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Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning

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SUMMARY

Most terrestrial plants form arbuscular mycorrhiza (AM), mutualistic associations with soil fungi of the order *Glomeromycota.* The obligate biotrophic fungi trade mineral nutrients, mainly phosphate (P_i), for carbohydrates from the plants. Under conditions of high exogenous phosphate supply, when the plant can meet its own P requirements without the fungus, AM are suppressed, an effect which could be interpreted as an active strategy of the plant to limit carbohydrate consumption of the fungus by inhibiting its proliferation in the roots. However, the mechanisms involved in fungal inhibition are poorly understood. Here, we employ a transcriptomic approach to get insight into potential shifts in metabolic activity and symbiotic signalling, and in the defence status of plants exposed to high P_i levels. We show that in mycorrhizal roots of petunia, a similar set of symbiosis-related genes is expressed as in mycorrhizal roots of *Medicago, Lotus* and rice. P_i acts systemically to repress symbiotic gene expression and AM colonization in the root. In established mycorrhizal roots, P_i repressed symbiotic gene expression rapidly, whereas the inhibition of colonization followed with a lag of more than a week. Taken together, these results suggest that P_i acts by repressing essential symbiotic genes, in particular genes encoding enzymes of carotenoid and strigolactone biosynthesis, and symbiosis-associated phosphate transporters. The role of these effects in the suppression of symbiosis under high P_i conditions is discussed.

Keywords: arbuscular mycorrhiza, symbiosis, petunia, glomus, phosphate, carotenoid.

INTRODUCTION

Arbuscular mycorrhiza (AM) are mutualistic symbiotic associations between most vascular land plant species, and fungi of the phylum *Glomeromycota* (Smith and Read, 2008), in which the plant trades carbohydrates for mineral nutrients from the fungus, in particular phosphate (P_i). The factors involved in early communication between the symbiotic partners have been elucidated in considerable detail in recent years (reviewed in Parniske, 2008). The first known chemical signal in AM is the root-borne branching factor strigolactone, which promotes hyphal branching and metabolism (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). Conversely, an unknown diffusible fungal signal triggers the induction of symbiosis-associated genes in the root before the first physical contact is established (Kosuta *et al.*, 2003).

Once a fungal hyphopodium has been formed on the surface of the root epidermis, its position is detected by the subtending epidermal cell, which reacts with the establishment of an intracellular infection structure, the pre-penetration apparatus (PPA) that is necessary for fungal invasion (Genre *et al.*, 2005). These cellular adaptations of the host depend on a symbiotic signalling pathway referred to as the common SYM pathway, because it is shared with root nodule symbiosis (RNS). The SYM pathway is functionally conserved between monocot and dicot species (Chen *et al.*, 2007a, 2008; Gutjahr *et al.*, 2008), suggesting that it evolved in early land plants, and became secondarily recruited into RNS. After penetration of the epidermis and a subsequent intercellular expansion phase, the fungus resumes intracellular colonization of cortical cells, again with the help of a PPA-related cellular accommodation structure of the plant (Genre *et al.*, 2008). This results in the establishment of arbuscules and the associated symbiotic interface, over which nutrient exchange proceeds. Hence, the establishment of functional AM involves a series of steps which are under tight control mainly by the host plant.

The cellular adaptations during elaboration of AM are associated with pronounced physiological changes (Smith and Read, 2008), in particular the establishment of the symbiotic P_i-uptake system of the plant (Bucher, 2007). This involves the induction of symbiosis-specific P_i transporters (PTs), the expression of which is triggered by lyso-phosphatidylcholine (LPC) (Drissner *et al.*, 2007). Symbiosis-specific PTs are localized to the periarbuscular membrane where they absorb the P_i delivered over the symbiotic interface by the fungus (Harrison *et al.*, 2002). Induction of genes encoding H^+-ATPase in cells that harbour arbuscules (Gianinazzi-Pearson *et al.*, 2000; Krajinski *et al.*, 2002), and the acidification of the interface (Guttenberger, 2000) are indicative of an energized nutrient uptake mechanism.

Transcriptomic analyses in the legumes *M. truncatula* and *L. japonicus,* as well as in rice (*Oryza sativa*) have revealed that AM development is associated with a dramatic transcriptional switch (Liu *et al.*, 2003, 2007; Wulf *et al.*, 2003; Brechenmacher *et al.*, 2004; Güimil *et al.*, 2005; Hohnjec *et al.*, 2005; Kistner *et al.*, 2005; Grunwald *et al.*, 2009; Guether *et al.*, 2009). Based on these studies, a common set of AM-associated genes has emerged, which can serve as reliable markers of symbiosis. While most of these genes have not yet been functionally tested, knock-down analysis of the symbiosis-inducible PTs in *L. japonicus* and *M. truncatula* (*LjPT3* and *MtPT4,* respectively) has revealed their essential role in P_i-transfer and symbiotic development (Maeda *et al.*, 2006; Javot *et al.*, 2007). Furthermore, two genes encoding an AM-inducible apoplastic subtilase (Takeda *et al.*, 2009) and an ankyrin protein (Pumplin *et al.*, 2010), respectively, have been shown to be required for normal AM development.

Besides positive regulatory mechanisms, the plant has means to limit colonization by AM fungi. Root systems that have already been colonized by AM fungi exhibit a reduced tendency to be infected and colonized by further symbiotic propagules (Catford *et al.*, 2003). Interestingly, as in the case of the common SYM pathway, this effect shares common aspects with autoregulation of nodulation in legumes (Catford *et al.*, 2003), which involves a receptor kinase in the shoot (Meixner *et al.*, 2005; Magori and Kawaguchi, 2009). Another example of negative regulation by the plant is the suppression of AM symbiosis under high P_i levels (Menge *et al.*, 1978; Jasper *et al.*, 1979; Thomson *et al.*, 1986; Amijée *et al.*, 1989; Franken and Gnädinger, 1994; Nagy *et al.*, 2009). As P_i is the major nutrient delivered by the fungus, the suppressive effect of P_i could be interpreted as a negative regulatory feedback mechanism of the plant host to limit carbohydrate allocation to the symbiont under conditions of optimal P_i supply. This implies that the plant has means to control and limit fungal proliferation according to its nutrient status. The mechanisms involved, however, are unknown.

Considering the successive steps in the AM interaction, a number of hypothetical scenarios could potentially account for negative regulation of AM by P_i: (i) P_i could limit the biosynthesis or secretion of strigolactone or other signals involved in pre-symbiotic interaction; (ii) P_i could reduce the expression of components of the SYM pathway or stimulate negative autoregulation; (iii) P_i could increase the defence status in the roots, hence leading to the rejection of the symbiont; (iv) the plant could limit the delivery of essential nutrients (e.g. carbohydrates) to the symbiont, thereby slowing down its growth (Olsson *et al.*, 2006); or (v) P_i could downregulate essential components involved in downstream steps of root colonization and/or establishment of the symbiotic interface (Nagy *et al.*, 2009). Each of these scenarios, which are not mutually exclusive, would be likely to be associated with a characteristic shift in gene expression patterns of particular marker genes.

Here, we explore the transcriptional changes associated with AM development and with elevated P_i supply in *Petunia hybrida.* Microarray analysis reveals that the symbiosis-associated transcriptome of petunia involves a set of highly conserved genes that overlaps to a large extent with the complement of AM-associated genes of *Medicago, Lotus,* and rice. P_i-dependent changes in transcript levels involved mostly the down-regulation of symbiosis-responsive genes encoding PTs, pathogenesis-related (PR) proteins, and certain proteases. Most interestingly, the suppression of genes encoding enzymes involved in carotenoid and apocarotenoid biosynthesis indicates that these pathways are generally suppressed by P_i. These results suggest that high P_i levels trigger a complex anti-symbiotic syndrome, which results in strong repression of AM fungal colonization.

RESULTS

P_i inhibits intraradical proliferation and arbuscule development of *Glomus intraradices*

To determine the P_i sensitivity of AM in petunia, plants were inoculated with *G. intraradices* and weekly supplemented with increasing levels of KH_2PO_4 between 0.1 and 10 mM. Application of 0.5 mM P_i caused a reduction of colonization to approximately 50%, and the interaction was almost completely suppressed at 10 mM (Figure 1). Inspection of the rare colonized root segments showed that in addition to the reduction in total root colonization, high P_i supply caused qualitative differences in intraradical colonization patterns (Figure 2). In general, the fungus formed only small colonies that failed to extend along the root. Instead of the thick hypha formed in control roots (Figure 2a), thin short hyphae were formed at the periphery of the colonies, where they appeared to become arrested (Figure 2b). Confocal analysis of the intracellular stages indicated that the hyphal coils in epidermal cells were not affected by P_i in an obvious way (Figure 2c,d), whereas arbuscules were malformed and less branched and appeared less dense than in controls (Figure 2e,f).

To exclude potential effects of potassium in the KH_2PO_4 solution, and to test the sensitivity of the interaction to unspecific salt effects (osmotic stress), several control experiments were carried out. In the first experiment, inoculated plants were supplemented with the following salt solutions at a concentration of 5 mM each: KH_2PO_4, NaH_2PO_4, K_2SO_4, $MgSO_4$ and KCl (Figure S1). Root colonization was reduced only in plants supplemented with KH_2PO_4 or NaH_2PO_4 (Figure S1a). Shoot and root fresh weight was only marginally affected by the treatments (Figure S1b, white and black bars, respectively), indicating that the plants did not suffer from salt stress. Treatments with nitrate (up to 5 mM KNO_3) did not alter AM, indicating that the P_i effect is specific (data not shown). High salt supply (up to 30 mM KCl and K_2SO_4) did not affect AM colonization significantly, documenting a pronounced robustness of the symbiosis to osmotic stress (data not shown). Taken together, these results establish P_i as the suppressive agent in KH_2PO_4.

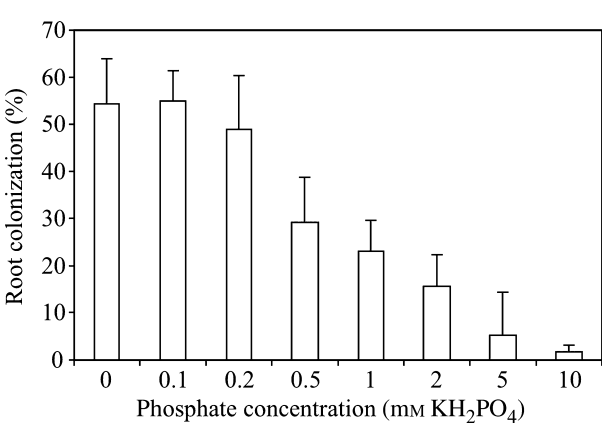

Figure 1. Mycorrhizal colonization as a function of fertiliser phosphate concentration.
Petunia hybrida plantlets were inoculated with *Glomus intraradices* and grown in pot cultures with different phosphate concentrations in the fertiliser solution. Roots were sampled after 5 weeks, stained with trypan blue and mycorrhizal titer was quantified. Shown are means ± SD (n = 3).

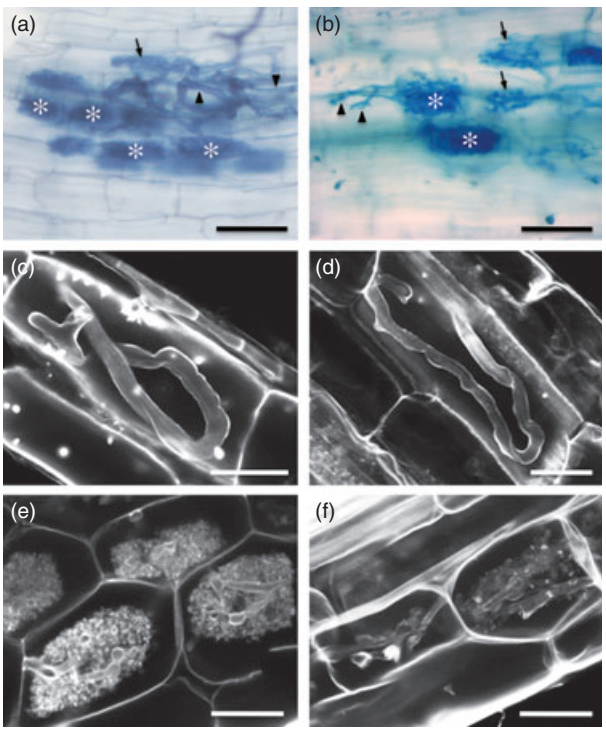

Figure 2. Fungal intraradical morphology as a function of fertiliser phosphate concentrations.
Mycorrhizal roots of *P. hybrida* plants fertilised with 30 μM (a, c, e) or 5 mM KH_2PO_4 (b, d, f) were analysed by light microscopy after trypan blue staining (a, b) or by confocal microscopy after acid fuchsin staining (c–f). Intercellular hyphae (arrowheads), arbuscules at various developmental stages (arrows) and fully developed arbuscules (asterisks) are indicated. (Black bars, 50 μM; white bars 25 μM).

Phosphate acts systemically through improved P-status in the shoot

Phosphate may act directly on fungal development in the soil, or indirectly by changing plant physiology to suppress fungal development in the root. To distinguish between these possibilities we performed split-root experiments in which plants were treated with high P_i levels on one side, and the effects on the AM interaction was assessed on the other half of the root system, which was supplemented with low P_i levels. As controls, plants with split roots were treated on both sides with either low or high P_i levels. High P_i levels exerted a systemic inhibitory effect on AM colonization in roots exposed to low P_i levels (Figure 3a). This effect was

associated with a systemic inhibitory effect on the expression of the AM marker gene *PhPT4* (Figure 3b) (Wegmüller *et al.*, 2008). Interestingly, repression of AM colonization and *PhPT4* expression in the roots did not correlate with P levels in the respective roots, which was not significantly altered (Figure 3c), but rather correlated negatively with shoot P levels, indicating that the symbiotic status of mycorrhizal plants may depend on the P status of the shoot. Relatively high P levels in plants treated with low P_i levels (Figure 3c, left; compare with Figure 4) can be explained with the plants having access to two pots instead of one, hence doubling the absolute P_i supply per plant. The fact that relatively small differences in shoot P levels (Figure 3c) correlated with large effects on AM colonization and gene expression (Figure 3a,b) points to a pronounced threshold effect in P sensing or response. Taken together, our split root experiments suggest that P_i acts primarily through the plant rather than directly on the fungus, although direct effects of P_i on AM fungi cannot be excluded.

Generation of a petunia microarray and experimental setup

The adverse effects of high exogenous P_i levels on AM may be associated with induction of a defence response or with repression of symbiotic functions. To distinguish between these possibilities, we interrogated the transcriptomic response of petunia roots to AM and to high P_i supply using a custom made microarray. First, a set of 45 783 EST sequences was generated from cDNA libraries derived from petunia control roots, mycorrhizal roots, and P_i-treated roots (Table 1; see Supporting information for details) and assembled into a set of 10 150 contigs and 7793 singletons. A complete list of the clustered sequences (referred to as drpoolB) is provided at http://est.molgen.mpg.de/plantDR (User: DidierReinhardt, Password: Al8JJ9mt). Distribution of the sequences to functional groups based on GO-annotation of the closest homologue of *A. thaliana* is shown in Figure S2. This sequence information was combined with the EST sequences raised from cuttings during adventitious root formation, and with all accessible *P. hybrida* and *P. axillaris* nucleotide sequences retrieved from public databases (see Table S1 at http://pgrc.ipk-gatersleben.de/petunia_array). These sequences, which comprise the entire known petunia transcriptome, were clustered to generate a set of 24 816 unigene sequences for the design of a custom microarray by NimbleGen (see Supporting information).

A total of four independent experiments were carried out to determine the set of genes responding to AM at low P_i levels (30 μM KH_2PO_4). Two different petunia lines (W115 and W138) were used and plants were harvested 5, 7 and 8 weeks after inoculation. The rational of comparing gene expression of different petunia lines harvested at different time points of mycorrhizal development was to apply a stringent filter and to retain only genes that are robustly and consistently regulated in well established AM, irrespective of the petunia cultivar. Furthermore, early time points were not considered as P_i appeared to act at a relatively late stage of the symbiotic interaction, based on the appearance of residual AM colonization in cortical tissues (Figure 2). To establish the effects of high P_i levels on gene expression, two additional treatments were included in the two-first experiments (harvested 5 weeks after inoculation). In these treatments, inoculated and mock-inoculated plants were weekly supplemented with 5 mM KH_2PO_4. The following colonization levels were observed in the four experiments.

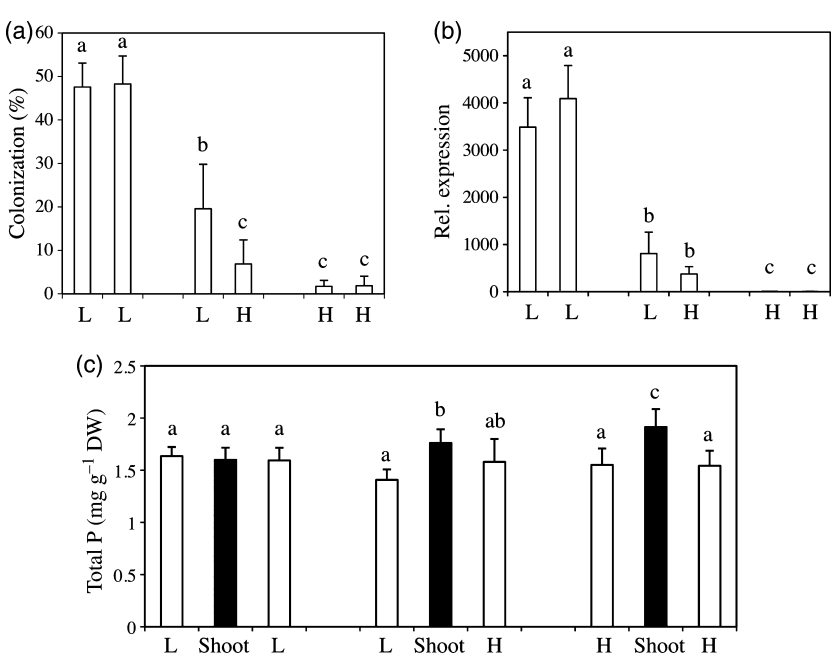

Figure 3. Systemic effects of exogenous phosphate on AM colonization, *PhPT4* expression and phosphorus levels in roots and the shoot.
Plants with split roots systems were fertilised with 30 μM KH_2PO_4 (L) or with 5 mM KH_2PO_4 (H) as indicated.
(a) In plants treated differently in their two root compartments (L H), AM colonization was intermediate between plants grown entirely at low (L L) or high (H H) P_i conditions.
(b) *PhPT4* expression in both compartments of L/H plants was intermediate between plants grown only at low (L/L) or high (H/H) P_i concentration.
(c) Exogenous P_i application increased shoot total P levels (black columns) independently of whether only one (L/H) or both (H/H) root compartments were fertilised with high Pi concentrations. However, P levels in all roots were unaffected (white columns). Shown are means ± SD (n = 7). Different letters indicate significant differences (Student's *t*-test, $P \leq 0.05$).

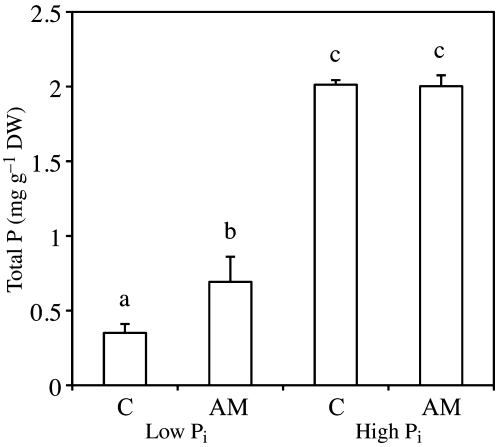

Figure 4. Leaf phosphorus content as a function of mycorrhizal colonisation and phosphate fertilisation.
Plantlets of *P. hybrida* were inoculated (AM) or not (C) with *G. intraradices* and grown with 30 μM KH_2PO_4 (low P_i) or 5 mM KH_2PO_4 (high P_i) in the fertiliser solution. Total phosphorus content of leaves was determined 5 weeks after inoculation. Shown are means ± SD (n = 3). Different letters indicate significant differences (Student's t-test, $P \leq 0.05$).

Table 1 cDNA libraries representing different petunia tissues grown under various conditions

Suffix in ID names	Library
dr001	Normalized root cDNA library (control conditions)
drs12	Substractive root cDNA library (control minus AM)
drs21	Substractive root cDNA library (AM minus control)
drs13	Substractive root cDNA library (control minus P_i-treated)
drs31	Substractive root cDNA library (P_i-treated minus control)
dr004	Normalized petal cDNA library (*Petunia axillaris*)

All libraries except for dr004 were prepared from *Petunia hybrida* Mitchell root tissues grown under different conditions as follows: control (30 MM KH_2PO_4), P_i-treated (5 mM KH_2PO_4), and inoculated with *G. intraradices* under control P_i conditions (AM). Substractive libraries were prepared by substracting mRNA from these three conditions in the indicated directions.

Experiment 1: 74.3 ± 4.4% at low P_i levels, and 2.9 ± 1.2% at high P_i levels; experiment 2: 56.0 ± 2.0% at low P_i levels, and 1.7 ± 0.58% at high P_i levels; experiment 3: 66.3 ± 10.0%; and experiment 4: 72.3 ± 12.3%, respectively. Shoot P content of mycorrhizal plants was increased compared with controls under conditions of low P_i supply whereas high P_i supply caused generally high shoot P content, irrespective of AM inoculation (Figure 4). Total RNA was extracted from roots and used for microarray analysis according to the manufacturer's guidelines. A complete list of all gene IDs of the array, their expression signal, and their induction ratio in all treatments is presented in Table S2.

AM-specific genes of plant and fungal origin

For comparative expression analysis, the microarray analysis tool Fire2.2 (Garcion *et al.*, 2006) was used to extract genes with particular expression patterns. First, we identified genes of which the expression level was in the range of the background in controls and in the treatment with P_i alone (<100), and which were at least twp-fold induced in all four mycorrhizal samples. These genes were classified as AM-specific (Table S3a–c). As reliable induction ratios cannot be derived if the control expression levels are at the detection limit, expression values are shown for the AM-specific genes (Table S3a–c). IDs were assigned to functional groups according to Journet *et al.* (2002). Many AM-specific genes were homologous to plant AM markers identified in previous studies on *M. truncatula* (Journet *et al.*, 2002; Liu *et al.*, 2003, 2007; Wulf *et al.*, 2003; Brechenmacher *et al.*, 2004; Hohnjec *et al.*, 2005; Grunwald *et al.*, 2009), *O. sativa* (rice) (Güimil *et al.*, 2005), and *L. japonicus* (Kistner *et al.*, 2005; Guether *et al.*, 2009) (Table S4). They encode PHPT4 and several other transporters (class III), proteases (class IX), glutathione-*S*-transferase (GST), and class III chitinase (class XII.A). An AM-specific homologue of the gene encoding the carotenoid cleavage dioxygenase 4b (CCD4b) of *Chrysanthemum morifolium* (Ohmiya *et al.*, 2006) points to an involvement of apocarotenoids that are known to accumulate in mycorrhizal roots (Fester *et al.*, 2007). For 11 IDs, that did not match a sequence in the protein database of NCBI, homologous ESTs from various plant tissues and species were identified (class XIII.A), whereas for further 25 IDs, only EST sequences from mycorrhizal roots of *M. truncatula* or *L. japonicus* were identified (class XIII.B). Hence, the latter could represent sequences of plant or fungal origin. Although the AM-specific genes were not induced above background levels by P_i alone, they were induced to low levels in mycorrhizal roots at high P_i levels (AM-P_i), presumably reflecting the residual colonization level of 2.9% and 1.7%, respectively.

Consistent with the mixed contribution of plant and fungal RNA to the mycorrhizal samples used for EST sequencing and microarray analysis, a significant number of AM-specific IDs were homologous to sequences of fungal origin (Table 3b). A further 7 AM-specific IDs showed homology to organisms other than plants and fungi (Table S3c), and 40 IDs did not match any sequence of the public databases. These genes may represent either new unknown AM-specific plant genes or fungal sequences (Table S3c).

AM-induced genes and their response to P_i

Many IDs exhibited moderate to intermediate expression levels in control roots, and induced levels in mycorrhizal roots (Table S3d). This category included, among others, genes encoding symbiotic PTs (*PhPT3* and *PhPT5*), several ABC transporters, the aquaporin NOD26 (class III), and a

number of proteases of various types (class IX). Interestingly, many sequences encoded homologues of defence-related proteins (class XII.A), including PR10, barwin-related glucanases, glutathione-*S*-transferase (GST), peroxidases, chitinases, and germin-like proteins (Table 2). Very few AM-inducible genes were also induced by P_i alone, indicating that the improved P status of mycorrhizal plants (Figure 4) is not reflected by P_i-inducible genes in the root. Notably, a considerable fraction of transporters (group III), proteases (group IX), and stress-related genes (group XII.A) were repressed by P_i alone and/or in mycorrhizal roots at high P_i (Table S3d).

AM-repressed genes

As observed in previous studies, fewer genes were repressed by AM, and the extent of regulation was weaker than in the case of the induced genes (Table S3e). Interestingly, several genes encoding mineral nutrient transporters were repressed, in particular nitrate transporters and a zinc/iron transporter (class III), presumably reflecting the improved nutrient status in mycorrhizal roots. Notably, in contrast to the poor overlap between AM-inducible and P_i-inducible genes (see above) many AM-repressed genes were also repressed by P_i. This was particularly evident for several IDs encoding phospholipase D and SPX-proteins which are involved in P-starvation response and signalling (see below). In most cases, repression by P_i was even stronger than by AM, correlating with the P levels in the shoot (Figure 4). These results indicate that improved P status is generally associated with repression of P-starvation responsive genes.

Genes induced by P_i

We next looked for markers of defence which may become induced by P_i alone (Table S3f), or in mycorrhizal roots at high P_i (AM-P_i) (Table S3g). In general, the two lists overlapped to a large degree with slightly more genes being induced by the combined AM-P_i treatment. Most of these additional genes were AM-inducible genes, for which weak levels of induction in the AM-P_i treatment likely reflect the residual colonization level. Notably, only few genes of class XII.A (defence) were induced by P_i alone (1.8%) or by AM-P_i (1.1%), and their induction was weak (Table 2). By comparison, a relatively high number of genes (13%) among the AM-inducible genes were classified as defence markers and they were strongly induced (Table S3d; Table 2). Finally, as stated above (Table S3d), very little overlap between P_i-inducible and AM-inducible genes was observed. Taken together, our data lend little support to the hypothesis that P_i may induce defence mechanisms.

Genes repressed by P_i alone or in inoculated roots at high P_i

To explore whether essential symbiosis-related functions were affected by high P_i levels, the genes repressed by P_i alone, or by P_i in mycorrhizal roots (AM-P_i) were determined. 464 and 492 genes, respectively, were repressed by the two treatments, and the lists were largely overlapping (Table S3h,i). Many repressed genes encoded known markers of P-starvation such as, purple acid phosphatases (PAPs), phytase, RNase, PEP carboxylase, SPX domain-containing proteins, the constitutive *PTs PhPT2* and *PhPT7,* and genes encoding enzymes of fatty acid biosynthesis (Wasaki *et al.*, 2003; Misson *et al.*, 2005; Hernandez *et al.*, 2007). Interestingly, a miRNA399 homologue was induced, whereas the expression of phosphate starvation regulator PHR1 (Bari *et al.*, 2006) was not affected by P_i. A role for miR399 in the regulation of AM has recently been postulated by Branscheid *et al.* (2010). The petunia homologues of the phosphate transport regulator PHO_2 and of At4/Mt4 are not known and were therefore not represented on the array. Taken together, this gene expression pattern is indicative of a general repression of the P-starvation response. Consistently, genes encoding enzymes involved in the biosynthesis of sulfolipids and galactolipids, which replace phospholipids under P-deprivation (Essigmann *et al.*, 1998; Andersson *et al.*, 2003), were repressed, together with lipolytic enzymes involved in the recycling of phospholipids (phospholipase C, phospholipase D, glycerophosphoryl diester phosphoesterase) (Li *et al.*, 2006).

We next considered genes the repression of which may potentially affect AM colonization. Strong gene repression by P_i was observed for the symbiotic *PhPT5,* like in tomato (Nagy *et al.*, 2009), as well as for the constitutive *PhPT2* and *PhPT7,* and for other transporters (Table S3h,i, Class III). Considering genes with a potential function in secondary metabolism and hormone pathways (class VI), a large fraction encoded enzymes involved in carotenoid production and processing (Table 2, Figure 5). In particular, enzymes of the plastidial MEP pathway and of carotenoid biosynthesis, as well as enzymes involved in biosynthesis of the diterpene-derived hormone gibberellic acid and of the strigolactones were downregulated (for review see Lu and Li, 2008). Genes encoding the ABA biosynthetic enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) were induced. A homologue of jasmonic acid-inducible JA2 was slightly induced, whereas genes encoding components of ethylene biosynthesis and signal transduction were slightly repressed (Table 2).

In the group of signalling components (class X), we observed a strong repression of various kinases, phosphatases, SPX domain-containing proteins with homology to the Arabidopsis P-starvation gene At-SPX3 (Duan *et al.*, 2008), and, notably, of *SYM10* of pea (*P. sativum*), the orthologue of the nod factor receptor *NFR5* in *L. japonicus* (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). However, no effect of P_i on the expression of the *SYM* gene homologues *PhSYMRK, PhCASTOR, PhPOLLUX, PhCCaMK,* and *PhNUP133,* and of homologues of other components of the

Table 2 AM regulation of genes encoding enzymes involved in carotenoid biosynthesis and processing, in hormone biosynthesis and signalling, and in defence and cell rescue

		Expression ratios					
		AM, – P_i		+ P_i		AM, + P_i	
Sequence ID	Putative function	5W1	5W2	5W1	5W2	5W1	5W2
MEP pathway							
cn4671	Transketolase, chloroplast precursor	0.18	0.4	0.04	0.04	0.04	0.04
cn8419	Transketolase, chloroplast precursor	0.23	0.42	0.05	0.04	0.05	0.06
cn8324	1-deoxy-D-xylulose 5-phosphate synthase 2 (DXS2)	0.88	0.84	0.39	0.45	0.38	0.27
cn4868	1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)	0.35	0.76	0.41	0.41	0.29	0.81
cn9083	4-(cytidine 5′-diphospho)-2C-methyl-D-erythritol kinase (CMK)	1.02	0.39	0.28	0.47	0.28	0.32
cn9082	4-(cytidine 5′-diphospho)-2C-methyl-D-erythritol kinase (CMK)	1.12	0.77	0.16	0.48	0.17	0.47
Carotenoid and terpenoid metabolism							
CL1919Contig1	Geranylgeranyl pyrophosphate synthase 1 (GGPS)	0.19	0.9	0.06	0.31	0.06	0.29
CL1028Contig1	Geranylgeranyl pyrophosphate synthase 1 (GGPS)	0.62	0.84	0.07	0.49	0.07	0.22
CL8749Contig1	Phytoene synthase (PSY)	1.03	1.25	0.15	0.47	0.11	0.31
cn8042	Carotenoid isomerase (CRTISO)	0.64	0.74	0.28	0.29	0.31	0.39
cn3078	Carotenoid cleavage dioxygenase 4	71.32	51.13	0.87	0.68	1.08	1.21
CL6596Contig1	Terpene synthase	0.12	0.29	0.02	0.02	0.02	0.01
Strigolactone biosynthesis							
CL5144Contig1	Dad1/CCD8	0.93	1.07	0.1	0.88	0.08	0.33
ABA synthesis							
CL9680Contig1	9-*cis*-epoxy-carotenoid dioxygenase 1	10.37	3.22	4.65	2.43	8.35	4.2
cn9068	9-*cis*-epoxycarotenoid dioxygenase	3.8	2.24	4.5	2.09	7.2	2.71
cn9067	9-*cis*-epoxycarotenoid dioxygenase	15.78	3.08	3.77	4.09	6.39	4.62
cn8538	9-*cis*-epoxycarotenoid dioxygenase	0.26	0.63	0.17	0.46	0.19	0.45
Gibberellin biosynthesis and metabolism							
cn8481	Copalyl diphosphate synthase	0.33	0.65	0.03	0.21	0.03	0.09
CL841Contig1	Copalyl diphosphate synthase	0.26	0.49	0.06	0.24	0.1	0.17
CL9774Contig1	Copalyl diphosphate synthase	0.45	0.81	0.09	0.39	0.07	0.18
CL590Contig1	Gibberellin 20 oxidase	3.3	13.15	65.2	3.57	75.23	9.02
Ethylene biosynthesis and signalling							
cn1901	1-aminocyclopropane-1-carboxylate oxidase 4	0.14	0.23	0.19	0.2	0.21	0.43
cn4574	Ethylene response factor 4	0.55	0.58	0.24	0.2	0.26	0.24
Jasmonic acid signalling							
cn502	Jasmonic acid 2	8.03	2.28	2	2.37	4.38	2.05
Defence and cell rescue							
CL687Contig1	Glutathione-*S*-transferase	959.5	726.5	1.06	1.86	23.94	6.03
CL4772Contig1	Barwin-related endoglucanase	483.9	443.1	1.02	1.14	7.57	2.28
CL6207Contig1	Nectarin-1 precursor	424.4	171.3	0.74	1.38	4.54	1.74
cn8393	Glutathione-*S*-transferase GST 34	364.6	305.5	1.58	1.73	5.64	3.41
cn8660	Class III chitinase (hevamine-A precursor)	292.2	221.0	0.74	2.28	4.59	1.23
CL3731Contig1	Pathogenesis-related protein PR10a	102.0	38.29	0.35	1.87	1.41	0.87
CL542Contig1	Chitinase 1 precursor	100.7	69.13	1.69	1.18	5.79	1.26
cn8323	Barwin-related endoglucanase	82.85	77.19	0.97	1.05	5.63	1.50
cn8322	Barwin-related endoglucanase	75.67	74.24	0.53	0.59	2.98	1.20
cn8321	Barwin-related endoglucanase	59.61	36.58	0.83	0.79	5.92	1.04
dr001P0005J09.F.ab1	Plant pathogenesis related protein PR10	31.75	6.28	0.37	0.22	1.77	0.32
cn7357	Plant pathogen related protein PR10	13.90	49.48	0.04	0.44	0.93	1.00
cn8547	Germin like protein/Rhicadhesin receptor precursor	12.15	93.55	0.08	0.41	0.95	1.51
cn8455	Chitinase 1 precursor	10.88	48.90	1.61	1.31	5.34	1.22
CL5846Contig1	Haem peroxidase, plant/fungal/bacterial	7.46	37.32	0.10	0.72	0.27	0.53
dr001P0003J03.F.ab1	Ntprp27	4.85	11.76	0.53	1.11	0.39	0.49
CL1481Contig1	Haem peroxidase, plant/fungal/bacterial	4.57	33.89	0.05	0.88	0.15	0.62
drs21P0007L08.R.ab1	Pathogenesis-related protein PR10	4.25	35.94	0.02	0.36	0.62	0.97
cn1351	Wound-induced protein 1	2.90	6.51	0.18	0.85	0.20	0.40
dr001P0001A01.F.ab1	Remorin	2.58	0.71	4.65	2.45	5.03	1.87
cn10012	Pathogenesis-related 10 protein PR10–2	2.40	11.09	0.14	0.96	0.20	0.64
dr004P0024F10.F.ab1	Glutathione *S*-transferase	2.31	2.26	3.54	2.97	3.93	2.00
CL6557Contig1	Wound/stress protein	1.88	0.92	0.09	0.13	0.20	0.10

Table 2 (Continued)

		Expression ratios					
		AM, – P_i		+ P_i		AM, + P_i	
Sequence ID	Putative function	5W1	5W2	5W1	5W2	5W1	5W2
dr001P0009M08.F.ab1	Peroxidase ATP23a	1.68	1.84	5.01	4.19	2.96	3.33
CL3749Contig1	Elicitor-inducible protein EIG-J7	1.49	0.68	0.23	0.30	0.24	0.17
CL7348Contig1	Cationic peroxidase 1 precursor	1.45	0.64	0.23	0.49	0.37	0.45
drpoolB-CL514Contig1	Prb-1b	1.36	0.57	0.20	0.38	0.30	0.29
drpoolB-CL5121Contig1	Elicitor-inducible protein EIG-J7	1.35	1.28	0.14	0.13	0.16	0.10
drpoolB-CL4847Contig1	Protein disulfide isomerase (PDI)-like protein 2	1.34	1.93	0.12	0.39	0.17	0.30
cn4853	Aci112	1.22	0.25	0.27	0.51	0.15	0.26
cn9719	Remorin 2	1.20	1.37	2.17	3.48	1.87	2.77
CL2794Contig1	Peroxidase	1.01	0.48	0.16	0.30	0.32	0.21
CL71Contig1	Patatin-like protein 1	0.98	1.35	2.95	0.57	2.82	2.08
cn8497	Peroxidase	0.98	0.80	0.34	0.44	0.50	0.56
cn8819	Macrophage migration inhibitory factor family protein	0.97	1.88	2.70	2.54	2.10	1.73
cn3721	Haemolysin-III related family protein	0.96	1.32	0.36	0.46	0.32	0.39
cn3722	Hemolysin III-related family protein	0.95	1.27	0.25	0.47	0.31	0.49
cn1201	Wound-induced protein 1	0.94	0.84	0.28	0.42	0.29	0.41
cn3166	Peroxidase	0.93	0.85	0.23	0.33	0.30	0.46
dr004P0021L02.F.ab1	Germin-like protein	0.93	3.74	0.27	0.93	0.23	0.45
cn8670	Basic 30 kDa endochitinase precursor chitinase	0.92	0.79	0.21	0.73	0.22	0.45
CL3407Contig1	Ntprp27	0.81	0.45	0.18	0.37	0.23	0.23
cn1137	Hydrogen peroxide-induced 1	0.73	0.43	0.17	0.23	0.22	0.31
drpoolB-CL730Contig1	TMV induced protein 1–2	0.72	0.56	0.20	1.02	0.18	0.43
IP.PHBS009B22u	Peroxidase	0.72	0.69	0.21	0.52	0.40	0.44
CL9435Contig1	PR1 protein	0.70	0.47	0.31	0.23	0.52	0.65
CL5857Contig1	Wound-induced protein WIN2 precursor WIN2 protein	0.69	0.43	0.26	0.18	0.18	0.12
cn8671	Basic chitinase	0.56	0.51	0.18	0.43	0.16	0.37
CL1532Contig1	Transmembrane BAX inhibitor motif-containing protein 4	0.49	0.42	0.22	0.49	0.12	0.51
CL6343Contig1	Vestitone reductase-related	0.40	0.34	0.45	0.48	0.31	0.37
CL4280Contig1	Superoxide dismutase [Fe]	0.31	0.21	0.33	0.29	0.48	0.27
cn1044	Peroxidase 2	0.18	0.60	0.54	0.77	0.39	0.47
cn517	Chloroplast thioredoxin f	0.14	0.40	0.09	0.15	0.12	0.30

Putative function, expression levels, and expression ratios are shown for genes which encode potential carotenoid biosynthetic or processing enzymes, and for genes encoding enzymes implicated in the biosynthesis and metabolism of ABA, GA, ethylene and strigolactone, or components in ethylene and JA signaling. Furthermore, defence-related sequences such as genes encoding PR protein homologues are listed.

nuclear pore complex were found (Table S5). The repression of *PAM1,* a gene required for intracellular accommodation of AM fungi in cortical cells (Feddermann *et al.*, 2010), is consistent with the defects in arbuscule development in P_i-treated roots (Figure 2b,f). Notably, genes encoding homologues of the AM-inducible PR10 and germin-like proteins, and numerous other defence-related genes that are not affected by AM, were repressed by P_i (Table 2).

Real time PCR confirms P_i-mediated repression of AM-inducible genes

To corroborate the results obtained from array analysis, we analyzed the expression of a number of selected AM-responsive genes, and of the constitutively expressed *PhPT1* and *SYMRK,* by quantitative real time PCR (qPCR). First, qPCR was carried out using the RNA that had already been used for microarray analysis (qPCR1 and qPCR2). The resulting data confirmed the induction in AM, but the extent of regulation was in many cases larger than deduced from microarray analysis, consistent with the larger dynamic range of qPCR analysis compared with microarray analysis (Table S6). Interestingly, not only the induction ratios were larger than anticipated from array data, but also the repression by P_i, in particular of *PhPT5* and a *terpene synthase,* was stronger than in the case of microarray analysis. Subsequently, an independent biological replicate experiment was carried out, which confirmed the general trends (Table S6, qPCR3).

The results from qPCR analysis allowed us to quantitatively assess the degree of gene repression by P_i in the samples inoculated by *G. intraradices* with and without high P_i supply (Table S6). According to the reduction in colonization of approximately 25-fold, a gene whose expression is reduced about 25-fold would be expressed proportional to

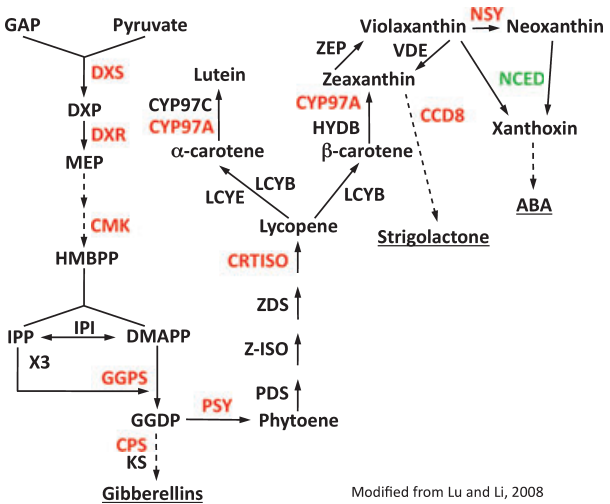

Figure 5. Regulation by phosphate of genes encoding enzymes in carotenoid biosynthesis and processing.
Downregulated enzymes are indicated in red, upregulated enzymes are indicated in green (Compare with Table 2). Abbreviations: ABA, abscisic acid; CPS, *ent*-copalyl diphosphate synthase; CRTISO, carotene isomerase; CYP97A, carotene β-hydroxylase (cytochrome P450 type); CYP97C, carotene ε-hydroxylase (cytochrome P450 type); DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GAP, glyceraldehyde-3-phosphate; GGDP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; GGR, geranylgeranyl diphosphate reductase; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; HYDB, carotene β-hydroxylase (non-heme di-iron type); IPI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; KS, *ent*-kaurene synthase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NSY, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; ZEP, zeaxanthin epoxidase.

the colonization level, and could therefore be defined as a quantitative marker for colonization. Consequently, genes that are repressed >25-fold in at least two of the three replicate experiments (bold repression values and shaded IDs) may be repressed by P_i in a direct way, not just as a result of reduced colonization. Based on this reasoning, most of the genes are repressed directly by P_i, in particular *PhPT4* and *PhPT5*.

P_i-related repression of AM cannot be assigned solely to strigolactone deficiency

One of the major results of the microarray experiments was that P_i represses several enzymes involved in carotenoid biosynthesis and processing. Hence, we suspected that P_i may repress AM by inhibiting the biosynthesis of the apocarotenoid strigolactone. Strigolactone exudation is known to be negatively correlated with P_i supply in red clover (Yoneyama *et al.*, 2007) and tomato (Lopez-Raez *et al.*, 2008). Unfortunately, the strigolactones of petunia are not known preventing the direct determination of strigolactone levels in our experiments. We therefore attempted to complement the P_i-dependent repression of AM development by exogenous supply of the synthetic strigolactone GR24 (Gomez-Roldan *et al.*, 2008) using the strigolactone defective petunia mutant *dad1* (Snowden *et al.*, 2005) as a control. Four weeks after inoculation, wild type plants had reached an intraradical colonization level of more than 70% in the presence or absence of GR24 (Figure 6). *Dad1* mutants exhibited substantially lower levels of colonization in all categories (extraradical hyphae, hyphopodia, total colonization, and arbuscules) with only 22% total intraradical colonization. All aspects of colonization in *dad1* were improved by GR24 (10 nM), although only extraradical and intraradical colonization reached the significance threshold of 0.05%. In contrast, all P_i-treated plants exhibited very low levels of colonization, irrespective of an additional supply of GR24. Even 10-fold higher GR24 concentrations (100 nM) did not enhance AM fungal colonization in P_i-treated roots (Figure 6).

Repression of *PT* genes by P_i in established AM precedes the reduction in colonization

Compared with the relatively mild effects of downregulation of carotenoid biosynthesis on arbuscule turnover (Floss *et al.*, 2008a,b), mutation or downregulation of AM-specific *PTs* resulted in pronounced reduction of intraradical AM colonization, presumably due to accelerated arbuscule senescence (Maeda *et al.*, 2006; Javot *et al.*, 2007). Based on these observations, we reasoned that the repression of *PT* genes by P_i in our experiments may represent a cause of reduced AM colonization, rather than merely its consequence. To establish the sequence of events, plants with colonized roots were treated with high P_i levels, and were subsequently tested over time for reduction in colonization and for repression of *PT* genes. Little effects on AM colonization were observed during the first week after P_i treatment (Figure 7a), and only after 2 weeks, a significant reduction in colonization was detected. In contrast, a reduction of *PhPT5* gene expression was already evident after two days, and after 2 weeks, *PhPT5* expression was almost completely repressed (Figure 7b). Similar results were observed for *PhPT3* and *PhPT4* (Figure S3). Hence, the inhibitory effect on *PT* gene expression clearly preceeded the reduction of root colonization. This finding suggests that reduced *PT* gene expression is more likely to be the cause than the consequence of reduced AM colonization in roots supplied with high P_i concentrations.

DISCUSSION

While numerous studies have dealt with the effects of P_i limitation on plants and on their strategies to cope with this condition (Franco-Zorrilla *et al.*, 2004; Desnos, 2008; Fang *et al.*, 2009), much less is known about how high P_i levels influence plants. High P_i-fertilisation limits AM in the field (He and Nara, 2007), which in turn results in the loss of

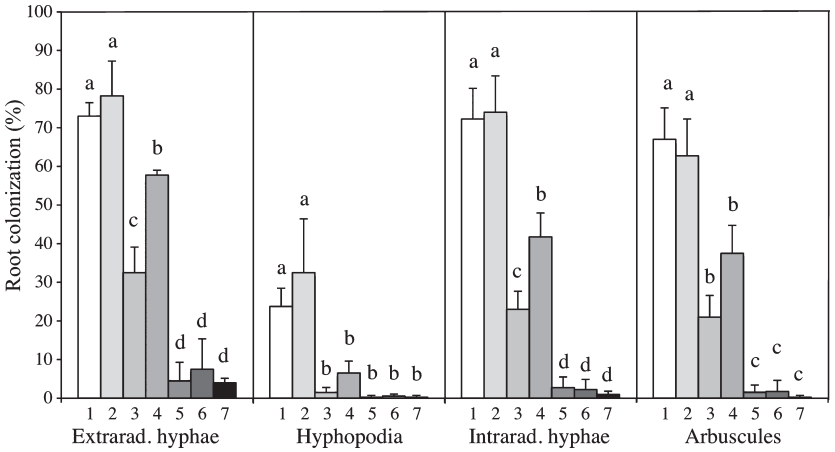

Figure 6. AM colonization in roots treated with combinations of P_i and the synthetic strigolactone GR24.
Wild type plants (columns 1, 2, 5, 6 and 7) or *dad1* mutants (columns 3 and 4) were inoculated with *G. intraradices* and treated with fertiliser containing 30 μM KH_2PO_4 (columns 1–4) or with fertiliser containing 5 mM KH_2PO_4 (columns 5–7). GR24 was supplied at 10 nM (columns 2, 4 and 6) or at 100 nM (column 7). Total intraradical colonization was determined in four biological replicates. Shown are means ± SD (n = 4). Different letters indicate significant differences (Student's t-test, $P \leq 0.05$). Statistical analysis was performed independently for the four categories of colonization.

AM-related benefits other than P_i supply, such as the supply of nitrogen (Govindarajulu *et al.*, 2005), sulfur (Allen and Shachar-Hill, 2009) and microelements (reviewed in George, 2000), increased pathogen resistance, and improved stress tolerance (reviewed in Pozo and Azcon-Aguilar, 2007). Hence, a better understanding of the mechanisms of AM repression by high P_i may help reconcile the advantages of AM and mineral fertilisation.

In our experiments, AM colonization was inhibited by solutions containing 0.5 mM or more soluble orthophosphate (Figure 1), which is in the range of P_i in the soil solution of arable soils (e.g. McDowell and Sharpley, 2001). Our split root experiments show that the effect of P_i on AM symbiosis and on gene expression is systemic (Figure 3), indicating that a systemic signal may relate phosphorus (P) status throughout the plant. However, it should be noted that P_i itself is mobile within the plant (Vierheilig *et al.*, 2000), and tends to accumulate in the shoot where it regulates the expression of P-signalling genes (Burleigh and Harrison, 1999). Interestingly, P_i application through the leaves is sufficient to inhibit AM colonization in the roots (Sanders, 1975). These observations are compatible with a scenario in which P_i from the roots is translocated to the shoot, where a mobile signal is generated to alter the physiology of the roots (Doerner, 2008), and thereby their competence to engage into AM symbiosis.

AM symbiosis in petunia is accompanied by expression of conserved marker genes

We have chosen a transcriptomic approach to reveal the pathways that are affected by P_i. As no transcriptomic study on AM in petunia has been described to date, we first discuss the AM-related changes in gene expression, and then examine how P_i interferes with regulation of gene expression, and how this may influence the interaction between petunia and *G. intraradices.*

Mycorrhizal petunia roots expressed a conserved complement of AM-associated marker genes (Table S4). The best-studied AM-induced genes, in terms of regulation and function, are the symbiotic *PT*s, which are essential for P_i transfer from the fungus to the plant, and for effective symbiotic development (Maeda *et al.*, 2006; Javot *et al.*, 2007). In petunia, the symbiotic *PhPT3, PhPT4,* and *PhPT5,* representatives of the three conserved symbiotic *PT* genes of *Solanaceae* (Nagy *et al.*, 2005; Chen *et al.*, 2007b; Reddy *et al.*, 2008), were induced in mycorrhizal petunia (Table S3a,d) as observed in previous reports (Wegmüller *et al.*, 2008). A characteristic response observed in all transcriptomic studies on AM is the strong induction of genes encoding proteases of various types (Takeda *et al.*, 2007). We have detected AM-specific and AM-induced members of subtilases, cysteine proteases, legumains, and serine carboxypeptidases (Table S3a,d). Furthermore, the petunia AM-induced transcriptome comprised the following known AM-inducible genes: ABC-transporters, aquaporin (*NOD26*), and various signalling components (Table S3a,d).

Several AM-responsive genes encoded homologues of PR proteins (Table 2) such as chitinase III, which has been recognized previously as AM-specific marker (Salzer *et al.*, 2000), PR10, glutathion-S-transferases (GST), barwin-related endoglucanases, germin-like proteins, and peroxidases. For PR10 and barwin-related glucanases homologues have been identified in various monocot and dicot species, but not in *Arabidopsis thaliana* (data not shown). This leads us to speculate that AM-induced PR gene homologues are not induced as part of a transient defence response (that is repressed at later stages of symbiosis) but as components of a conserved symbiosis program. Hence, they may serve dedicated functions in symbiosis, which have been under positive selection during the evolution of AM, and were secondarily lost in the non-symbiotic species *A. thaliana.*

Transcriptional effects of high P_i on defence and on hormonal stress pathways

A central question of this study was, whether inhibition of AM by P_i is related to the induction of a defence response. However, unexpectedly, the percentage of defence-related genes among P_i-induced genes (1.8%) and AM-P_i-induced

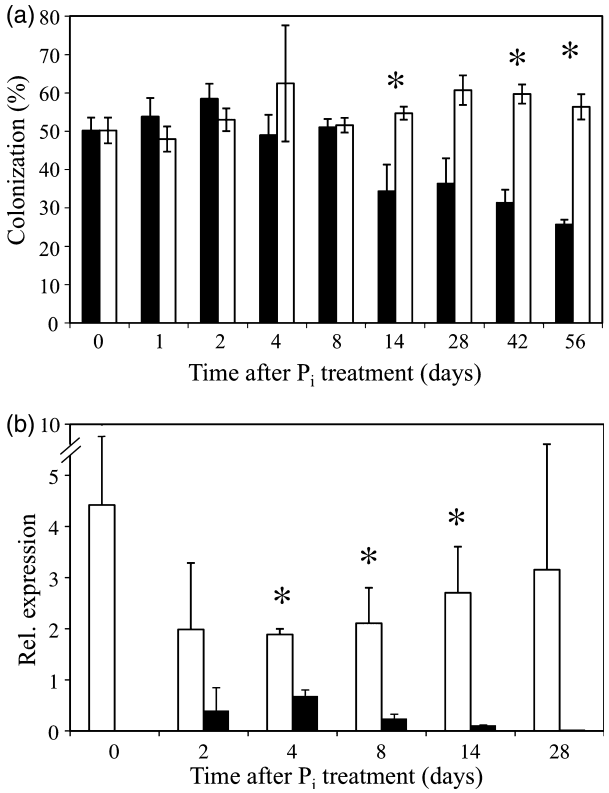

Figure 7. AM colonization and *PhPT5* expression in plants treated sequentially with AMF inoculum and with P_i.
Plants were first inoculated with *G. intraradices* and grown for 4 weeks with 30 μM KH_2PO_4, followed by application of 5 mM KH_2PO_4 (t = 0) for various times.
(a) Total root colonization in samples taken at the indicated times after addition of high P_i.
(b) *PhPT5* expression relative to *GAPDH* in the same samples as analyzed in (a). White columns represent plants that continued to be fertilised with 30 μM KH_2PO_4, black columns represent plants treated with 5 mM KH_2PO_4. Shown are means ± SD (n = 3). Asterisks represent significant difference between treatments (Student's t-test, $P \leq 0.05$).

genes (1.2%) was nearly 10-fold smaller than in the set of AM-inducible genes (13%), and the few potential defence genes induced by P_i exhibited only weak induction ratios, hence, the induction of homologues of PR proteins appears to be associated with the active symbiosis rather than with its inhibition.

Hormonal pathways have been shown to influence AM development (Hause *et al.*, 2007), with ABA and JA positively influencing colonization (Isayenkov *et al.*, 2005; Herrera-Medina *et al.*, 2007), and ethylene limiting infection (Penmetsa *et al.*, 2008). Our data indicate that ABA biosynthesis and JA signalling may be induced by P_i, whereas ethylene biosynthesis and signalling was reduced (Table 2), hence, being compatible with promotion rather than reduction of AM colonization. Taken together, these results do not support the hypothesis that P_i may limit AM development by inducing defence or modifying the balance of stress hormones. Consistent with this notion, the microscopic appearance of the interaction at high P_i levels did not resemble a hypersensitive response, a hallmark of resistance reactions of plants. Rather, fungal colonization appeared to be slowed down during colonization of the root cortex (Figure 2), arguing for a gradual decline and a relatively late abortion of the interaction at the level of arbuscule development and intercellular hyphal spreading in the cortex.

Transcriptional effects of high P_i on symbiotic signalling and carbohydrate metabolism

At least seven common *SYM* genes are required for progression of AM infection (Parniske, 2008). A reduction of expression of any of these genes by P_i could therefore potentially result in a suppression of colonization. However, the expression of petunia common *SYM* gene homologues was not affected by P_i (Table S5). This is consistent with the observation that the phenotype of AM inhibition by P_i in the cortex (Figure 2) is considerably different from the phenotype of *sym* mutants, in which epidermal entry by AM fungi is strongly inhibited (Parniske, 2008). Similarly, the homologue of a receptor kinase which negatively regulates rhizobial and AM fungal colonization in legume roots (Meixner *et al.*, 2005; Magori and Kawaguchi, 2009), was not affected by P_i supply (Table S5). In contrast, a homologue of the *L. japonicus* receptor kinase NFR5, that was repressed by P_i (Table S5), could represent an important regulator of AM symbiosis. LjNFR1 and LjNFR5 are essential components of nod factor perception and signalling (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). In analogy, PhNFR5 could play a role as a receptor for a fungal signal.

P_i can interfere with carbohydrate metabolism and transport (Hammond and White, 2007; Müller *et al.*, 2007), and high P_i levels have been hypothesized to lead to reduced carbohydrate allocation to the strictly biotrophic fungal symbiont, thereby providing a plausible mechanism by which plants could limit fungal proliferation under conditions of P_i saturation (Olsson *et al.*, 2006). However, apart from weakly regulated genes encoding sucrose-6-phosphate phosphatase, sucrose-phosphate synthase isoform C, and a fructose-bisphosphate aldolase-like protein, there was no evidence for significant changes in sugar metabolism or transport. Repression of two IDs with homology to genes encoding cell wall invertase inhibitors could potentially lead to increased invertase activity in the apoplast, however, this would not be expected to affect fungal growth (Schaarschmidt *et al.*, 2007). Hence, our data do not support a role of sugar allocation in P_i-related suppression of AM.

P_i represses carotenoid biosynthetic pathways and AM-associated genes

Combined evidence from biochemical and genetic studies suggests that carotenoids and their derivatives (apocarotenoids), which include abscisic acid (ABA) and strigolactones, play

prominent roles in AM symbiosis. Apocarotenoids such as mycorradicin accumulate in mycorrhizal roots (reviewed in Strack and Fester, 2006), and maize mutants defective in carotenoid biosynthesis exhibited decreased AM fungal colonization levels (Fester *et al.*, 2002). A root-borne carotenoid-derived signal (Matusova *et al.*, 2005), which stimulates AM fungal branching and metabolism (Buée *et al.*, 2000; Besserer *et al.*, 2006), was identified as strigolactone (Akiyama *et al.*, 2005). Carotenoid biosynthesis occurs in the plastids via the MEP pathway involving DXS2 (Walter *et al.*, 2007; Lu and Li, 2008; Phillips *et al.*, 2008). An alternative potential route of 1-deoxy-D-xylulose 5-phosphate biosynthesis is provided by plastidial transketolase (Bouvier *et al.*, 1998). Inhibition of plastidial carotenoid biosynthesis by knockdown of *DXS2,* and of *CCD1,* which encodes a carotenoid processing enzyme, has been shown to result in premature arbuscule senescence in *M. truncatula* (Floss *et al.*, 2008a,b).

In our experiments, P_i repressed both, *transketolase* and *DXS2* and numerous genes that encode enzymes catalysing subsequent steps of carotenoid biosynthesis and processing (Table 2; Figure 5). In addition, the expression of *DAD1,* the orthologue of *MAX4* in *A. thaliana* and *RMS1* in pea, which encode the strigolactone biosynthetic enzyme CCD8 (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), was repressed by P_i. These results are consistent with the observed negative correlation between P_i levels and strigolactone biosynthesis (Yoneyama *et al.*, 2007; Lopez-Raez *et al.*, 2008). Indeed, mutation of *RMS1* (Gomez-Roldan *et al.*, 2008), or of *DAD1* (Figure 6), results in reduced AM colonization. Hence, reduced strigolactone production may contribute to P_i-dependent repression of AM. However, this effect cannot be the principle bottleneck, since the exogenous supply of the synthetic strigolactone GR24 did not alleviate the repression of AM by P_i. In this context it should be noted that the P_i effect was much stronger than the symbiotic mutant phenotypes of strigolactone biosynthetic mutants (Gomez-Roldan *et al.*, 2008; Figure 6) supporting the conclusion, that strigolactone deficiency alone cannot explain the P_i effects.

P_i repressed the constitutive levels of AM-inducible *PhPT5,* and abolished the induction of AM-specific *PhPT4* (Table S6), potentially by reducing the responsiveness to the symbiotic signal lyso-phosphatidylcholine (Drissner *et al.*, 2007; Nagy *et al.*, 2009). Interestingly, when high P_i levels were supplied to plants that had already been colonized for 4 weeks (approximately 50% colonization), repression of all symbiotic *PTs* was observed within 2 days (Figures 7 and S3), while reduction in colonization was not observed before 2 weeks after P_i addition. Hence, reduced *PT* gene expression cannot be the consequence of reduced colonization. In light of the observation that mutations in the symbiotic *PT* genes of *L. japonicus* (Maeda *et al.*, 2006) and *M. truncatula* (Javot *et al.*, 2007) caused defective AM interactions, the rapid repression of all symbiotic *PTs* of petunia may well represent one of the causes for reduced colonization.

Besides *PTs,* P_i repressed several AM-inducible proteases (Table S3h,i). Interestingly, AM-inducible proteases of the subtilase type are involved in AM development in *L. japonicus* (Takeda *et al.*, 2009). Hence, P_i could be envisaged to impinge on symbiosis by repression of proteases. However, it should be noted that in petunia, P_i repressed only serine carboxypeptidases, but not the other protease types (subtilase, cysteine protease, legumain). Interestingly, P_i repressed *PAM1,* a gene that is essential for intracellular development of AM fungi in petunia (Feddermann *et al.*, 2010). Downregulation of the *PAM1* orthologue *VAPYRIN* in *M. truncatula,* caused similar defects as mutation of *PAM1* (Pumplin *et al.*, 2010), emphasizing its conserved role in intracellular accommodation of AM fungi. Hence, the downregulation of *PAM1* by P_i could potentially be responsible for some of the defects in cortical colonization of roots supplied with high P_i levels.

In conclusion, taken together, comparative transcriptomic analysis in petunia, *Medicago, Lotus,* and rice reveals a conserved complement of AM-regulated genes, which may serve essential functions in establishment and functioning of AM symbiosis. High exogenous P_i supply caused a strong systemic inhibition of AM colonization. We have proposed a number of hypothetical mechanisms, by which P_i could interfere with AM. An involvement of a defence response or interference with the common SYM pathway is not supported by our data, nor is P_i likely to act through modification of carbohydrate relations. Rather, P_i appears to act through repression of (apo)carotenoid biosynthetic genes and of various genes with a role in symbiosis, such as phosphate transporters, proteases and genes involved in intracellular accommodation. Future studies should further address the mechanisms involved in the multi-facetted syndrome of P_i-related inhibition of AM. This will help to develop strategies aimed at reducing its adverse effects towards symbiosis, thereby allowing reconciliation of the advantages of P_i-fertilisation with the multiple benefits of the AM interaction.

EXPERIMENTAL PROCEDURES

Plant and fungal material, plant treatments, and evaluation of root colonization

Petunia hybrida (lines W115 and W138) and *Glomus intraradices* (MUCL 43204) were grown as described (Reddy *et al.*, 2007). For the treatments with nutrient solutions, plants were grown in pots containing a mixture of sand and soil (3:1 v/v) and watered weekly with 50 ml of the indicated solutions prepared with a basic nutrient solution (Reddy *et al.*, 2007). Inoculations of petunia with *G. intraradices* were performed as described (Reddy *et al.*, 2007). Generation of cuttings from *P. hybrida* W115 was done according to Ahkami *et al.* (2009).

Plants for split root experiments were first grown in the presence of low P_i levels (30 μM KH_2PO_4) for 4 weeks. Then each plant was transplanted to two pots with inoculum, whereby the root system

was split between the two pots. To evaluate the dynamics of the P_i effect in colonized roots, plants were first inoculated with *G. intraradices.* After 4 weeks of culture with low P_i supply, the plants had reached a colonization level of approximately 50% and were treated with high P_i solution for 1–56 days. Treatments with GR24 were carried out thrice weekly for 5 weeks after inoculation. For the determination of colonization levels, roots were harvested at indicated time points and stained with trypan blue according to Reddy *et al.* (2007). Acid fuchsin staining was carried out following the protocol of Floss *et al.* (2008a), except that 0.1% acid fuchsin was used. Mycorrhizal colonization levels were determined as described (Reddy *et al.*, 2007).

Root and petal cDNA library construction and EST sequencing

Total RNA was extracted from roots of *P. hybrida* (W138 and W115) and from petals of *P. axillaris* using the hot phenol protocol as described (Verwoerd *et al.*, 1989). mRNA purification and concentration was performed using Dynal oligo-dT magnetic beads (Invitrogen; http://www.invitrogen.com). To optimize the random sequencing from cDNA libraries, normalized cDNA libraries were generated by equilibrating abundant and rare transcripts. Normalized libraries were constructed using the cDNA SMART-kit (Clontech; http://www.clontech.com/) and the thermostable duplex-specific nuclease (Zhu *et al.*, 2001). The suffix dr001 in the EST names refers to the normalized library from roots grown at 30 μM KH_2PO_4, the suffix dr004 refers to the normalized library from petunia petals (corolla tubes) (Table 1). The preparation of subtractive cDNA libraries was carried out with the PCR-Select cDNA subtraction kit (Clontech) according to the manufacturer's specifications (for further details see Table 1 and Supporting information). The resulting cDNAs were cloned into libraries dsr12, drs21, and drs13 using the TOPO TA cloning kit (Invitrogen) or into library dsr31 using CloneJET PCR Cloning Kit (Fermentas, http://www.fermentas.com). This material was electroporated into ElectroMAX DH10B cells (Invitrogen). Randomly picked clones from cDNA libraries were sequenced at Max-Planck-Institute for Molecular Genetics (Berlin-Dahlem, Germany) using Capillary Sequencer systems: ABI 3730 XL and GE Healthcare (formerly Amersham-Pharmacia, http://www.gelifescience.com/) MegaBace 4500 equipped with Caddy system (Watrex, http://www.megabace.net/megabace/index.html/) and Sequencing kit ABI BigDye Terminator v.3.1 for both sequencing systems.

Construction, sequencing and clustering of a normalized cDNA library of petunia cuttings

RNA was extracted from cuttings at various developmental stages as described (Ahkami *et al.*, 2009) and poly (A) RNA was prepared using oligo(dT) cellulose Type7 according to manufacturer's instruction (Amersham Pharmacia, Germany). Construction of the normalized cDNA library was performed as described (Lein *et al.*, 2008), with minor modifications, using 5 μg poly(A) RNA. The cDNA library was subjected to three rounds of normalization, involving the denaturation, reassociation and removal of double-stranded cDNAs and the isolation and amplification of single-stranded cDNAs via polymerase chain reaction (PCR). After normalization, equalized cDNAs were ligated at random into the pCRblunt vector and transformed into competent *E. coli* cells followed by the selection of blue/white colonies. Clones were picked and sequenced by at GATC Biotech AG using Capillary Sequencer systems ABI 3730 XL (Konstanz, Germany). Approximately 4700 sequences were obtained after processing, a success rate of approximately 94%. The average reading length was 495 bp.

Microarray design, hybridization and analysis

The EST sequences generated in this study were clustered together with all sequences of *P. hybrida* and *P. axillaris* available at Genbank (15 713), TIGR (4466), and the Solanaceae genomics network SGN (5135) to generate a set of 24 816 non-redundant unique sequences (unigenes) for microarray design. The entire set of sequences used for this clustering is listed in Table 1 (available at http://pgrc.ipk-gatersleben.de/petunia_array). Design of a four-plex microarray with 72 000 features was carried out using the ArrayScribe 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. Shorter sequences were represented by two probes. Array design, probe synthesis, hybridization, analysis, and data normalization was carried out by Nimblegen. Analysis of expression data sets was carried out with Fire2.2 (Garcion *et al.*, 2006). Quantitative real-time polymerase chain reaction coupled to reverse transcription (qPCR) was carried out as described (Reddy *et al.*, 2007) using the primers listed in Table S7. A complete list of the gene IDs represented on the microarray, and the corresponding expression values in controls and the induction ratios in all treatments is provided in Table S2. For further experimental details see Supporting information.

Shoot phosphorous determination

Shoot phosphorus content was determined in three individual mature leaves per data point as described (Reddy *et al.*, 2007).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of various salt solutions on AM root colonization in petunia.

Figure S2. Gene Ontology (GO) classification of the EST sequences from petunia roots and petals.

Figure S3. Effects of exogenous P_i supply on the expression of *PhPT3, PhPT4,* and *PhPT5* in petunia roots previously colonized by *G. intraradices.*

Table S1. List of all petunia sequences generated in this study and from public databases, which were clustered to give rise to the 24 816 unigene sequences used for array design.

Table S2. Complete list of gene IDs represented on the microarray, with Blast hits from plant and fungal databases, with their expression levels in controls, and with expression ratios in all treatments.

Table S3. List of genes regulated by AM and P_i, sorted according to their expression pattern.

Table S4. Comparison of AM-inducible genes of petunia with homologues described in other plant species.

Table S5. List of genes with a putative function in symbiotic signalling, and their expression ratios.

Table S6. qPCR analysis of selected genes.

Table S7. List of primers used for qPCR analysis.

Appendix S1. Experimental procedures.

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