2013

Arbuscular mycorrhizal fungi differ in their ability to regulate the expression of phosphate transporters in maize (*Zea mays* L.)

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Mycorrhiza (2013) 23:507–514
DOI 10.1007/s00572-013-0491-1

SHORT NOTE

Arbuscular mycorrhizal fungi differ in their ability to regulate the expression of phosphate transporters in maize (*Zea mays* L.)

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Received: 12 November 2012 / Accepted: 18 February 2013 / Published online: 7 March 2013

Abstract Previous studies have found that some phosphate (Pi) starvation inducible transporter genes are downregulated and arbuscular mycorrhizal (AM) inducible Pi transporter genes are upregulated in maize roots associated with the fungus *Glomus intraradices*. However, little is known about the functional diversity of different AM fungal species in influencing the expression of Pi transporters in maize roots. Here, we studied the expression of two Pi transporter genes *ZEAma:Pht1;3* (Pi starvation inducible) and *ZEAma:Pht1;6* (AM inducible) in maize root colonized by different AM fungal inoculants. Non-mycorrhizal maize, maize colonized by *Glomus deserticola* (CA113), *Glomus intraradices* (IA506), *Glomus mosseae* (CA201), *Gigaspora gigantea* (MN922A) and the co-inoculation of all four species were established. The expression patterns of the two genes were quantified using real-time, reverse transcription polymerase chain reaction. The expression level of *ZEAma:Pht1;6* was 26–135 times higher in AM plants than in non-mycorrhizal maize roots, whereas the expression level of *ZEAma:Pht1;3* was five to 44 times lower in AM plants than in non-mycorrhizal plants. Expression of the two genes differed with inoculation treatment, and increasing the diversity of AM fungi in maize roots led to greater expression of *ZEAma:Pht1;6* as well as Pi uptake in shoots. The expression of *ZEAma:Pht1;6* was significantly positively correlated with AM colonization rate, concentration of AM biomarkers in maize roots, Pi uptake and dry weight of shoot, but negatively correlated with the expression of *ZEAma:Pht1;3*. Addition of Pi fertilizer at a low concentration significantly increased the expression of *ZEAma:Pht1;6* but had no effect on the expression of *ZEAma:Pht1;3*.

Keywords Arbuscular mycorrhizal fungi · Maize · Pi transporter gene · Phosphorus · Real-time RT-PCR · Fatty acid biomarker

Introduction

Arbuscular mycorrhizal (AM) fungi are known to form mutualistic associations with the roots of the majority of terrestrial plant species, and stimulate host plant growth mainly by enhancing the uptake of phosphorus (Smith and Read 2008). The symbioses are potentially able to acquire phosphorus via two pathways: directly via root epidermal cells and root hairs, and via the AM fungi (Smith et al. 2003). Phosphate (Pi) transporters play important roles in Pi transfer for both pathways (Benedetto et al. 2005; Javot et al. 2007). It is reported that there are three types of genes encoding Pht1 transporters involved in Pi uptake regulation by AM plant associations: AM fungal Pi transporter genes, plant Pi transporter genes, and AM-inducible plant Pi transporter genes (Javot et al. 2007).

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The plant Pi transporters and AM-inducible Pi transporters are mainly expressed in roots, especially in rhizodermal cells (Bucher 2007); however, the AM-inducible Pi transporters are predominantly or exclusively expressed in root cells containing arbuscules when roots are colonized by AM fungi (Javot et al. 2007). It is believed that the AM-inducible plant Pi transporters are involved in the acquisition of Pi released by the AM fungus at the symbiotic interface and can be used as markers for the symbiotic Pi uptake pathway (Grace et al. 2009). A limited number of AM-inducible plant Pi transporters have been identified in *Medicago truncatula* (Harrison et al. 2002), rice (Paszkowski et al. 2002), potato (Nagy et al. 2005), wheat (Glassop et al. 2005), and tomato (Nagy et al. 2005). In maize, at least eight Pi transporters have been cloned up to now, they are *ZEAma;Pht1;1–6* (AY974041-AY974046), ZmPT3 (AY639021), and ZmPT4 (AY639022) (Nagy et al. 2006; Wright et al. 2005). The gene *ZEAma;Pht1;6* has been identified as an AM-inducible Pi transporter in maize (Glassop et al. 2005). This gene was strongly expressed in mycorrhizal roots, with limited expression in non-mycorrhizal plants (Glassop et al. 2005) and older leaves under low Pi conditions (Nagy et al. 2006). Although *ZEAma;Pht1;6* may not be a strict AM specific-induced Pi transporter (Javot et al. 2007), evidence suggests that this gene is involved in Pi uptake via the mycorrhizal uptake pathway (Nagy et al. 2006).

Few studies have focused on the expression of Pi transporters in maize root colonized by AM fungi (Glassop et al. 2005; Nagy et al. 2006; Wright et al. 2005). These studies concluded that when maize roots were colonized by AM fungi, the gene *ZEAma;Pht1;6* had increased expression while the expression of several other Pi starvation-inducible Pi transporters, such as, *ZEAma;Pht1;1*, decreased. These studies mainly used one AM species (*Glomus intraradices*); thus, it is unclear how different AM species induce expression of Pi transporters in maize roots. It has been reported that different AM species or isolates have varying influence on the expression of Pi transporters in plant species such as *M. truncatula* and tomato (Burleigh et al. 2002; Poulsen et al. 2005). Furthermore, little is known about the relationship between the expression of Pi transporters in maize roots colonized by AM fungi and the growth or Pi uptake of maize. It is well-known that AM species show functional diversity in plant growth or Pi uptake; however, the molecular mechanisms underlying functional diversity are still not clear for many AM associations (Burleigh et al. 2002).

Therefore, the aim of the present study was to determine whether the expression patterns of Pi transporters using, reverse transcription polymerase chain reaction (Real-time RT-PCR) in maize roots varies among AM inoculants and whether this translates to functional diversity in Pi uptake.

Methods and materials

Production of AM fungal inoculum

Four AM fungal species, *Glomus deserticola* (CA113), *Glomus intraradices* (IA506), *Glomus mosseae* (CA201), and *Gigaspora gigantea* (MN922A), were ordered from the International Culture Collection of Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu) and were propagated on maize (*Zea mays* L.) in pasteurized soil–sand mix (containing 25 % soil and 75 % quartz sand). Plants were cultured in the greenhouse for 4 months under 28 °C in the daytime and 25 °C in the night. The root colonization by AM fungi was checked during the culture, and the presence of spores was confirmed by sieving.

Plant growth conditions

Maize plants (local, but unknown commercial hybrid) were cultured in 3-L cylinder pots filled with 3-L pasteurized soil–quartz sand mixture (1:3) (*v*/*v*). Treatments of non-mycorrhizal, single-inoculations of *G. deserticola*, *G. intraradices*, *G. mosseae*, *Gigaspora gigantea*, and the co-inoculation of all four AM species were set up and planted to maize. To inoculate the pots, 100 g of each AM fungal inoculum (for the mixture inoculation, 25 g each of the four AM species) was mixed into a 2.5-kg mixture. The AM fungal inoculum contains AM spores, AM extra-radical hyphae as well as root fragments. For the non-mycorrhizal treatment, 100 g of pasteurized soil–quartz sand mixture (1:3) was mixed into the quartz sand mixture instead of AM fungal inoculum. There were eight replicates for each AM treatment. Two pre-germinated maize seeds were sown in each pot on November 2, 2009. After the seedlings emerged, the plants were thinned to one per pot, and the pots were placed in a greenhouse with 16/8-h light/dark cycle at 28/25 °C. Four of the eight replicates for each AM treatment were watered with 400 mL of Hoagland nutrient solution with Pi (1 mM KH_2PO_4) (+Pi treatment) and without Pi (−Pi treatment) in each pot, respectively. The pots were watered with Hoagland nutrient solution once a week from November 8 for 9 weeks. Each pot received 435.4 mg of KH_2PO_4 in total for the +Pi treatment.

Harvest

Plants were harvested on 5 January 2010. Shoots were cut, oven-dried at 75 °C for 72 h, and then weighed. Dry shoots were crushed using a blender into a powder to determine Pi content using the method of van Aarle et al. (2002). The roots were washed with distilled water and divided into three parts: one part was stored in liquid nitrogen for RNA extraction; one

part was stored at −20 °C for the measurement of percent root colonization by AMF; and the remaining part was freeze dried at −50 °C for 48 h then frozen at −20 °C until measurement of AM fungal fatty acid biomarkers.

AM colonization and concentration of AM biomarkers in maize roots

Roots were cut into about 1-cm long segments and stained with 0.05 % (*w*/*v*) Trypan blue (Phillips and Hayman 1970), and the percent root colonization by AM fungi was calculated as described by Trouvelot et al. (1986). To quantify AM fatty acid biomarkers in maize roots, freeze-dried maize roots were roller-milled for 12 h at room temperature. Fatty acids were extracted from 30- to 50-mg roller-milled roots using the method of Grigera et al. (2007a). The fatty acids C16:1*cis*11 and C18:1*cis*11 were selected as biomarkers for the AM fungi (Grigera et al. 2007a), although C16:1*cis*11 has also been reported in a few bacteria (Zelles 1999) and some zygomycetous fungi (Amano et al. 1992), and C18:1*cis*11 is common to several gram-negative bacteria (Zelles 1999). The extraction procedure we used on freeze-dried and ground roots recovers ester-linked fatty acids from neutral, glycol- and phospho-lipid pools within the intra-radical hyphae, vesicles, spores, and arbuscules in maize roots as well as extra-radical structures remaining on root surfaces (Grigera et al. 2007b).

Real-time RT-PCR analysis of Pi transporter genes

Total RNA was extracted from about 80-mg maize roots using the RNeasy Mini Kit (QIAGEN). Approximately 1 μg of the total RNA was used as a template for first-strand cDNA synthesis using QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacture's instructions. Random primers supplied by the kit were used during the reverse transcription reaction. The cDNA was stored at −20 °C until analysis.

Before real-time RT-PCR was conducted, gene-specific primers for AM-inducible Pi transporter *ZEAma:Pht1;6* (forward: 5′-ACATAAACGCCCTCAAGGAG-3′ and reverse: 5′-GACGGTGACCCAGTAGCC-3′), which produced a 92-bp-long fragment, were designed using Primer Premier (version 5.0) software (Palo Alto, CA). For other Pi transporters, the Pi starvation-inducible Pi transporter gene *ZEAma:Pht1;3* (Nagy et al. 2006) was selected. Quantification of additional Pi transporters is needed, but the design of gene-specific primers for real-time PCR is problematic due to a high degree of similarity (Nagy et al. 2006). The specific primers for *ZEAma:Pht1;3* was forward: 5′-GCATGCATGCATG CAAATG-3′ and reverse: 5′- CGAAAACAACAGTA CTTGATTGATC-3′) (Nagy et al. 2006), and a 354-bp-long fragment was amplified. Primers were tested by PCR experiments on genomic DNA of AM fungi to exclude cross-hybridization with fungal templates (data not shown). To quantify the expression of Pi transporter genes, standard curves were made for both *ZEAma:Pht1;6* and *ZEAma:Pht1;3*. For the gene *ZEAma:Pht1;6*, the PCR thermal program consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and one cycle of 72 °C for 10 min. For *ZEAma:Pht1;3*, the PCR program was one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and one cycle of 72 °C for 10 min. The PCR products were purified and cloned into TOPO 2.1 vector using TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions. Plasmid DNA was purified from positive clones using the Wizard Plus Minipreps DNA Purification System (Promega). The occurrence of target genes in the plasmid DNA was verified by sequencing. The concentration of purified plasmid DNA was determined by spectrophotometry (Model: Biomate 3) at 260 nm, and the molecular weight of DNA was calculated using the formula $NC = (K \times Na)/(660 \times L)$, where L is the average DNA fragment length for each gene, K is the DNA concentration determined from its absorbance at 260 nm, 660 is the molecular mass of DNA in Da per base pair, and Na is Avogadro's constant (6.023×10^{23}). The plasmid DNA was then serially diluted with sterile water to obtain 10^9–10^2 copies per microliter.

One microliter of first strand cDNA or standard plasmid DNA mixed with QuantiFast SYBR Green Master Mix (QIAGEN) following the manufacture's instructions was used for real-time RT-PCR. Real-time RT-PCR was conducted using the iCycler iQ (Bio-Rad), and the thermal cycling consisted of an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing/extension at 60 °C for 30 s (for the gene *ZEAma:Pht1;6*) (60 °C for 60 s for *ZEAma:Pht1;3*), and 80 °C for 15 s. The fluorescence signal data were detected at the third step of the cycle to eliminate the signal of possible primer dimmers. Each reaction was performed in triplicate, and the average threshold cycle value was calculated for each sample. There were four biological replicates for each treatment. To calibrate gene expression data, a maize housekeeping gene, γ-tubulin, was used as a control (Ding et al. 2009). This gene showed a stable expression throughout the course of our experiment, although re-arrangement of the cytoskeleton may occur during symbiosis development (Genre and Bonfante 1998). The primers for this gene, which can amplify a 131-bp fragment, was: forward: 5′-GTCCTGTGCCACTCTATTGC-3′ and reverse: 5′-CTTGTTTCCACCTGATTTGG-3′. The standard curve for the γ-tubulin gene followed the same protocol as the Pi transporter genes, with a thermal program of one cycle of 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and one cycle of 72 °C for 10 min. Real-time

RT-PCR was performed under the same conditions as the Pi transporter gene *ZEAma:Pht1;6*.

Statistics

The influence of AM inoculation and Pi treatment on AM colonization, concentration of AM biomarkers in maize root, maize growth parameters, and expression of Pi transporters were analyzed by factorial ANOVA, and the means compared by least significant difference at the 0.05 level using SAS 8.2 (SAS Institute 1999). Pearson correlation coefficients were used to determine relationships among AM colonization, AM biomarkers, maize growth parameters, and Pi transporter expression parameters.

Results

AM colonization and concentration of AM biomarkers in maize roots

AM fungi colonized maize roots in all five inoculated treatments but not in the non-mycorrhizal control. Both AM inoculation ($P<0.0001$) and Pi treatment ($P<0.05$) influenced percent root colonized by AM fungi (Table 1). The percent root colonized by *Gigaspora gigantea* was significantly lower than the other four AM inoculations, which were not significantly different from one another in either Pi treatment (Table 1). Overall, the addition of Pi significantly decreased percent root colonized by AM fungi.

The influence of AM inoculation ($P<0.001$) and the interaction of AM inoculation and Pi treatment ($P<0.01$) on AM fatty acid biomarkers (C16:1*cis*11 and C18:1*cis*11) were significant (Table 1). The highest concentration of the AM biomarker C16:1*cis*11 was found in roots colonized by *G. intraradices*, and the concentration of this fatty acid was also significantly higher in roots inoculated with the AM mix than with *G. deserticola*, *G. mosseae* or *Gigaspora gigantea* in both Pi treatments (Table 1). This fatty acid biomarker was not detected in non-mycorrhizal roots. The AM biomarker C18:1*cis*11 was highest in roots colonized by *G. deserticola* and lowest in non-mycorrhizal roots in both Pi treatments. The concentration of C18:1*cis*11 was significantly lower in roots colonized by *Gigaspora gigantea* than by *G. mosseae* or the AM mix (Table 1). The addition of Pi did not significantly influence the concentration of AM biomarkers in maize roots ($P>0.05$).

Plant shoot dry weight and Pi uptake

Different AM species showed functional diversity in either shoot dry weight or Pi uptake. AM inoculation ($P<0.0001$), Pi treatment ($P<0.0001$) as well as their interaction ($P<0.0001$)

Table 1 Influence of different AM and phosphate (Pi) treatments to maize on AM colonization, shoot dry weight, Pi uptake, and AM fungal fatty acid biomarkers (C16:1*cis*11, C18:1*cis*11)

Pi treatment	AM inoculation	Colonization rate (%)	Shoot dry weight (g per plant)	Pi uptake (mg per plant)	C16:1*cis*11 (nmol mg^{-1})	C18:1*cis*11 (nmol mg^{-1})
−Pi	Non-mycorrhizal	0	6.9±0.8d	6.55±1.4d	0	0.35±0.2d
	Glomus deserticola	72.4±5.8a	16.5±1.1c	37.9±2.1bc	0.7±0.06c	3.5±0.29a
	Glomus intraradices	84.6±1.9a	21.7±0.5a	35.8±2.1c	11.6±1.2a	1.47±0.19bc
	Glomus mosseae	73.7±3.3a	18.7±0.5bc	45.5±5.1ab	1.0±0.05c	1.61±0.08b
	Gigaspora gigantea	28±2.9b	20.3±0.8ab	37.1±3.1bc	1.5±0.09c	0.94±0.25c
	Co-inoculation	77±6.6a	20.0±0.5ab	53.1±2.7a	3.8±0.4b	1.24±0.23bc
+Pi	Non-mycorrhizal	0	17.2±0.9b	19.8±2.5d	0	0.54±0.26d
	Glomus deserticola	64.9±7.3a	19.3±1.1ab	42.6±3.2bc	0.29±0.03c	2.06±0.1a
	Glomus intraradices	71.8±4.6a	21.6±0.6a	45.1±1.3bc	10.0±0.1a	1.88±0.08ab
	Glomus mosseae	72.0±2.7a	20.1±1.1a	51.2±5.9ab	1.1±0.03c	1.74±0.15b
	Gigaspora gigantea	20.3±1.4b	20.7±0.8a	38.0±5.0c	1.2±0.17c	1.31±0.06c
	Co-inoculation	76.6±2.0a	20.9±0.7a	63.1±5.7a	6.6±0.4b	1.65±0.02b
Significance[a] due to						
AM inoculation		***	***	***	***	***
Pi treatments		*	***	***	NS	NS
AM inoculation × Pi treatment		NS	***	NS	**	***

NS not significant

*$P<0.05$; **$P<0.01$; ***$P<0.001$

[a] By analysis of variance

influenced shoot dry weight (Table 1). In the absence of Pi, the dry weight of maize shoots colonized by *G. intraradices* was significantly higher than that colonized by *G. mosseae* and *G. deserticola*. There was no significant difference in dry weight among the five AM treatments on the addition of Pi. Non-mycorrhizal plants had significantly lower dry weights than AM inoculated plants under both Pi treatments (Table 1). Overall, Pi addition increased the dry weight of maize (Table 1).

Phosphate uptake was influenced by both AM inoculation ($P<0.0001$) and Pi treatment ($P<0.001$) (Table 1). The colonization of roots by AM fungi increased Pi uptake in maize shoots under both Pi treatments compared with non-mycorrhizal controls. Inoculation with a mixture of AM fungi led to the greatest Pi uptake. Pi uptake in plants colonized by *G. intraradices* or *Gigaspora gigantea* was lower than that colonized by *G. mosseae* regardless of Pi treatment. Overall, the addition of Pi increased Pi uptake by maize (Table 1).

Expression of Pi transporter genes

The relative expression of the AM-inducible Pi transporter gene *ZEAma:Pht1;6* was influenced by AM inoculation ($P<0.0001$), P treatment ($P<0.0001$) and their interaction ($P<0.001$) (Table 2). Colonization of maize roots by AM fungi greatly increased the relative expression of *ZEAma:Pht1;6* compared to non-mycorrhizal roots under both Pi treatments. The expression of *ZEAma:Pht1;6* in mycorrhizal roots was 26–135 times higher than that in non-mycorrhizal roots. The different AM inoculations showed varying expression of *ZEAma:Pht1;6*. In the absence of Pi, the relative expression of *ZEAma:Pht1;6* was higher in the AM mixture compared to the other four inoculations. The expression was also higher in *G. mosseae* or *G. intraradices* compared to *G. deserticola* or *Gigaspora gigantea*. The addition of Pi led to high expression of *ZEAma:Pht1;6* in roots colonized by the AM mix, and *G. mosseae* was higher than that of *G. deserticola* and *G. intraradices*. Overall, the addition of Pi significantly increased the relative expression of *ZEAma:Pht1;6* in plants colonized by AM fungi (Table 2).

Inoculation with AM fungi significantly decreased the relative expression of the plant root Pi transporter *ZEAma:Pht1;3* (Table 2). The expression of *ZEAma:Pht1;3* in non-mycorrhizal roots was five to 44 times higher than that in mycorrhizal roots. The greatest repression occurred in roots colonized by *G. mosseae*, *G. deserticola*, and the AM mix. The addition of Pi increased the expression of *ZEAma:Pht1;3* in non-mycorrhizal roots; however, the effect was not significant due to high variation (Table 2).

Relationship among AM colonization, AM biomarkers, maize growth parameters, and Pi transporter expression

The degree of AM colonization was positively correlated with the concentration of AM biomarkers, plant growth parameters, and the expression of *ZEAma:Pht1;6* but negatively correlated with the expression of *ZEAma:Pht1;3* (Table 3). The two AM biomarkers correlated positively with plant dry weight, Pi uptake, and the expression of *ZEAma:Pht1;6*, but

Table 2 Influence of different AM and phosphate (Pi) treatments on the relative expression levels of *ZEAma:Pht1;6* and *ZEAma:Pht1;3* in maize roots

Pi treatment	AM inoculation	Relative Expression level of *ZEAma:Pht1;6* ($\times10^{-2}$)	Relative Expression level of *ZEAma:Pht1;3* ($\times10^{-2}$)
−Pi	Non-mycorrhizal	0.23±0.09e	2.27±0.50a
	Glomus deserticola	5.92±0.5d	0.14±0.05c
	Glomus intraradices	16.3±1.3b	0.49±0.21b
	Glomus mosseae	12.1±1.7bc	0.04±0.03c
	Gigaspora gigantea	8.63±2.7 cd	0.19±0.04bc
	Co-inoculation	30.7±2.9a	0.30±0.14bc
+Pi	Non-mycorrhizal	0.50±0.06d	3.73±1.18a
	Glomus deserticola	15.7±4.6c	0.10±0.01 cd
	Glomus intraradices	19.5±1.8bc	0.60±0.13b
	Glomus mosseae	30.1±2.3a	0.08±0.01d
	Gigaspora gigantea	24.5±3.2ab	0.20±0.03c
	Co-inoculation	27.3±1.3a	0.22±0.09 cd
Significance[a] due to			
AM inoculation		***	***
Pi treatments		***	NS
AM inoculation × Pi treatment		***	NS

NS not significant

*$P<0.05$; **$P<0.01$; ***$P<0.001$

[a]By analysis of variance

Table 3 Pearson correlations among AM colonization, AM fungal fatty acid biomarker concentrations, maize growth, and Pi transporter expression parameters

	AM colonization	C16:1*cis*11	C18:1*cis*11	Pi uptake	Dry weight	*ZEAma:Pht1;3*
C16:1*cis*11	0.53**	–	–	–	–	–
C18:1*cis*11	0.53**	0.04	–	–	–	–
Pi uptake	0.76***	0.30	0.39*	–	–	–
Dry weight	0.56***	0.51**	0.51**	0.67***	–	–
ZEAma:Pht1;3	−0.64***	0.20	−0.45**	−0.59***	−0.41*	–
ZEAma:Pht1;6	0.58***	0.36*	0.13	0.70***	0.53**	−0.55**

*$P<0.05$; **$P<0.01$; ***$P<0.001$

negatively with the expression of *ZEAma:Pht1;3* (Table 3). The expression of *ZEAma:Pht1;6* was positively correlated with plant Pi uptake and dry weight, indicating the importance of this gene to Pi uptake in maize. The expression of *ZEAma:Pht1;3* was negatively correlated with plant growth parameters (Table 3).

Discussion

The present study demonstrated that different AM species show functional diversity in terms of colonization, maize growth, Pi uptake as well as Pi transporter gene expression. The lower degree of colonization found for *Gigaspora gigantea* reflected different colonization strategies between fungi belonging to Glomaceae and Gigasporaceae (Feddermann et al. 2010). The AM biomarkers varied greatly among the different AM inoculants, and differed from AM colonization data. Variation of AM biomarker data among different AM inoculants has been noted in other studies (Graham et al. 1995; Madan et al. 2002), and apparently, a single fatty acid biomarker is not adequate to represent the biomass of all AM species. Maize colonized by AM fungi showed positive growth response compared to non-mycorrhizal maize, supporting the high AM dependency of maize (Hamel and Smith 1991).

All AM inoculations significantly increased the expression level of the AM-inducible Pi transporter (*ZEAma:Pht1;6*) and decreased the Pi starvation-inducible Pi transporter (*ZEAma:Pht1;3*) in maize roots. The increased expression of *ZEAma:Pht1;6* in AM-colonized maize has been found in previous studies (Glassop et al. 2005; Nagy et al. 2006). For the Pi starvation-inducible Pi transporter genes, the expression of *ZEAma:Pht1;1*, *ZEAma:Pht1;4*, and *ZmPT3* was found to decrease in maize root colonized by *G. intraradices* (Nagy et al. 2006; Wright et al. 2005), while the expression of *ZEAma:Pht1;3* remained relatively constant (Nagy et al. 2006). This is inconsistent with our data and may be due to: (1) different methods used in quantifying gene expression level and (2) different maize hybrids. In the study of Nagy et al. (2006), the gene expression level was quantified using RNA gel blot method, which is a qualitative or semi-quantitative method (Mirucki 2009). Using real-time RT-PCR the expression of *ZEAma:Pht1;3* was decreased by colonization with *G. intraradices*, but the relative decrease (~5 times vs CK) was much lower compared to other AM species (~50 times for *G. mosseae*). The study of Wright et al. (2005) found that the expression of *ZEAma:Pht1;1* and *ZEAma:Pht1;4* was decreased in maize root colonized by *G. intraradices*, but the relative decrease differed between the two maize hybrids (River and H511). Thus, different maize hybrids (a local commercial hybrid in our study and Gaspé Flint in the study by Nagy et al. (2006)) may also explain the inconsistency.

AM fungi were found to differ in their ability to induce the expression of *ZEAma:Pht1;6*. Although *G. deserticola*, *G. intraradices*, and *G. mosseae* had similar AM colonization, the expression of *ZEAma:Pht1;6* was significantly lower in maize roots colonized by *G. deserticola* than either *G. intraradices* or *G. mosseae* in the −Pi treatment. In contrast, *Gigaspora gigantea* had a relatively high expression level of *ZEAma:Pht1;6* at a much lower AM colonization. It is believed that the AM-inducible Pi transporters are involved in plant uptake of Pi released by the fungus in the plant root (Smith et al. 2011). A positive correlation between the expression of *ZEAma:Pht1;6* and Pi uptake was found in our study and is supported by the work of Grace et al. (2009). In tomato, the expression of AM-inducible Pi transporter genes (*LePT3* and *LePT4*) was positively correlated to the fungal contribution to plant Pi content (Poulsen et al. 2005), consistent with our study. Thus, the variation in expression of AM-inducible Pi transporter genes may reflect differing amounts of Pi released from the intra-radical hyphae by different fungal species. Co-inoculation resulted in the highest expression level of *ZEAma:Pht1;6* (especially in the −Pi treatment) as well as the highest Pi uptake, suggesting a high diversity of AM colonization may transfer more Pi to the intra-radical hyphae within maize roots. The present data provide additional support for AM-inducible Pi transporter genes as markers for a functional mycorrhizal Pi uptake pathway in plants (Javot et al. 2007; Poulsen et al. 2005).

The expression of *ZEAma:Pht1;3* also varied among AM species, with the greatest reduction in expression on colonization by *G. mosseae*. Burleigh et al. (2002) also found that *G. mosseae* resulted in the greatest reduction in *MtPT2* (a Pi

starvation-inducible gene) in *M. truncatula*. However, the mechanisms by which AM colonization reduces expression of plant Pi starvation-inducible Pi transporters are still unclear. Smith et al. (2011) suggested that the upregulation of Pi transporters in non-mycorrhizal plants may be part of "stress responses," and Burleigh and Bechmann (2002) suggested that factors other than those involving the Pi nutrition of plant may play important roles in influencing gene expression. It has been hypothesized that changes in carbon allocation during the symbiosis may influence expression of plant Pi transporter genes (Burleigh and Bechmann 2002); however, it is not clear whether downregulation of plant Pi transporters can also be triggered by the plant in response to fungal contact (Javot et al. 2007).

The addition of Pi significantly increased the relative expression of *ZEAma:Pht1;6* in AM plants but not that of *ZEAma:Pht1;3*. This is not surprising given AM fungi would transfer more Pi to the intra-radical hyphae after the addition of Pi. Although, *ZEAma:Pht1;3* was reported to be a Pi starvation-inducible gene (Nagy et al. 2006), the Pi concentration used in our study was sufficiently low to have no effect on the expression of *ZEAma:Pht1;3*.

In conclusion, different AM fungal inoculations display functional diversity in Pi uptake by maize. The colonization of maize by AM fungi significantly decreased the expression level of the Pi starvation-inducible Pi transporter gene *ZEAma:Pht1;3*, but increased the expression of the AM-inducible Pi transporter gene *ZEAma:Pht1;6*. AM species showed variable effects on expression of the two genes, and increasing the diversity of AM fungi in maize roots through co-inoculation with all four AM species led to higher expression of *ZEAma:Pht1;6* as well as Pi uptake in shoots. The expression of *ZEAma:Pht1;6* was positively correlated with AM colonization rate, concentration of AM biomarkers, Pi uptake and dry weight of shoot, supporting its use as an indicator of AM function in maize. Addition of low levels of Pi fertilizer significantly increased the expression of *ZEAma:Pht1;6*.

Acknowledgments The work was funded by the National Natural Science Foundation of China (Program no. 41201239), the Natural Science Basic Research Plan in Shaanxi Province of China (program no. 2012JQ3011), and the University of Nebraska. We thank Elizabeth Jeske for the assistance in the AM biomarker analysis, and Ryan McGhee and Jennifer McGhee for the assistance in the molecular analyses. We also thank Ruth Miller for the assistance in the greenhouse.

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