

Arbuscular mycorrhizal growth responses are fungal specific but do not differ between soybean genotypes with different phosphate efficiency

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• **Background and Aims** Arbuscular mycorrhizal (AM) fungi play a key role in the phosphate (P) uptake of many important crop species, but the mechanisms that control their efficiency and their contribution to the P nutrition of the host plant are only poorly understood.

• **Methods** The P uptake and growth potential of two soybean genotypes that differ in their root architectural traits and P acquisition efficiency were studied after colonization with different AM fungi and the transcript levels of plant P transporters involved in the plant or mycorrhizal P uptake pathway were examined.

• **Key Results** The mycorrhizal growth responses of both soybean genotypes ranged from highly beneficial to detrimental, and were dependent on the P supply conditions, and the fungal species involved. Only the colonization with *Rhizophagus irregularis* increased the growth and P uptake of both soybean genotypes. The expression of *GmPT4* was downregulated, while the mycorrhiza-inducible P transporter *GmPT10* was upregulated by colonization with *R. irregularis*. Colonization with both fungi also led to higher transcript levels of the mycorrhiza-inducible P transporter *GmPT9*, but only in plants colonized with *R. irregularis* were the higher transcript levels correlated to a better P supply.

• **Conclusions** The results suggest that AM fungi can also significantly contribute to the P uptake and growth potential of genotypes with a higher P acquisition efficiency, but that mycorrhizal P benefits depend strongly on the P supply conditions and the fungal species involved.

Key words: Arbuscular mycorrhizal symbiosis, *Glycine max*, *Glomus aggregatum*, *Glomus custos*, mycorrhizal growth response, mycorrhizal uptake pathway, gene expression, phosphate acquisition efficiency, phosphate transporter, *Rhizophagus irregularis*.

INTRODUCTION

In addition to nitrogen, phosphate (P) is the nutrient that most often limits crop productivity and plant growth. Although the total soil contents of P may be high, P is often present in forms (e.g. organic P) that are unavailable for plant uptake. Phosphate has a low mobility in the soil (10^{-12} – 10^{-15} m² s⁻¹; Schachtman *et al.*, 1998), and rapid uptake of inorganic phosphate (P_i) from the rhizosphere leads to depletion zones around the roots that limit further P_i uptake to the slow rate of diffusion. Crop productivity in agricultural systems depends on regular applications of P fertilizers that are produced from processed ground phosphate rock, but the known global reserves of this non-renewable resource are expected to be depleted in 50–100 years (Cordell *et al.*, 2011; Schroder *et al.*, 2011). Therefore, the improvement of the P efficiency of major crops represents an urgent research priority (Wang *et al.*, 2010b; Schroder *et al.*, 2011).

The P efficiency depends on the ability of plants to take up P_i from low-P soils (P acquisition efficiency; PAE) or the ability of plants to produce biomass or yield per unit of acquired P (P utilization efficiency; PUE) (Wang *et al.*, 2010a, b). The PAE of crops can be improved by changes in the root morphology and architecture, an efficient symbiosis with AM fungi or changes in the root exudate composition leading, for example,

to an enhanced release of organic acids or phosphatases or to an acidification of the root rhizosphere (Wang *et al.*, 2010b). A better PUE of the plant is characterized, for example, by an increased efficiency with which plants are able to re-translocate P, or to re-use stored P, or to operate with lower tissue P concentrations (Wang *et al.*, 2010a).

The majority of land plants, including soybeans and other crops, form a mutualistic symbiosis with arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota. The extraradical mycelium (ERM) of the fungus acts as an extension of the root system and is able to explore a large soil matrix in the search for nutrients. The nutrients that are taken up by the fungus are then transferred to the intraradical mycelium (IRM) in the root cortex, where these nutrients are transferred across the mycorrhizal interface to the host. In return for these nutritional benefits, the host plant transfers significant amounts of its photosynthetically fixed carbon to the fungus (Wright *et al.*, 1998).

The AM colonization of the root system leads to a change in the nutrient acquisition strategy of the plant and shifts the surface for nutrient uptake from the root–soil interface to the mycorrhizal interface. In many crops, the mycorrhizal uptake pathway via the ERM and IRM plays a more important role in mycorrhizal roots for P supply than the plant uptake pathway via the epidermis and root hairs (Smith *et al.*, 2003, 2004; Li

et al., 2006). The mycorrhizal uptake pathway can, for example, account for 100 % of the total P uptake in AM-responsive flax plants, and even contributes >80 % to the total P uptake in non-responsive tomato or wheat plants (Smith et al., 2003, 2004; Li et al., 2006). High-affinity P_i uptake transporters of the plant that are involved in the P_i uptake from the root–soil interface are often downregulated in mycorrhizal roots (Grunwald et al., 2009), while P_i transporters that are involved in the P_i uptake from the mycorrhizal interface are upregulated. These mycorrhiza-inducible P_i transporters have been identified in many crop species (Rosewarne et al., 2000; Harrison et al., 2002; Nagy et al., 2005; Pumplin et al., 2012; Sisaphaithong et al., 2012), and the expression of *MtPT4*, the AM-inducible P_i transporter of *Medicago truncatula*, has been shown to be positively correlated to the P transport activity in colonized roots (Fellbaum et al., 2014). However, there are also contradictory reports in which the transcript levels of these P_i transporters were not related to mycorrhizal P uptake benefits (Grace et al., 2009; Sisaphaithong et al., 2012).

Mycorrhizal growth responses (MGRs) can range from highly beneficial to detrimental, and it has been suggested that they follow a parasitism to mutualism continuum (Johnson and Graham, 2013; Smith and Smith, 2013). The reasons for this high variability are not known, and it has been suggested that negative MGRs are mainly the result of the high carbon costs of the symbiosis for the host plant, particularly under high P supply conditions (Peng et al., 1993). However, it has also been hypothesized that negative MGRs could be the result of the suppression of the plant uptake pathway that is not compensated for by an increase in the P uptake via the mycorrhizal uptake pathway (Smith and Smith, 2011; Smith et al., 2011). The mycorrhizal P responsiveness (improvement of the P nutrition in mycorrhizal plants compared with non-mycorrhizal controls) can also be affected by the P efficiency of the plant, and it has been shown that in barley and wheat, the mycorrhizal P responsiveness was negatively correlated to the PUE of the plant (Baon et al., 1993; Zhu et al., 2001).

We studied here the mycorrhizal growth and P uptake response of two soybean [*Glycine max* (L.) Merr.] genotypes, HN112 and HN89, after colonization with three different AM fungal species, and examined the effect of these fungi on the transcript levels of P_i transporters involved in the plant or mycorrhizal uptake pathway. The genotypes differ in their root morphology and architecture. The genotype HN89 has a shallower root architecture and shows an increased exudation of malate compared with the more deep-rooted genotype HN112 (Wang et al., 2010b, 2011; Liang et al., 2013). It has been shown that HN89 has a higher PAE than HN112, because this genotype was able to outperform HN112 both under P-deficient conditions on acid soils and under field conditions with P fertilizer applications (Zhao et al., 2004; Wang et al., 2011). The goal of the study is to better understand how P-efficient plant genotypes will respond to the colonization with AM fungi. Hypothetically, it is possible that the AM colonization could nullify the advantage of P-efficient soybean genotypes (e.g. by a downregulation of P_i transporters of the plant uptake pathway, or changes in the root exudate composition), and that P-inefficient soybean genotypes with a successful AM symbiosis could outcompete their P-efficient counterparts on low P soils. Our aim is to

better understand how traits of P-efficient genotypes can successfully be combined with beneficial AM fungal communities to increase the P uptake of soybean – one of the most important leguminous crop species worldwide.

MATERIALS AND METHODS

Plant and fungal materials

The experiments were conducted with the two soybean (*Glycine max*) genotypes HN112 and HN89 that are characterized by their deep or shallow root architecture, and their low or high PAE, respectively (Zhao et al., 2004; Liao et al., 2006). The seeds were surface sterilized for 15 min in 3 % sodium hypochlorite and rinsed several times in deionized water. The seeds were then sown into pots filled with an autoclaved (121 °C for 1 h) growth substrate of sand, organic soil and perlite (80:10:10, v/v/v) with a pH of 8.2 and a plant-available P level of 0.63 mg kg⁻¹ soil (Olsen et al., 1954). After 1 week of germination, the seedlings were inoculated by adding 1000 spores of *Rhizophagus irregularis* (previously classified as *Glomus intraradices*, Schenck & Smith; DAOM 197198; Biosystematics Research Center), *Glomus aggregatum* [Schenck & Smith; isolate 0165 collected from the Long Term Mycorrhizal Research Site (LTRMS), University of Guelph, Canada] or *Glomus custos* (Cano & Dalpé; isolate 010 collected from Southwest Spain by Mycovitro S.L., Granada, Spain) into the soil close to the roots. An additional treatment without AM fungal inoculum (NM) was used as a control. The inoculum was produced by growing the fungi in root organ cultures with Ri T-DNA-transformed carrot (*Daucus carota*, clone DCI) roots in Petri dishes filled with mineral medium (St-Arnaud et al., 1996). The roots were cultured for approx. 8 weeks and the fungal spores were isolated from the growth medium by blending the medium in 10 mM sodium citrate buffer (Doner and Bécard, 1991).

Experimental design and growth conditions

First, the growth response and P efficiency of the two soybean genotypes after colonization with the different AM fungi was tested in a greenhouse experiment. The diurnal air temperatures ranged from 22 to 25 °C, and the natural light intensity from 400 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The pots were randomly placed on a single bench and regularly rearranged when watered. The plants were grown in 3.0 L pots, and supplied with three different P concentrations: 25, 100 or 500 μM P as KH_2PO_4 that will be referred to as low (LP), medium (MP) or high phosphate (HP) supply, respectively. The different P treatments were applied by watering the plants every week with a solution containing the following nutrients (in μM): KNO_3 (2.5×10^3), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (2.5×10^3), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1×10^3), K_2SO_4 (250), Fe-EDTA(Na) (80), H_3BO_3 (20), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (4.50), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.30), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.16) and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.16). The plants were harvested 50 d after transplanting, and prepared for further analysis.

Secondly, a radiotracer experiment was conducted to follow the P_i uptake and transport of *R. irregularis* and *G. aggregatum* to both soybean genotypes, and to study the expression of plant P_i transporters that are involved in the plant or mycorrhizal P

uptake pathway. For this experiment, the plants were grown in two-compartment systems made out of plastic boxes (series 500/80, Ultraplant, Espergærde, Denmark) with one root compartment (RC) filled with 500 g of substrate (see above) and one hyphal compartment (HC) filled with 100 g of substrate. The HC was separated from the RC by a plastic divider with a central hole closed by two layers of a 50 nm nylon mesh with a 1 mm air gap in between to prevent ion diffusion from the HC to the RC. The membrane allowed fungal hyphae to cross from the RC into the HC, but confined the roots to the RC. Non-mycorrhizal plants were used to confirm that there was no leakage of P from the HC into the RC, and as reference for the gene expression studies. The plants were grown in a growth chamber with a 14 h photoperiod, a 25 °C/20 °C day/night cycle and a light intensity of 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The plants were regularly watered, and every other week a nutrient solution (see above) containing 25 μM P as KH_2PO_4 (LP) was added to both compartments. The plants were harvested after 7 weeks and, 5 d before the harvest, 1.2 MBq of [^{33}P]orthophosphate (Perkin Elmer, Boston, MA, USA) was added to the HC in a sufficient amount of H_2O to ensure a homogeneous penetration of the label into the substrate of the HC.

Plant harvest

After harvest, the plant roots were cleaned with tap water, and root morphological traits, such as total root length, root surface area, root volume and root average diameter, were determined. For the analysis, the computer image analysis software Win-Rhizo Pro (Régent Instruments, Québec, Canada) was used. After the analysis, the roots were cut into segments of 1–2 cm length, and stained with 5% ink–vinegar solution as described previously (Vierheilig *et al.*, 1998). The mycorrhizal colonization was determined as the percentage of root length colonized by AM fungi using the grid intersection method (McGonigle *et al.*, 1990). The same method was also used to determine the percentage of mycorrhizal roots that were colonized with arbuscules, vesicles or intraradical hyphae. Roots and shoots were dried at 70 °C to determine the dry weight and prepared for the P analysis. The MGRs were calculated using the following formula (Hetrick *et al.*, 1992): $\text{MGR} = 100 \times (\text{AM} - \text{NM})/\text{NM}$, in which AM and NM refer to the total dry biomass of individual mycorrhizal plants or the average of non-mycorrhizal plants, respectively. Similarly, we determined the effect of the different AM fungi on the P content of the plants (mycorrhizal P response; MPR).

For the radiotracer experiment, the plants were harvested 5 d after ^{33}P labelling. At harvest, the shoots were cut off and dried to determine the biomass, and analysed for their ^{33}P content. The roots were washed, weighed and divided into three aliquots; one aliquot was used to determine the fresh to dry weight ratio, to calculate the root biomass and to determine the ^{33}P content of the root; one was used for the AM colonization assay; and one was frozen in liquid nitrogen, and later used for the expression studies of P transporter genes.

P analysis

To measure the non-labelled P in the plant samples, the dried plant material was ground using a tissue homogenizer (Precellys

24, Cayman Chemical Company, Ann Arbor, MI, USA) and an aliquot of the plant material was weighed and digested in 2 M HCl for 2 h at 95 °C. After digestion, P was determined in the samples spectrophotometrically at 436 nm after addition of ammonium molybdate vanadate solution (Fisher Scientific, Pittsburgh, PA, USA). For the ^{33}P analysis, the dried plant material was ground and an aliquot was weighed and digested in the tissue solubilizer TS-2 (Research Product International, Mount Prospect, IL, USA) for 2 h at 45 °C. Then, 150 μL of glacial acetic acid and 2 mL of liquid scintillation cocktail (Biosafe II; Research Product International) were added to each sample. The ^{33}P was measured with a liquid scintillation counter (LS-6500 Multi-Purpose Scintillation Counter, Beckman Instruments, Pullerton, CA, USA), and an internal standard was used to correct the data for differences in counting efficiency.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed according to the manufacturer's instructions. Briefly, total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA), and the supernatant was purified with the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA) and eluted into 1 μL of an RNase inhibitor (Murne, New England Biolabs, Ipswich, MA, USA). The first-strand cDNA was synthesized using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). The qRT-PCRs were performed in the ABI 7900 system (Applied Biosystems®, Life Technologies, St Aubin, France) in a final volume of 20 μL using the QuantiTect SYBR Green PCR Kit (Qiagen), 0.6 μM of gene-specific primers and 2 μL of the cDNA template diluted 5-fold. We analysed the expression of all 14 P transporter genes in soybean roots by using the specific primer pairs as shown in Qin *et al.* (2012). To normalize the data, the soybean housekeeping gene *TefS1* (X56856; Qin *et al.*, 2012) was used as a reference gene. The reaction conditions were as follows: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Fluorescence data were collected at 72 °C. Changes in gene expression were compared with non-mycorrhizal control plants, and fold induction was calculated using the $\Delta\Delta\text{CT}$ method (Winer *et al.*, 1999).

Statistical analyses

Means and standard errors of five biological replicates were calculated using Microsoft Excel 2003 (Microsoft Company, Seattle, WA, USA). The statistical software SAS (SAS Institute Inc., Cary, NC, USA) was used for the two- or three-way analyses of variance (ANOVA; the results are given in Supplementary Data Tables S1–S5). Significant differences among treatments were assessed by Duncan's multiple comparison test ($P \leq 0.05$) using SAS. Significant differences are shown in the figures as different letters on the bars.

RESULTS

Effect of AM fungi on biomass of two soybean genotypes under different P supply conditions

The growth of both soybean genotypes was affected by the P availability and the mycorrhizal fungal species that colonized

the root system (Fig. 1). Both shoot and root dry weights significantly increased with higher P availabilities (Fig. 1; Table S1). The genotype HN112 showed a stronger biomass response after an increase in the P supply conditions. Compared with LP conditions, the HP treatment increased the shoot biomass of non-mycorrhizal HN112 plants by 166.5%, but that of non-mycorrhizal HN89 plants only by 100.7%. The genotype HN89 had a significantly higher root biomass than HN112 under LP conditions, but the shoot biomass did not differ between the genotypes.

Both soybean genotypes showed consistent growth responses after inoculation with the different AM fungi (Fig. 1). Compared with the NM control, *R. irregularis* led to a significant increase in the shoot biomass of both genotypes under LP and MP availabilities, and of HN89 also under HP supply conditions. In contrast, the inoculation with *G. aggregatum* had no effect on the shoot biomass under LP conditions, but reduced the shoot biomass of HN112 and HN89 plants under MP conditions. The shoot biomass was generally not affected by an inoculation with *G. custos*; only at MP did we observe a decrease in the shoot biomass of HN89 plants. The root biomass was only slightly affected by an AM colonization; only plants that were

colonized with *G. aggregatum* had a lower root biomass under MP than non-mycorrhizal control plants (Fig. 1C, D).

The two soybean genotypes differed in their root architectural traits particularly under HP supply conditions, and HN112 was characterized by a longer total root length and a larger root surface area than HN89 (Supplementary Data Fig. S1A, B; Table S2). In contrast, HN89 plants had a slightly larger root average diameter than HN112 plants (Supplementary Fig. S1C). Root architectural traits were affected by the P supply conditions, and HP availabilities led to an increase in the total root length, the root surface area, the root volume and the root average diameter (Fig. S1). In contrast, AM fungal inoculations generally did not have an effect on root architectural traits, and only the colonization of the roots with *G. aggregatum* led to a reduction in the total root length, the root surface area and the root volume in HN112 under MP conditions (Fig. S1A–D).

The addition of P significantly increased the total P content in roots and shoots of non-mycorrhizal and mycorrhizal plants of both soybean genotypes (Fig. 2; Table S1). Of the AM fungal species that were tested, only the inoculation with *R. irregularis* led, under all P supply conditions, to a significant increase in the shoot and root P contents of both soybean genotypes

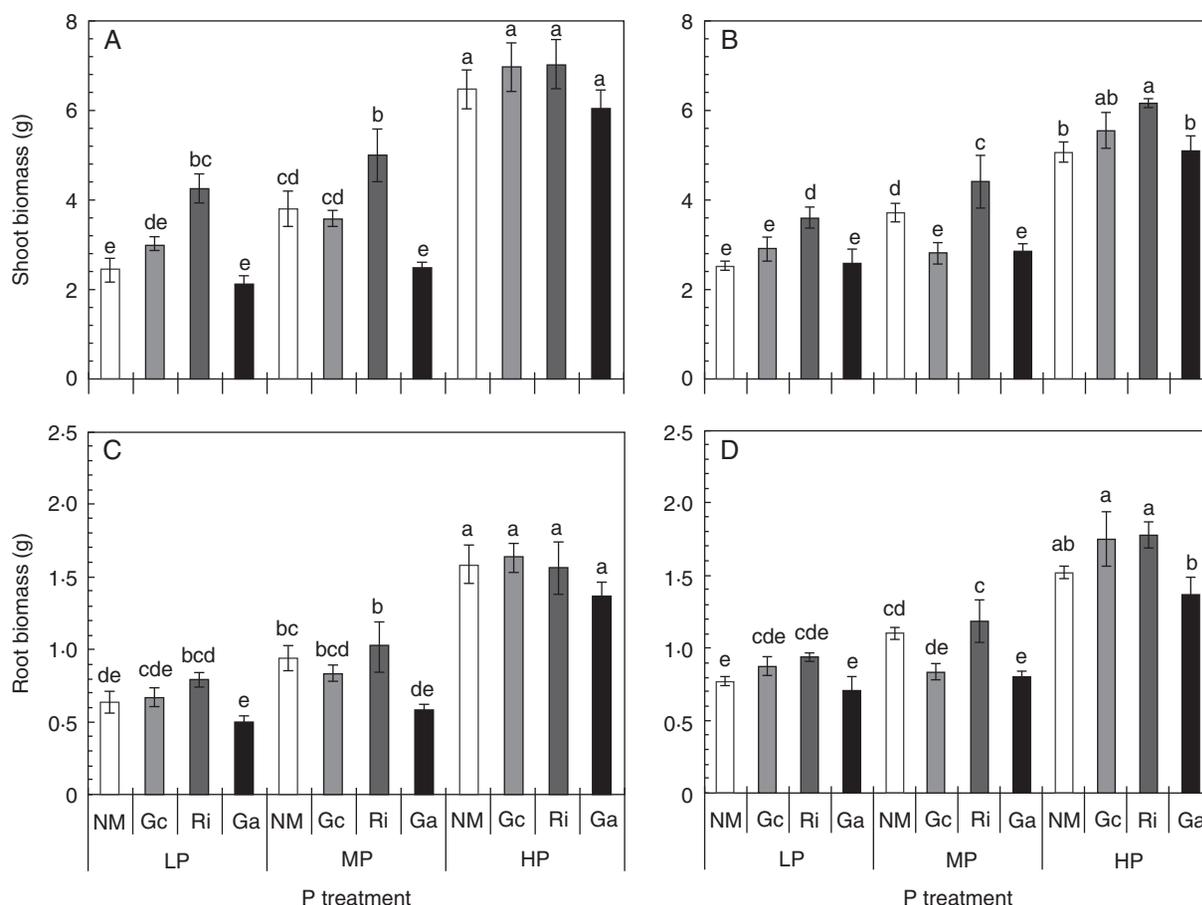


Fig. 1. Shoot (A, B) and root (C, D) biomass of the two soybean genotypes HN112 (A, C) and HN89 (B, D) after inoculation with three different AM fungal species. The plants were grown for 7 weeks under low (LP), medium (MP) or high (HP) P supply conditions. Shown in the figure are the means of five biological replicates \pm s.e.m. NM, non-mycorrhizal controls; Gc, Ri and Ga, plants colonized with *Glomus custos*, *Rhizophagus irregularis* or *Glomus aggregatum*, respectively. ANOVA results are shown in Table S1. Different letters on the bars indicate significant differences among treatments of one soybean genotype according to Duncan's multiple comparison test ($P \leq 0.05$).

relative to non-mycorrhizal controls. Plants that were colonized with *G. aggregatum* or *G. custos* did not differ in their root or shoot P contents from non-mycorrhizal controls (Fig. 2). The mycorrhizal colonization with *R. irregularis* also led to an increase in the P tissue concentration (P per unit plant biomass) in roots and shoots under LP and MP conditions. However, there was no difference in the shoot P concentrations between non-mycorrhizal or mycorrhizal plants when the plants were supplied with HP (Fig. S2; Table S2).

In the first experiment, the non-mycorrhizal plants of both soybean genotypes did not differ in their biomass or their P contents in the shoot under LP conditions (Figs 1 and 2), and both soybean genotypes showed a similar mycorrhizal responsiveness in terms of biomass (Fig. 3A, B) or P contents (Fig. 3C, D). Only at LP, the P-inefficient genotype HN112 showed a slightly higher MGR and MPR than the P-efficient genotype HN89. *Rhizophagus irregularis* clearly increased the biomass and P uptake of both soybean genotypes under all P supply conditions, while *G. aggregatum* and *G. custos* generally led to neutral MGR or MPR (Fig. 3A–D). The colonization with *G. aggregatum* caused a negative MGR only at MP supply. The positive effects of *R. irregularis* on soybean growth and P uptake decreased with increasing P availabilities in the soil (Fig. 3A–D).

We also analysed the percentage root length that was colonized by AM fungi, and found that all AM fungal species formed typical mycorrhizal structures in both soybean genotypes, including ERM, IRM, vesicles and arbuscules. However, while *R. irregularis* reached colonization rates of up to 70%, the mycorrhizal colonization rates of *G. aggregatum* or *G. custos* were significantly lower in both soybean genotypes (Fig. 3E, F). Increasing P availabilities in the soil led to a reduction in the colonization rates of HN112 with *G. custos* and *R. irregularis* (Fig. 3E).

Transport of P to both soybean genotypes via the mycorrhizal uptake pathway

To explore whether the differences in the MGR after the colonization with *R. irregularis* or *G. aggregatum* were due to differences in the plant or mycorrhizal P uptake pathway, the transport of P from the ERM to the host plant was followed in two compartment systems. The plants of this experiment were grown in growth chambers under LP conditions, and in this experiment the biomass and the P contents of roots and shoots of HN89 plants were significantly larger than those of HN112 plants (Supplementary Data Fig. S3; Table S1). In contrast to the growth response experiment that was conducted in the

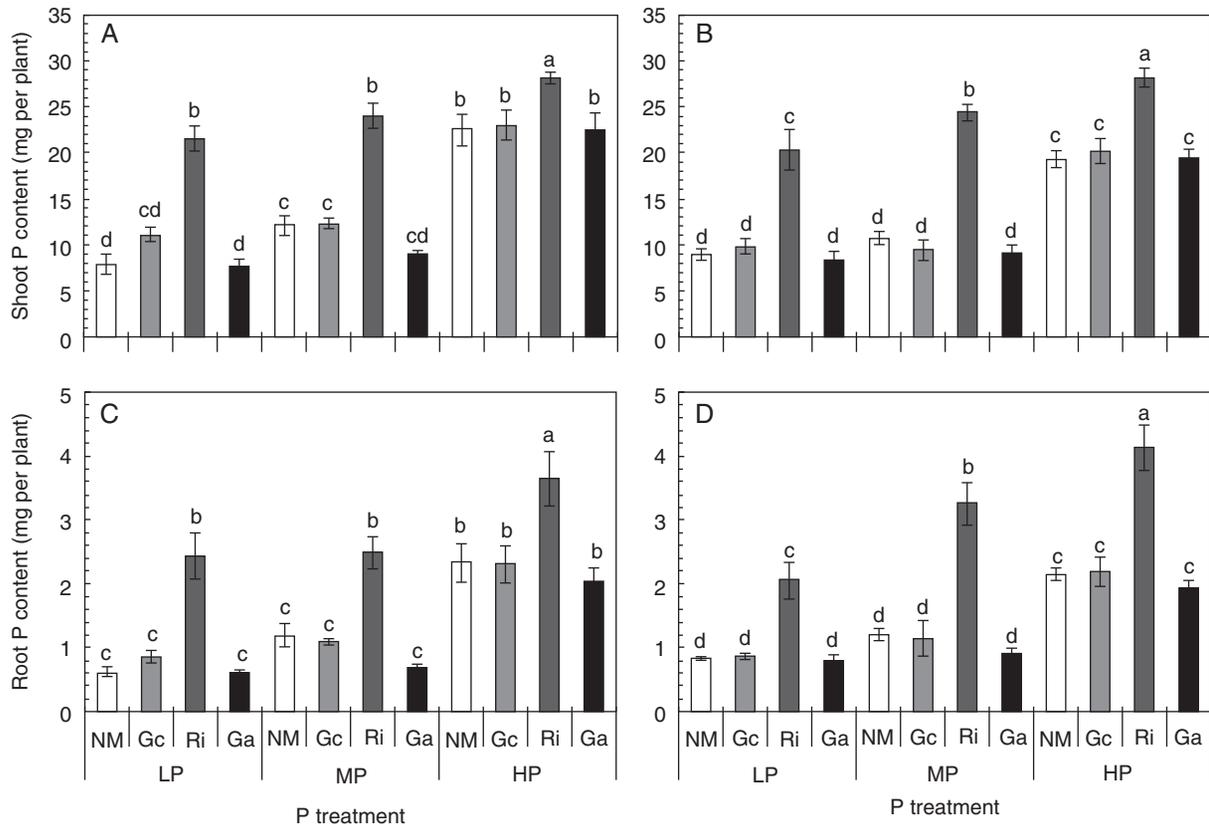


FIG. 2. P contents in shoots (A, B) and roots (C, D) of the two soybean genotypes HN112 (A, C) and HN89 (B, D) after inoculation with three different AM fungal species under different P supply conditions. The plants were grown for 7 weeks under low (LP), medium (MP) and high (HP) P supply conditions. Shown in the figures are the means of five biological replicates \pm s.e.m. NM, non-mycorrhizal controls, Gc, Ri and Ga, plants colonized with *Glomus custos*, *Rhizophagus irregularis* or *Glomus aggregatum*, respectively. ANOVA results are shown in Table S1. Different letters on the bars indicate significant differences among treatments of one soybean genotype according to Duncan's multiple comparison test ($P \leq 0.05$).

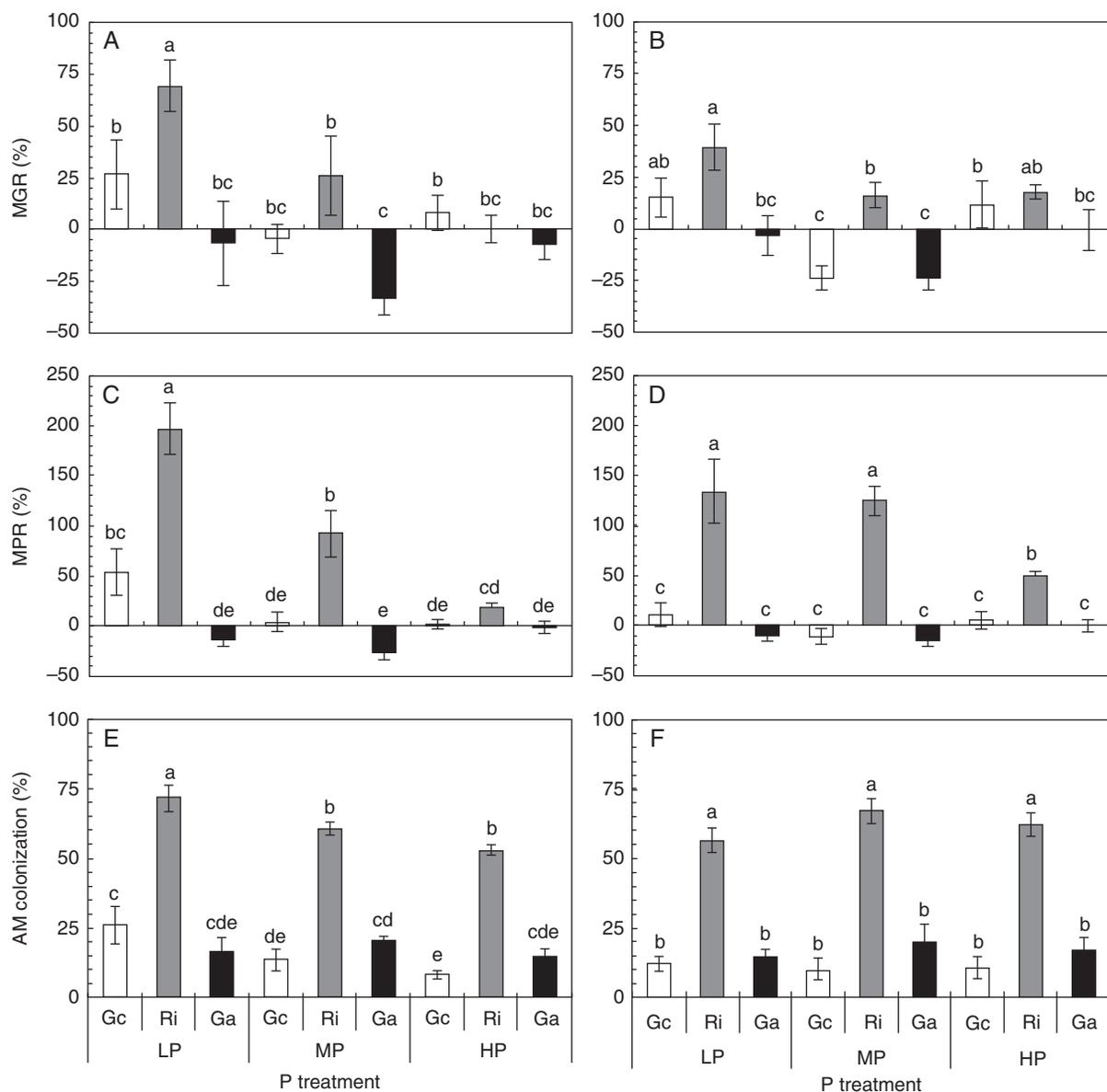


Fig. 3. Mycorrhizal growth response (A, B), mycorrhizal phosphate response (C, D) and AM colonization (E, F) of the two soybean genotypes HN112 (A, C, E) and HN89 (B, D, F) after inoculation with three different AM fungal species. The plants were grown for 7 weeks under low (LP), medium (MP) and high (HP) P supply conditions. Shown in the figures are the means of five biological replicates \pm s.e.m. Gc, Ri and Ga, plants colonized with *Glomus custos*, *Rhizophagus irregularis* or *Glomus aggregatum*, respectively. ANOVA results are shown in Table S1. Different letters on the bars indicate significant differences among treatments of one soybean genotype according to Duncan's multiple comparison test ($P \leq 0.05$).

greenhouse, the plants in this experiment were exposed to shorter daylengths, and HN112 plants had already started to flower and to develop seed pods. In this experiment, the effect of *R. irregularis* on biomass and P content was not as large as in the growth response experiment. However, an inoculation with *R. irregularis* increased the shoot biomass and P content of HN112 plants (Fig. S3A, C; Table S3), and led to a higher root biomass of HN89 plants and higher root P contents in both soybean genotypes compared with NM control plants (Fig. S3B, D; Table S3). Consistent with the previous experiment, an inoculation with *G. aggregatum* did not have an effect on the biomass and the P contents of both soybean genotypes.

The root colonization rate with *R. irregularis* was very high in HN112 and HN89 plants (82.5 and 79.5%, respectively), but the colonization of the root systems with *G. aggregatum* was significantly lower (12.5% for HN89 and 67.9% for HN112) (Supplementary Data Fig. S4A; Table S3). Consistent with the higher root colonization, we found a higher number of arbuscules and vesicles in the mycorrhizal roots of both soybean genotypes that were colonized with *R. irregularis* (Fig. S4B, C; Table S3), but the root colonization with intraradical hyphae did not differ between both fungi in HN112 (Fig. S4D).

Both fungi translocated P from the ERM to the host plants, but *R. irregularis* transferred more P than *G.*

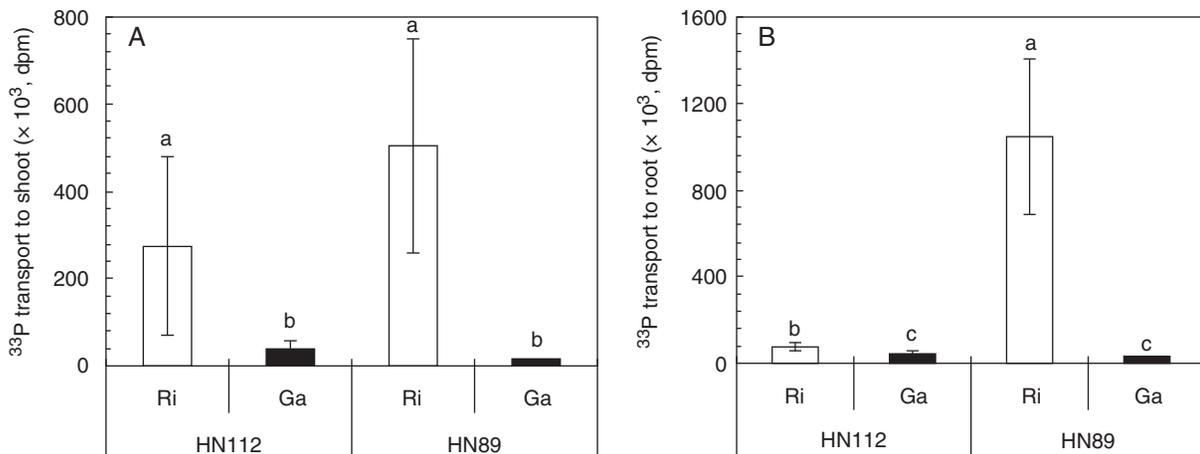


FIG. 4. ^{33}P transport by the ERM of *R. irregularis* (Ri) or *G. aggregatum* (Ga) to the shoot (A) or the roots (B) of the two soybean genotypes HN112 or HN89. The plants were inoculated with *Rhizophagus irregularis* (Ri) or *Glomus aggregatum* (Ga). Shown in the figures are the means of three biological replicates \pm s.e.m. ANOVA results are shown in Table S4. Different letters on the bars indicate significant differences among different AM treatments and soybean genotypes according to Duncan's multiple comparison test ($P \leq 0.05$).

aggregatum (Fig. 4) (Table S4). HN112 and HN89 plants that were colonized with *R. irregularis* had higher count rates in their roots and shoots than plants that were colonized with *G. aggregatum*. While in HN89 plants a significant proportion of the P that was transferred remained in the root system, the majority of the transferred P was detectable in the shoot of HN112 plants.

Transcript levels of P_i transporter (PHT1) genes

We measured the expression of 14 P_i transporter genes in mycorrhizal and non-mycorrhizal roots of both soybean genotypes under LP conditions and found that the transcript levels of 13 of these transporter genes were affected by the AM colonization. Several transporter genes were downregulated (*GmPT1*, *GmPT2* and *GmPT11*) by both AM fungi, while others were upregulated by *R. irregularis* alone (*GmPT10*) or by both AM fungi (*GmPT9*) (Fig. 5; Supplementary Data Fig. S5). Other transporter genes such as *GmPT4* were induced by a colonization with *G. aggregatum*, but suppressed by a colonization with *R. irregularis* in both soybean genotypes (Fig. 5A). The mycorrhiza-inducible P_i transporter gene *GmPT9* on the other hand was upregulated in the mycorrhizal roots of both soybean genotypes, but the transcript levels were much higher in HN89 than in HN112 (Fig. 5B). While we found an up to 1100-fold induction of *GmPT9* in HN89, the transcript levels were only induced by up to 42-fold in HN112. In contrast, *GmPT10*, another mycorrhiza-inducible P transporter, was only upregulated (up to 17-fold) in roots that were colonized with *R. irregularis*, but not in roots that were colonized with *G. aggregatum* (Fig. 5C). Several other P_i transporter genes, including *GmPT3*, *GmPT5*, *GmPT6*, *GmPT8*, *GmPT12*, *GmPT13* and *GmPT14*, were induced by a colonization with *G. aggregatum* in the P-efficient soybean genotype HN89, but were downregulated or unaffected by *R. irregularis* (Supplementary Data Fig. S5; Table S5).

DISCUSSION

Mycorrhizal growth responses can range from highly beneficial to detrimental, and have been suggested to follow a mutualism to parasitism continuum (Johnson *et al.*, 1997; Johnson and Graham, 2013; Smith and Smith, 2013). Mycorrhizal growth responses are context dependent and are affected by the nutrient supply conditions (Peng *et al.*, 1993), and/or the fungal or plant species involved (Smith *et al.*, 2004; Koch *et al.*, 2006), but the mechanisms that are responsible for the high variability in mycorrhizal benefits are only poorly understood. We studied the effect of different AM fungi on the mycorrhizal growth response and P uptake of two soybean genotypes that differ in their root architecture and PAE to determine whether and how the mycorrhizal dependency of the host affects mycorrhizal growth and P uptake benefits. The MGRs under our experimental conditions ranged from highly positive to negative, but the mycorrhizal growth or P uptake benefits demonstrated a stronger dependency on the fungal partner involved or the P supply conditions than on the PAE of the host (Figs 1–3).

It has been suggested that HN89 with its shallow root system has a higher PAE, because HN89 outperformed HN112 under P-deficient conditions on acid soils (Zhao *et al.*, 2004; Wang *et al.*, 2011) and in soils with toxic aluminium concentrations (Liao *et al.*, 2006). In our experiments, the growth or P uptake responses of non-mycorrhizal HN89 plants were higher than those of HN112 plants under LP conditions (Supplementary Data Fig. S3). Accordingly, the P-inefficient genotype HN112 showed, after an inoculation with *R. irregularis*, a slightly higher growth and P uptake response under LP supply conditions than the P-efficient genotype HN89 (Fig. 3; Fig. S3A). This is consistent with the findings of Wang *et al.* (2011), who reported that HN112 benefited more strongly from the co-inoculation with N-fixing bacteria and AM fungi than HN89. Mycorrhizal growth benefits, however, depended more strongly on the fungal species involved, and we found that only *R. irregularis* increased the plant biomass and P uptake of both soybean genotypes under all P supply conditions. In contrast, *G. custos*

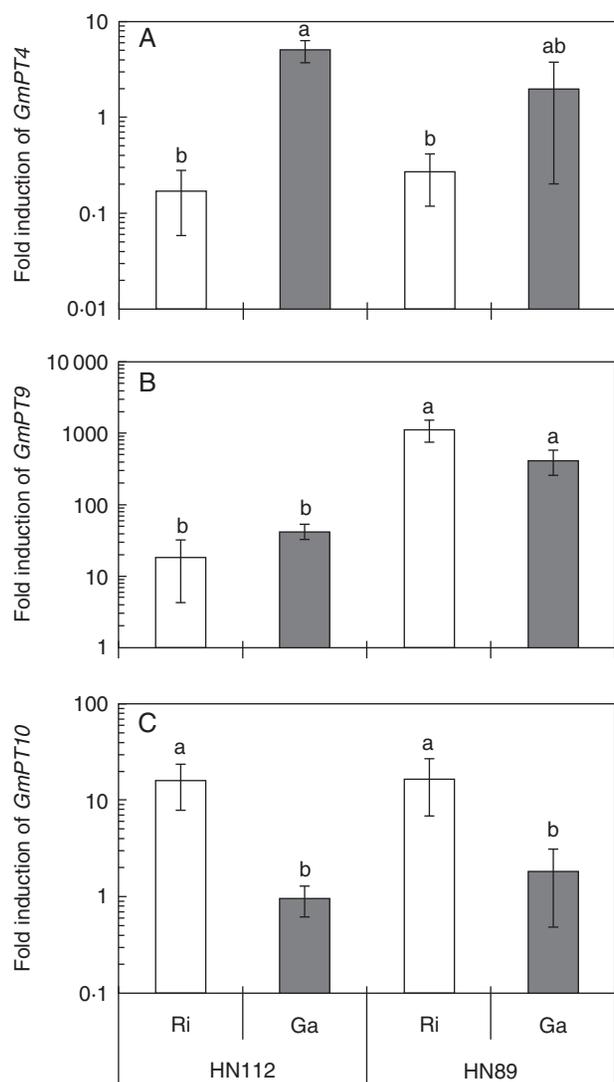


FIG. 5. Expression of the high affinity P_i transporter genes *GmPT4* (A), and the two mycorrhiza-inducible plant P_i transporter genes *GmPT9* (B) and *GmPT10* (C) in mycorrhizal roots of the two soybean genotypes HN112 and HN89 after inoculation with *Rhizophagus irregularis* (Ri) or *Glomus aggregatum* (Ga). Shown in the figures are the means of three biological replicates \pm s.e.m. Different letters on the bars indicate significant differences among different AM treatments and soybean genotypes according to Duncan's multiple comparison test ($P \leq 0.05$). ANOVA results are shown in Table S5. Values <1 represent a decrease and values >1 an increase in transcript levels relative to the NM control.

and *G. aggregatum* did not lead to positive growth or P uptake benefits of either soybean genotype, and *G. aggregatum* even led to growth depression under MP supply conditions. Fungal species or isolates of one species differ in the efficiency with which they provide nutritional benefits to their host (Koch et al., 2006; Avio et al., 2009; Ehinger et al., 2012; Mensah et al., 2015), and it has been suggested that the compatibility between the fungus and the host plant plays a significant role in these performance differences among various fungal species (Smith et al., 2004). *Glomus custos* and *G. aggregatum*, however, also led to smaller growth responses than *R. irregularis* in *Allium porrum* and *M. truncatula*. This supports the results of Kiers

et al. (2011), who characterized these *G. custos* and *G. aggregatum* isolates as less beneficial compared with *R. irregularis*. Root organ culture experiments demonstrated that *G. aggregatum* transfers less P per unit carbon to mycorrhizal roots than *R. irregularis* (Kiers et al., 2011).

The mycorrhizal colonization of both soybean genotypes with *R. irregularis* was higher than that with *G. custos* or *G. aggregatum*. It seems unlikely, however, that the lack of positive MGR and MPR by an inoculation with *G. custos* or *G. aggregatum* was the result of the lower mycorrhizal colonization rates with these fungi. Though *R. irregularis* reached higher colonization rates than *G. custos* or *G. aggregatum* in both experiments (Fig. 3E, F; Supplementary Data Fig. S4A), the colonization rate of HN112 with *G. aggregatum* was higher and reached 67.9% in the radiotracer experiment. The higher colonization with *G. aggregatum*, however, also had no effect on biomass or P content in this experiment (Fig. S3). The results of a recent meta-analysis suggest that while the extent of mycorrhizal colonization can lead to higher MGR or MPR, fungal taxa differ considerably in their degree of host plant benefit per root length colonized (Treseder, 2013). This is also supported by the fact that despite the relatively low colonization rate in the growth response experiment, *G. aggregatum* caused a significant growth depression under MP conditions (Fig. 1). Other authors have also observed a large negative MGR in plants with relatively low colonization rates (Hetrick et al., 1992; Li et al., 2006; Grace et al., 2009). The negative MGR could be a result of the changes in root architectural traits (Fig. S1) that we observed after an inoculation with *G. aggregatum* under MP conditions. While there were no significant effects of the AM colonization on the root architectural traits of both genotypes under low or high P supply conditions, an inoculation with *G. aggregatum* significantly reduced the total root length, the root surface area and the root volume of HN112 roots under MP conditions (HN89 roots showed the same trends, but the differences from the NM controls were not significant).

We observed a decrease in the MGR by *R. irregularis* with increasing P availabilities in the soil, and a mycorrhizal growth depression in both soybean genotypes after an inoculation with *G. aggregatum* when more P became available (Figs 1 and 3). Decreasing or negative MGRs have often been described as a result of high P availabilities in the soil (Peng et al., 1993; Kahiluoto et al., 2012). Negative MGRs at high P availabilities have often been attributed to the high carbon costs of the symbiosis (Wright et al., 1998; Graham, 2000) that are not counterbalanced by a net gain in P (Valentine et al., 2001; Jifon et al., 2002). However, to what extent the carbon costs of the symbiosis can explain negative or decreasing MGR under high P availabilities is still under debate (Li et al., 2008; Johnson and Graham, 2013; Smith and Smith, 2013), because the AM fungal colonization also increases the photosynthetic rate (Wright et al., 1998; Zhu et al., 2012), and it has been suggested that the host is able to regulate the carbon costs of the symbiosis, e.g. by a premature degradation of arbuscules under high P supply conditions (Javot et al., 2007; Breuillin et al., 2010). A shorter arbuscular life span could, for example, explain the decrease in the mycorrhizal colonization of HN112 plants with *R. irregularis* under HP supply conditions (Fig. 3E).

However, it has also been hypothesized that a negative MGR may be the result of a reduced P uptake via the plant uptake pathway (via the epidermis and root hairs) that is not compensated for by an increase in P uptake via the mycorrhizal uptake pathway (via the ERM and IRM), leading to a reduction in total P uptake and P deficiency of the plant (Li *et al.*, 2008; Smith and Smith 2011; Smith *et al.*, 2011). We studied this hypothesis by following the P uptake and transport in multicompartment systems and examined the expression of plant P_i transporters that are involved in the plant uptake pathway or the mycorrhizal uptake pathway. Consistent with the results of the growth response experiment, *R. irregularis* took up more P from the fungal compartment and transferred more P to both HN89 and HN112 plants than *G. aggregatum* (Fig. 4). The lower P contents in the shoots and roots of the plants that were colonized with *G. aggregatum* suggest that the lower efficiency with which this fungus takes up P from the soil is at least in part responsible for the lack of an effect of this fungus on P nutrition.

In soybean, 14 genes of the *Phl1* family have been identified (Qin *et al.*, 2012), and the results demonstrate that the expression of most of these transporter genes was affected by the AM symbiosis. Several of these P_i transporter genes, including *GmPT1*, *GmPT2*, *GmPT4* and *GmPT11*, are downregulated in mycorrhizal roots, particularly by the AM fungus *R. irregularis* (Fig. 5A; Supplementary Data Fig. S5). The P_i transporter gene *GmPT4*, for example, was upregulated in roots that were colonized with *G. aggregatum*, but downregulated in roots that were colonized with *R. irregularis* (Fig. 5A). This suggests that the plants that were colonized with *G. aggregatum* were P deficient compared with the plants that were colonized with *R. irregularis*. The P_i transporter genes *GmPT1*, *GmPT2*, *GmPT3*, *GmPT4*, *GmPT6*, *GmPT7*, *GmPT11*, *GmPT12* and *GmPT13* are predominantly expressed in soybean roots under P starvation (Qin *et al.*, 2012; Fan *et al.*, 2013). The downregulation of several of these P_i transporter genes particularly by *R. irregularis* could be associated with the P benefits of this fungus and confirms the findings of other authors who demonstrated that P_i transporters that are involved in the plant uptake pathway are often downregulated in mycorrhizal roots (Chiou *et al.*, 2001; Grunwald *et al.*, 2009; Tamura *et al.*, 2012). Consistent with the low P status of these plants, six *GmPT* genes were particularly induced by *G. aggregatum* in the P-efficient soybean genotype HN89, but downregulated in the P-inefficient soybean genotype HN112. However, the upregulation of these transporters by *G. aggregatum* did not lead to an increase in P uptake via the direct uptake pathway, and the P contents in these plants did not differ from that of the NM controls (Figs S3 and S5).

We also studied the expression of the mycorrhiza-inducible P_i transporter genes *GmPT8*, *GmPT9* and *GmPT10*. These transporter genes are particularly expressed in mycorrhizal roots under P limitation, and cluster with the mycorrhiza-inducible P_i transporter genes *OsPT11* from *Oryza sativa* (rice) and *MtPT4* from *M. truncatula* (Qin *et al.*, 2012; Tamura *et al.*, 2012). *GmPT8* and *GmPT9* were induced by colonization with *R. irregularis* and *G. aggregatum* (Fig. 5), but *GmPT10* was only upregulated by *R. irregularis*. The upregulation of *GmPT8*, *GmPT9* and *GmPT10* by *R. irregularis* is consistent with the positive impact of this AM fungus on the P nutrition of soybeans and suggests that these transporters play a role in the P uptake by the plant from the mycorrhizal interface.

Interestingly, *GmPT8* was only induced in the P-efficient genotype HN89, but was downregulated in the P-inefficient genotype HN112 (Fig. S5), and the transcript levels of *GmPT9* were more strongly induced in HN89 than in HN112 (Fig. 5). This indicates that the higher transcript levels of these transporters could contribute to the efficient AM symbiosis and P uptake of this genotype. However, *GmPT9* was also upregulated by *G. aggregatum*, a fungus that did not have a positive impact on P nutrition, and the genotype HN112 showed a slightly higher mycorrhizal growth and P uptake benefit than HN89 under our experimental conditions, but also in the experiments of other authors (Wang *et al.*, 2011). Tamara and co-authors (2012) examined the expression of *GmPT8*, *GmPT9* and *GmPT10* in a time course experiment, and found that *GmPT9* was induced much earlier than *GmPT8* and *GmPT10*; they concluded that these three transporter genes play different roles in AM roots. Our results also suggest that *GmPT8*, *GmPT9* and *GmPT10* may play different roles in the P transport across the mycorrhizal interface. Of all P_i transporter genes, the expression levels of *GmPT10* are most tightly aligned with the mycorrhizal P benefits, suggesting that *GmPT10* could serve as a molecular marker for the mycorrhizal growth or P uptake benefit of soybeans. However, more experimental work is needed to support this hypothesis.

Our results confirm that soybeans with a higher P efficiency can also benefit greatly from an AM symbiosis. The P-efficient genotype HN89 showed a slightly lower mycorrhizal MGR or MPR than the P-inefficient genotype HN112 but only under low P availabilities. This is in contrast to results in wheat and barley, in which a decrease in the MPR was found in genotypes with a higher PUE (Baon *et al.*, 1993; Zhu *et al.*, 2001). Our results should have important implications for the development of nutrient-efficient soybean cultivars. The P_i transporter expression clearly demonstrates that the colonization with *R. irregularis* shifts the P-absorbing surface of the plant from the soil–root interface with epidermis and root hairs to the plant–fungal interface inside the root in both soybean genotypes. There is increasing evidence suggesting that mycorrhizal- or other rhizosphere-related traits should be integrated into modern breeding programmes to identify plant and fungal traits that can be used synergistically to increase the P and nutrient uptake efficiency of important crop species, such as soybean.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: effect of AM fungi and P supply conditions on root traits. Figure S2: effect of AM fungi and P supply conditions on the P tissue concentration. Figure S3: biomass and P content of roots and shoots of the two soybean genotypes HN112 and HN89 in the ^{33}P transport experiment. Figure S4: AM colonization characteristics of the two soybean genotypes HN112 and HN89 in the ^{33}P transport experiment. Figure S5: expression of 11 high affinity P transporters in mycorrhizal roots of the two soybean genotypes HN112 and HN89. Table S1: results of the statistical analysis of the effect of P levels or arbuscular mycorrhizal treatments or interactions of the P level and the arbuscular mycorrhizal colonization on growth parameters of the two soybean

genotypes HN112 and HN89 in the greenhouse experiment. Table S2: results of the statistical analysis of the effects of P supply conditions, genotypes and arbuscular mycorrhizal treatments and interactions among these treatments on root architectural traits, and shoot or root P concentration in the greenhouse experiment. Table S3: results of the statistical analysis of the effects of soybean genotypes or arbuscular mycorrhizal treatments and interactions between soybean genotypes and mycorrhizal treatments on plant growth, mycorrhizal colonization and ^{33}P transport in the ^{33}P transport experiment. Table S4: results of the statistical analysis of the effects of the soybean genotype or the arbuscular mycorrhizal treatments or the interaction between soybean variety and arbuscular mycorrhizal treatment on ^{33}P transport and expression of plant phosphate transporter genes in the ^{33}P experiment. Table S5: results of the statistical analysis of the effects of the soybean genotypes or the arbuscular mycorrhizal treatments or the interaction between soybean genotype and arbuscular mycorrhizal treatment on expression of plant P transporter genes.

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