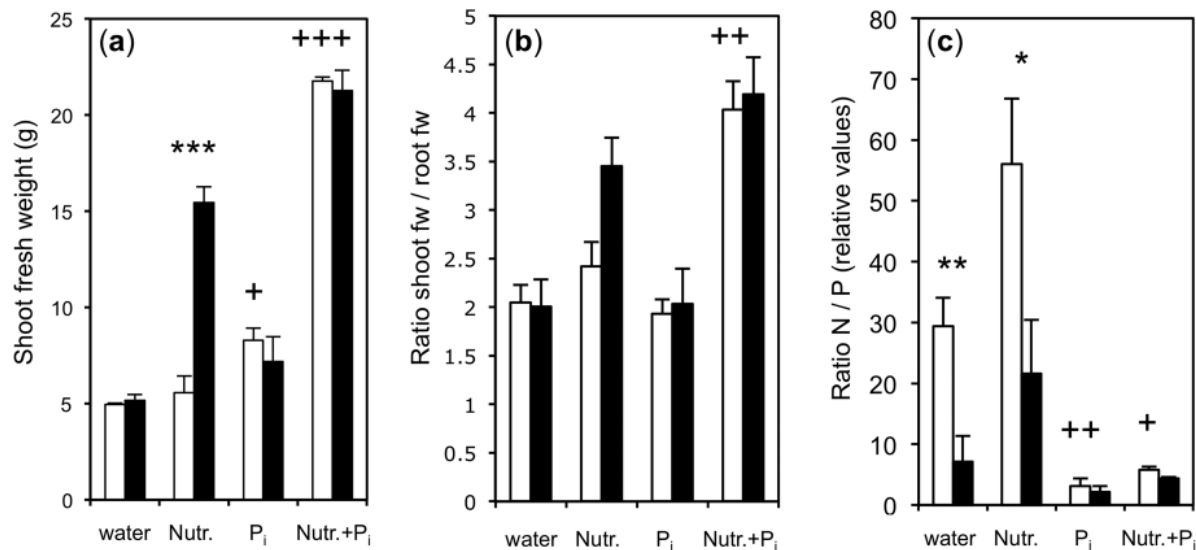
Finally, the relative levels of N and P (N/P ratio) in leaves were determined in the different treatments. Normally, the N/P ratio is in the range of 10–20 [50]. Plants treated only with water had a relatively high N/P ratio of ca. 30, and the supply with nutrient solution caused the ratio to increase to ca. 55 (Figure 6c), reflecting the effect of N supply via fertilization. Under both  $P_i$ -depleted nutrient regimes, mycorrhizal colonization dramatically reduced the N/P ratio reflecting the increased  $P_i$  supply through AM (Figure 6c). In this context, it is interesting to note that the improved P status in mycorrhizal plants supplied with only water

did not translate into an MGR (Figure 3d), indicating that these plants were starved for nutrients other than  $P_i$ . As expected, the treatments with high  $P_i$  dramatically reduced the N/P ratio in particular if  $P_i$  was applied alone (Figure 6c).

Taken together, these results indicate that only fertilized mycorrhizal plants and fertilized  $P_i$ -treated plants were well supplied with nutrients, whereas the other plants were limited for some nutrients other than  $P_i$ , conceivably N. In other words, exogenous  $P_i$  caused relative N-starvation. Furthermore, the N/P ratios of mycorrhizal plants suggest that AM promote P nutrition more than N nutrition.

### Microarray analysis of the $P_i$ effect indicates N-starvation

In order to obtain further insight into the nutritional status of the plants, microarray analysis was employed to assess the expression of genes involved in nutrient acquisition, which can serve as a diagnostic tool. In order to reveal the consequences of elevated  $P_i$  levels for plant physiology, the genes that responded to a treatment with  $P_i$  alone (in the absence of additional fertilizer) were first identified. Among a total of 660 genes that were induced at least 3-fold by  $P_i$ , the first ten contained three genes encoding predicted nitrate and nitrite transporters (data not shown). Focusing on only genes involved in mineral nutrient uptake revealed 28 genes induced 2-fold and 38 genes repressed 2-fold by  $P_i$  (Table S4). Interestingly, among the genes involved in nutrient acquisition, the ten IDs with the highest induction ratio comprised eight genes with a predicted role in nitrogen acquisition (Table 1, Table S4). Functional grouping of all  $P_i$ -regulated genes involved in nutrient acquisition confirmed that N-related genes were induced to the highest levels, followed by sulfur-related genes, whereas all PTs were repressed (Table S5). Interestingly, no ammonium transporter responded to high  $P_i$ .



**Figure 6. Shoot weight, shoot/root ratio and N/P ratio as indicators of nutritional status.** Treatments were as in Figure 3, shown are the values of the final time point (48 days after inoculation). Columns represent the average of three biological replicates, error bars represent standard deviations. Asterisks indicate significant differences between mycorrhizal and non-mycorrhizal plants (white vs. black columns), crosses indicate significant differences between the non-mycorrhizal nutrient treatments vs. the non-mycorrhizal water treatment (i.e. between the different white columns). (a) Shoot weight of plants grown with *R. irregularis* (black column) or without (white columns) under different nutritional conditions. (b) Shoot/root ratio of plants inoculated with *R. irregularis* (black columns) or without (white columns) under various nutritional conditions. A ratio of 3.5–4 indicates that plants are well supplied with mineral nutrients, whereas a ratio around 2 indicates that plants are starved and allocate relatively large amounts of resources to the root system to compensate nutritional deficits. (c) N/P ratio of the same plants as in (a),(b). In the absence of exogenous  $P_i$  supply, mycorrhizal plants (black columns) exhibited lower N/P ratios than non-mycorrhizal controls, reflecting increased mycorrhizal  $P_i$  supply. Administration of 5 mM  $KH_2PO_4$  reduced N/P ratio even stronger than AM, in particular if only  $P_i$  was supplied. doi:10.1371/journal.pone.0090841.g006



The N-related genes comprised nitrate transporters (NRTs) of both known subfamilies (NRT1 and NRT2) and a predicted nitrite transporter (Nitr1) (File S1; Figure 7). These genes were induced by  $P_i$ , irrespective of the presence of additional nutrients, and of the presence of the mycorrhizal fungus (Table 1, treatments 2, 3, 6, and 7), indicating that plants may experience relative N-limitation under all these conditions. In addition, they were induced in non-fertilized starved plants and, partially, in starved mycorrhizal roots (Table 1, treatments 4 and 5). This expression pattern is inversely correlated with the N/P ratio in the shoot (Table 1, first line; normalized to fertilized controls).

#### Quantitative real time RT-PCR (qPCR) analysis confirms results from microarray analysis

In order to independently evaluate the expression of the marker genes for N acquisition, a new experiment under equivalent conditions was performed and the expression of genes, identified by microarray analysis, was determined by quantitative real time RT-PCR (qPCR). Representative genes for the two NRT subfamilies and for the putative Nitr1 were selected (Figure 7), and their expression compared with the expression of the functional AM marker PT4 (Figure 8). In general, the expression of the N-related genes showed a similar trend as with microarray analysis. They were strongly induced in all treatments with  $KH_2PO_4$  (Figures 8a–c), and, to a lesser extent, in non-fertilized plants both, in mycorrhizal roots as well as non-mycorrhizal controls. PT4 was strongly induced in mycorrhizal plants supplied with low  $P_i$  levels, but only weakly in inoculated plants treated with  $KH_2PO_4$  (Figure 8d). Notably, the partially restored colonization in plants treated with  $P_i$  alone (28.2% colonization) was not

associated with a proportional restoration of PT4 induction (Figure 8d). This indicates that the MP for  $P_i$  remained inactive, hence, the level of AM colonization and the expression of PT4 are uncoupled under these conditions.

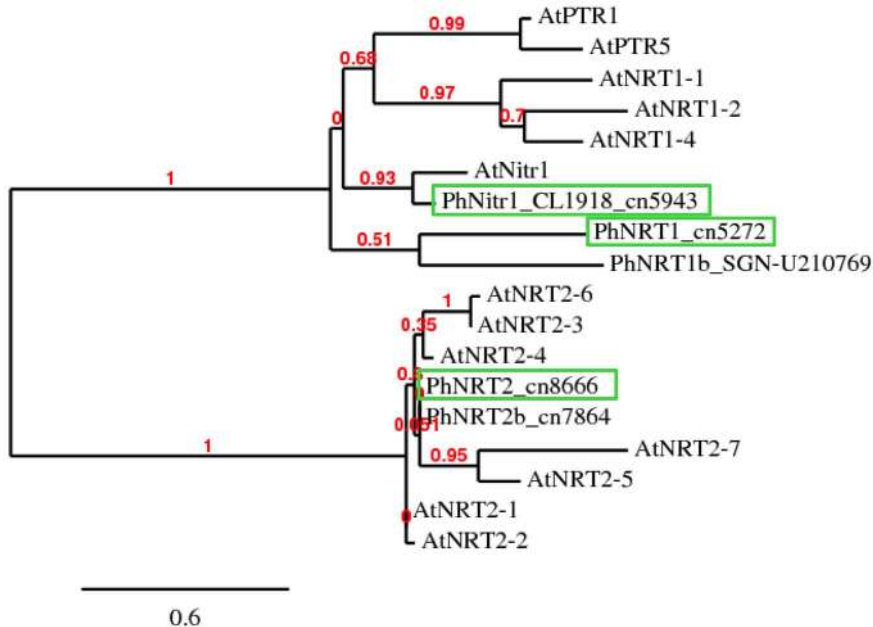
#### Suppression of AM colonization by $P_i$ depends on adequate nitrogen supply

So far, our results indicate that P caused relative N-starvation, in particular if it was applied alone (without further nutrients). Hence, it may have been the starvation for N that counteracted the inhibitory effect of high  $P_i$  and caused the recovery of root colonization. To test this possibility, several nutrients were removed individually by treating inoculated plants with high  $P_i$  together with basic nutrient solution from which individual nutrients were omitted (Figure 9; Table S2). While the treatment with all nutrients reduced AM colonization to 3%, the removal of nitrate from the nutrient solution resulted in a recovery to over 30% colonization, as in the case where all nutrients had been removed (compare with Figures 2,3). The individual removal of several other nutrient elements (S, K, Ca, Fe) also caused a partial recovery of AM colonization, although to a lesser extent.

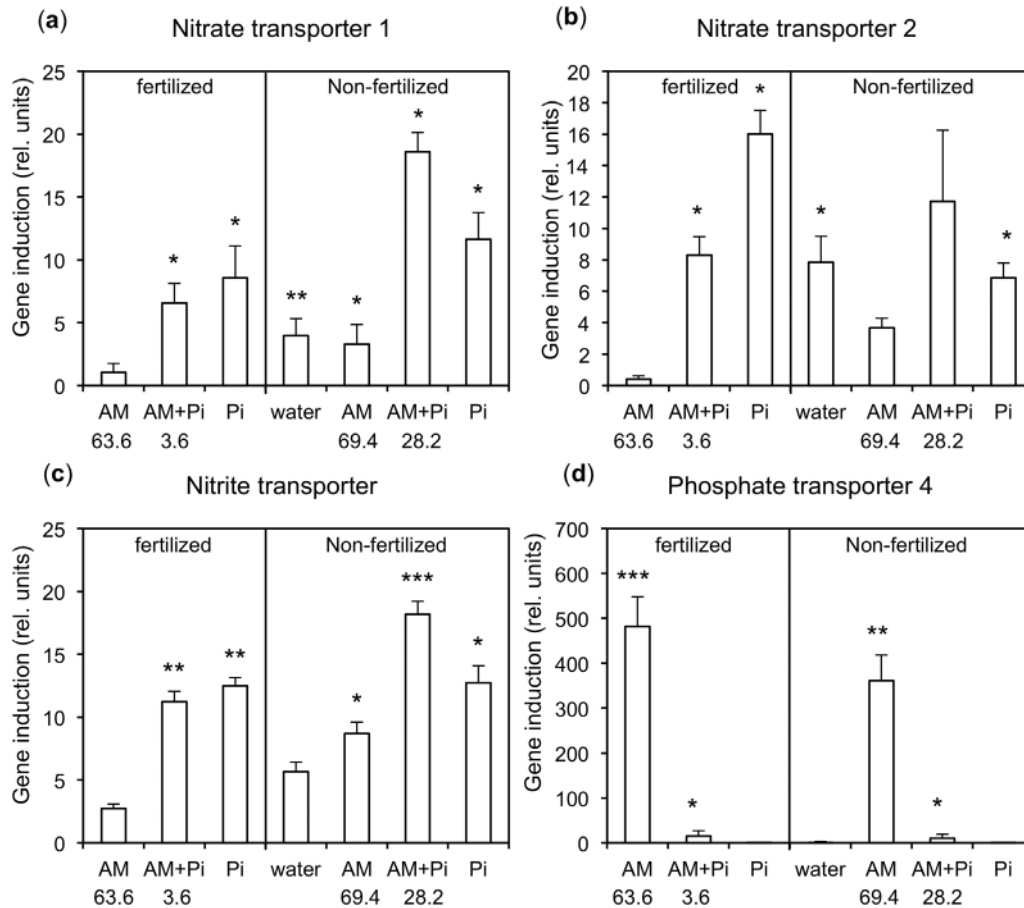
## Discussion

#### Genetic and nutritional control of AM symbiosis

During the past decade, genetic analysis of AM has revealed a suite of genes of the plant host that are essential for the establishment of the interaction and which are therefore referred to as symbiosis (SYM) genes [51]. They constitute a signaling pathway that triggers a characteristic calcium signal (calcium



**Figure 7. Phylogenetic analysis of  $P_i$ -responsive transporters of *Petunia hybrida* compared to *Arabidopsis* transporters for nitrate, nitrite, and peptides.** For the EST sequences listed in Table 1 the full-length predicted cDNA sequences were derived from the petunia genome sequence. Predicted petunia protein sequences were compared with *Arabidopsis thaliana* transporters for nitrate and nitrite (NRT and Nitr1, respectively), and for peptide transporters (PTR). Note the clear separation of the nitrate transporter subfamilies NRT1 and NRT2. The NRT1 family also comprises the nitrite transporter AtNitr1 and several peptide transporters, of which only two are represented (AtPTR2 and AtPTR5). *Petunia* has two very closely homologous representatives of the high affinity nitrate transporter family NRT2 (cn8666 and cn7864). In addition, there is a putative nitrite transporter (corresponding to the EST sequences CL1918 and cn5943), and two additional members of the low affinity NRT1 family. Genes boxed in green were analyzed by qPCR (Figure 8). Cn8665, which is almost identical to cn8666, and CL5245, which is predicted to encode a nitrogen limitation adaptation gene (see Table 1), were omitted from phylogenetic analysis. doi:10.1371/journal.pone.0090841.g007



**Figure 8. Quantitative real time PCR analysis (qPCR) of marker genes for nitrogen and phosphorus acquisition.** qPCR analysis was performed to determine the expression of the N transporters boxed in Figure 7, and of the AM-specific phosphate transporter PhPT4. Treatments were as in Figures 3–6 and Table 1. Expression values were first normalized to GAPDH and then expressed as induction ratios for the indicated treatments relative to the standard treatment (non-mycorrhizal fertilized plants), as in Table 1. Columns represent the mean of five biological replicates. Error bars represent the standard deviations derived from the two standard deviations of the compared treatments (see Materials and Methods). Numbers below the x-axis reflect the percentage of root colonization in the respective sample. doi:10.1371/journal.pone.0090841.g008

spiking) that is required for intracellular accommodation of the endosymbiont and for reprogramming of the host cells [51–53]. Apart from these endogenous factors, environmental factors influence AM in various ways. In particular, mineral nutrients are known to influence symbiosis. In general, low nutrient levels promote symbiosis, whereas high nutrient levels are inhibitory [3]. Since the primordial benefit of AM is the acquisition of mineral nutrients, the regulatory function of nutrients may represent a feedback mechanism to coordinate AM colonization with the nutritional requirements of the plant.

### Regulation of AM by phosphate

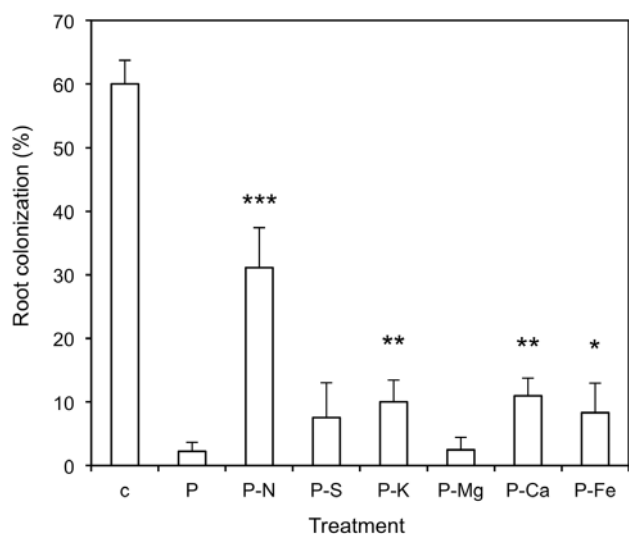
P<sub>i</sub> is thought to be the major currency with which the obligate symbiotic AM fungi pay for assimilates of the plant [54,55]. Accordingly, plants have long been known to respond particularly sensitive to high P<sub>i</sub> levels with suppression of the symbiosis [30–38]. Inhibition of AM development by P<sub>i</sub> is systemic and appears to depend on the P-status of the shoot [33,34]. Although an active role of the plant in the P<sub>i</sub>-related repression of AM is plausible, the mechanisms involved are elusive.

Transcriptomic analysis of the high P<sub>i</sub> response showed that besides the repression of the P<sub>i</sub>-starvation pathway, P<sub>i</sub> repressed carotenoid biosynthetic genes and symbiosis-related genes such as

PT4 [34]. Although production and secretion of the AM-promoting apocarotenoid strigolactone (SL) from the plant host is known to be inversely correlated with P<sub>i</sub> supply [33,56–58], reduced SL secretion alone cannot explain the inhibitory action of P<sub>i</sub>, since SL application does not alleviate the inhibition by P<sub>i</sub> [34]. A conceivable mechanism for P-related regulation of AM is the control of fungal growth through reduced supply of assimilates to the fungus [59].

### Regulation of AM by other nutrients

For optimal growth and functioning, plants rely on balanced nutrient supply, which implies some sort of coordination between the acquisition pathways for the different nutrients. In particular, the pathways for the nutrient elements required at highest amounts, namely N and P, are known to interact [50]. The present study describes a systematic analysis of the nutritional effects of P<sub>i</sub> and other nutrients on AM, and the interactions of P<sub>i</sub> with other nutrients. A first outcome is the finding that among all the tested nutrients, only P<sub>i</sub> and nitrate exerted a negative influence on AM, whereas other major nutrients such as potassium (K), calcium (Ca), magnesium (Mg), sulfate (SO<sub>4</sub>), and iron (Fe) did not influence AM at elevated concentrations (Figure 1; compare also with [34]). The relatively weak inhibitory



**Figure 9. Withdrawal of individual nutrients interferes with the inhibitory effect of  $P_i$ .** Effects of withdrawal of individual nutrients from the basic nutrient solution applied together with 5 mM  $KH_2PO_4$ . Omitted nutrients were replaced by other nutrients to maintain osmotic relationships. The strong inhibitory effect of  $P_i$  (P) was reduced particularly by removal of nitrate (P-N), but also to a lesser extent by removal of S, K, Ca, and Fe. The control treatment (c) represents fertilization with low  $P_i$  levels (0.03 mM). Columns represent the average of six biological replicates, error bars represent the standard deviations. Asterisks indicate significant differences between the treatments lacking individual nutrients and the treatment with basic nutrient solution and high  $P_i$ . doi:10.1371/journal.pone.0090841.g009

effect of nitrate suggests that this effect may not be of central importance for the regulation of AM in the case of petunia, although a significant decline of AM colonization has been associated with high nitrogen supply in other systems [60,61].

### Cross-talk of P with N and other nutritional pathways

Exogenous  $P_i$  supply in the absence of other nutrients resulted in a low S/R ratio (Figure 6b), indicative of starvation for a nutritional factor that causes the plant to prioritize root growth to compensate low nutrient supply [62]. Indeed,  $P_i$  supply resulted in strongly lowered N/P ratios (Figure 6c), i.e. in relative N-starvation. Nutrient starvation often leads to the induction of nutrient transporters to compensate for low nutrient supply [63]. For example,  $P_i$ -starvation induces  $P_i$ -transporters (PT) in *Arabidopsis* and tomato [64,65], whereas N-starvation induces various nitrate transporters (NRT) in rice and *Arabidopsis* [66,67].

Indeed, high  $P_i$  caused a strong induction of genes with a predicted role in N acquisition (Table 1; Tables S4 and S5), while transporters for other nutrients were affected to a lesser extent (Table S5). Interestingly, the  $P_i$ -related induction of genes involved in N acquisition was also observed in mycorrhizal plants, in particular in the non-fertilized ones (Table 1, treatment 5; Figure 8), consistent with the decrease of the N/P ratio in these plants (Figure 6c). Among the induced NRTs (Table S5; Figure 7), three very similar genes encoded predicted high-affinity transporters within the NRT2 family (cn8665, cn8666 and cn7864), with cn8665 and cn8666 being 100% identical at the amino acid level. Hence, only cn8666 was analyzed phylogenetically as a representative of this group (Figure 7). Cn8666 and cn7864 are particularly closely related to AtNRT2.1, which is essential for the response to nitrate starvation in the roots of *Arabidopsis* [68], and to

AtNRT2.4, which is induced by N-starvation in the root epidermis and contributes to nitrate acquisition under this condition [67]. Three further genes, represented by the EST sequences cn5272, cn5943, CL1918, and SG\_SGN-U210769, fall into the NRT1 family (Figure 7). Besides low-affinity nitrate transporters, the NRT1 family of *Arabidopsis* contains a predicted plastidial nitrite transporter (AtNitr1) [69], and a so-called nitrate transceptor [70,71], which is involved in both, the transport and the perception and signaling of nitrate [72]. The petunia EST consensus sequences cn5943 and CL1918 belong to the same petunia gene, which is most closely related to the *Arabidopsis* Nitr1 [69]. While several genes involved in N acquisition were induced by N starvation, two predicted nitrate transporters (cn8317 and cn8506) behaved in the opposite way, i.e. they were repressed by N starvation (Table S4 and S5). These genes may encode nitrate transporters that are induced by their own substrate, nitrate [73,74]. Taken together, these results indicate that  $P_i$  induces the entire nitrate acquisition pathway, and possibly nitrate sensing in petunia.

Besides the induction of nitrate transporters that are involved in the DP for N uptake, N starvation caused AM colonization to be partially restored under high  $P_i$  levels (Figures 3, 6, 9), conceivably to enable symbiotic N uptake despite the inhibitory levels of  $P_i$ . Interestingly, it has been observed in a natural environment as well that the repression of AM colonization by high  $P_i$  was relieved when N became limiting [40]. A similar interaction between P and N nutrition was shown on colonization of *Allium schoenoprasum* by *Glomus caledonium* in pot cultures [75]. These results are in line with the hypothesis that AM symbiosis evolved to ensure balanced nutrient levels in both partners and a stoichiometric relationship in the exchange of C, N, and P [76].

If AM colonization at high  $P_i$  levels was promoted to enable symbiotic N acquisition, ammonium transporters (AMT) of the plant would be expected to become induced to allow N from the fungus to be taken up in the form of ammonium [16]. However, no AMT was induced by high  $P_i$ , indicating that despite the restoration of root colonization, the symbiosis does not contribute to N uptake under these conditions, an assumption that is in line with the reduced N levels in these plants (Figure 5b). Surprisingly, the recovery of AM colonization observed in plants supplied with high  $P_i$  alone was not associated with a parallel induction of symbiotic PT expression (Figure 8d), although the colonization appeared morphologically normal. PT4 is commonly regarded as a marker for functional symbiotic cells with arbuscules, however, repression of PT4 by  $P_i$  appears to be stronger than induction of PT4 by the AM fungus. Taken together, these findings suggest that AM colonization under these conditions does not confer an obvious nutritional benefit to the plant host.

### Inhibition of AM development by high $P_i$ and by mutation of $P_i$ transporters: common mechanisms?

Interestingly, mutation of AM-related PTs in the plant causes a similar inhibitory effect on AM as high exogenous  $P_i$  supply [77–79]. However, in these cases, it is not the abundant supply, but the lack of  $P_i$  delivery that triggers inhibition. A possible explanation for this observation is that the plant can assess the costs (assimilate consumption of the fungus), and the benefits (fungal  $P_i$  supply) in the interaction, and inhibits fungal colonization if the balance is unfavorable. The fact that  $P_i$  strongly represses symbiotic PTs (Table S5) and other symbiosis-related genes [34] raises the possibility that the high  $P_i$  effect and the PT mutant phenotype may involve partially overlapping mechanisms: Although the two conditions have opposite consequences for the P status of the

plant, they both result in inhibition of the symbiotic  $P_i$  uptake pathway.

Inhibition of AM development by PT mutation has been observed in legumes and rice [77–79]. Mutation of a close homologue in tomato had no AM-related phenotype [80,81], conceivably because of functional redundancy among the three symbiotic PT genes commonly found in *Solanaceae* [81–83]. In *P. hybrida*, all three PTs were strongly repressed by  $P_i$ , thus resulting in a general conditional inactivation of the symbiotic  $P_i$  uptake pathway [34], potentially contributing to inhibition of AM development as in PT mutants of legumes and rice. An interesting parallel concerning the inhibitory effects of high  $P_i$  and of PT mutation on AM development extends to the fact that both conditions can be reversed by N-starvation (Figure 9; Javot et al., 2011).

### Mycorrhizal benefits depend on nutrient supply

The primary benefit of AM is thought to be the improved supply of macro- and micronutrients [21,84], in particular of  $P_i$ , which translates into increased growth rates of mycorrhizal plants, the so-called mycorrhizal growth response (MGR). Indeed, a robust MGR and enhanced nutrient levels in mycorrhizal plants were observed upon fertilization with basic nutrient solution (Figures 3d, 4a; Figure S1). Taking into account both the MGR and the increased nutrient content per gram of leaf tissue, mycorrhizal plants contained approximately 6-fold the amount of  $P_i$  compared to fertilized plants without AM, and approximately 4-fold the amount of the other nutrients.

Unfertilized mycorrhizal plants also had increased nutrient levels compared to non-mycorrhizal control plants, although to a lesser extent, and only  $P_i$  accumulation was substantial (Figure 4b), showing that despite the lack of an MGR (Figures 2, 3), these plants had gained a qualitative benefit from AM symbiosis. Although the growth of these plants was apparently limited by some factor, the higher nutrient content represents a potential advantage in fitness that can be translated into growth or in the production of additional offspring at a later stage of development.

On the other end of the scale, plants treated with only  $P_i$  alone were colonized to 30% but had no positive MGR and no induction of the symbiotic  $P_i$  uptake machinery. Notably, these plants even contained lower nutrient levels than non-mycorrhizal control plants treated with  $P_i$  only, hence, under these conditions, the interaction exhibited characteristics of a parasitic interaction.

MGR varies considerably with some plant species showing spectacular increases of >10-fold. Such cases are usually based on the bad performance of the plant in the absence of the fungal partner, a phenomenon that is expressed as ‘dependency’ of the plant on AM [85]. In contrast, many species, in particular cereals, show no (or even negative) MGR, and therefore are considered non-responsive [85,86]. However, it has been pointed out that such classifications represent an oversimplification [26], since the outcome of an interaction depends on both partners and involves environmental factors [87]. Indeed, our study shows that the environment has a strong influence on the MGR of *Petunia hybrida* with *R. irregularis*. Although plants in natural environments may experience less extreme conditions than in our experiments, our results show that depending on the nutritional conditions, an AM interaction can represent a range of outcomes along the continuum between strongly mutualistic and parasitic [88]. However, it should be kept in mind that mycorrhizal plants with neutral or negative MGR may profit from other benefits than improved nutrition, for example increased drought tolerance or disease resistance [7–9].

## Conclusions

Nutrient-dependent regulation of AM colonization provides an important feedback mechanism for plants to promote or limit fungal colonization according to their needs. We show here that the nutrients  $P_i$  and nitrate can potentially exert negative regulation on AM, whereas sulfate and the cations  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Fe^{3+}$  have no effect. On the other hand, starvation for several mineral nutrients, in particular for nitrate, reversed the inhibitory effect of  $P_i$  on AM, indicating that nutrient starvation triggers a dominant AM-promoting signal that counteracts the effects of high  $P_i$ . Future research should address the interplay of exogenous and endogenous factors in AM, in particular, how nutrients impinge on symbiotic signaling and on the subsequent cellular program in host cells.

## Supporting Information

**Figure S1 Nutrient content of mycorrhizal and non-mycorrhizal plants under different nutritional treatments.** Nutrient levels were determined in the leaves of plants treated with only water, with basic nutrient solution (Nutr.), with 5 mM  $KH_2PO_4$  (P), or with a combination of nutrient solution and 5 mM  $KH_2PO_4$  (Nutr.+P). Plants were harvested 36 days after inoculation with *R. irregularis* (black columns) or mock inoculation (white columns). Columns represent the average of three biological replicates, error bars represent the standard deviations.

(TIF)

**File S1 Predicted amino acid sequences of nitrate and nitrite transporters of *Arabidopsis thaliana* and *Petunia hybrida* used for phylogenetic analysis.**

(DOCX)

**Table S1** Nutrient content of plant growth substrate.

(XLSX)

**Table S2** Composition of nutrient solutions.

(XLSX)

**Table S3** Microarray data after first level analysis by Nimblegen. Mean gene expression values (Exprs) and variation coefficients (SE\_Exprs) resulting from the three probes per sequence ID are listed for the different nutritional regimes. All the induction ratios shown in Tables S4 and S5, and Table 1, are derived from the data listed in Table S3.

(XLSX)

**Table S4** Genes regulated by high  $P_i$  supply. Gene expression ratios above 2-fold and below 0.5-fold of genes with a predicted role in mineral nutrient acquisition are shown. Expression ratios were obtained by dividing the expression values from the treatment of interest (indicated combinations of basic nutrient solution, 5 mM  $KH_2PO_4$ , and *R. irregularis*) by the expression values of the reference treatment, i.e. non-mycorrhizal fertilized plants (see Table S3 for individual expression values). Expression ratios were sorted according to the treatment with high  $P_i$  alone (treatment 7). Note that the ten genes induced at highest levels comprise eight N-related genes (in bold), in particular transporters (see also Table 1).

(XLS)

**Table S5** Nutritional regulation of genes involved in nutrient acquisition. Expression ratios as in Table S4 but ordered according to predicted function.

(XLS)

**Table S6** Primers for quantitative real time RT-PCR (qPCR). (XLSX)

## Acknowledgements

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## Author Contributions

Conceived and designed the experiments: DR EN FBS UF. Performed the experiments: DR EN FBS UF. Analyzed the data: DR EN FBS UF. Contributed reagents/materials/analysis tools: DR UF. Wrote the paper: DR EN FBS UF.

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