Plant Mol Biol Rep (2012) 30:885–893
DOI 10.1007/s11105-011-0402-6

ORIGINAL PAPER

Expression of *NtPT5* Is Correlated with the Degree of Colonization in Tobacco Roots Inoculated with *Glomus etunicatum*

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Published online: 10 January 2012

Abstract An important characteristic of arbuscular mycorrhizal (AM) symbiosis is the transfer of phosphate (Pi) from AM fungi to plants, and this transfer is facilitated by plasma membrane-spanning phosphate transporter (PT) proteins. Five *Nicotiana tabacum PT* genes (*NtPT*), members of Pht1 family, express in the root. However, the ways these genes contribute to the development of AM is unclear. In this study, transcription analysis was performed to study the expression levels of *NtPT1–5* genes in tobacco roots. To investigate the expression pattern of these five genes during the development of AM from no inoculation to high colonization, we screened for differentiating transcriptional responses of non-mycorrhizal roots and AM roots by real-time PCR. Our results indicate that only *NtPT5* was mycorrhiza-specific phosphate transporter during arbuscular mycorrhizal symbiosis in tobacco roots, and the induction was tightly correlated with the degree of root colonization by *Glomus etunicatum*. In addition, based on an alignment and analysis of the evolution of Pht1 family members in six solanaceous plants, we developed the evolutionary pattern of the Pht1 gene family members in six solanaceous plants.

Keywords Arbuscular mycorrhiza · Phosphorus · Phosphate transporter · Tobacco · Solanaceous species

Abbreviations

AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungus
P	Phosphorus
PT	Phosphate transporter
W38	Wisconsin 38

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Introduction

Phosphorus is an indispensable nutrient for plants. The phosphorus concentration in soil solution rarely exceeds 10 μM (Schachtman et al. 1998), which limits the growth of plants (Coelho et al. 2010). The rate of absorption of phosphate (Pi) by growing roots is much higher than the rate of soil phosphorus diffusion. As a result, a depletion zone of phosphorus is formed at the root system level in the soil, limiting the supply of phosphorus to plants. However, plants have evolved elaborate mechanisms to facilitate phosphate uptake, including modification of the soil chemistry around the roots, activation of high-affinity phosphate transport with the use of the H^+ gradient to drive the transport process, and the formation of symbiotic associations with arbuscular mycorrhizal (AM) fungi (AMF) (Gianinazzi-Pearson et al. 2000; Karandashov and Bucher 2005; Rausch et al. 2001; Seufferheld and Curzi 2010). AM symbiosis is an ancient mutualistic symbiosis that originated more than 400 Ma ago in the roots of plants (Stubblefield et al. 1987). More than 80% of all terrestrial plants live in association with mycorrhizal fungi that facilitate mineral nutrient uptake, particularly phosphorus (Wang and Qiu 2006). The formation of intracellular fungal structures in the root cortex can trigger the expression of symbiosis-inducible phosphate transporters (Karandashov and Bucher 2005). Symbiosis-specific plant phosphate transporter genes have been identified in several plant species, such as tomato and rice (Chen et al. 2007; Drissner et al. 2007; Glassop et al. 2005; Harrison et al. 2002; Liu et al. 1998; Nagy et al. 2005; Paszkowski et al. 2002; Rausch et al. 2001; Wegmueller et al. 2008; Zhao et al. 2003). In addition, symbiosis-specific fungi phosphate transporter genes have been identified in fungi (Harrison and Vanbuuren 1995; Maldonado-Mendoza et al. 2001). With the growing number of PT sequences from different plant species, investigators have grouped PTs into

three families Pht1, Pht2, and Pht3. Pht1 proteins are localized in the plasma membrane, whereas Pht2 and Pht3 proteins are localized in the inner envelope of plastids and mitochondria, respectively (Bucher 2007; Karandashov and Bucher 2005; Mudge et al. 2002). To date, all the symbiosis-specific plant phosphate transporter genes belong to the Pht1 family (Karandashov and Bucher 2005).

Tobacco (*Nicotiana tabacum* L.) is a plant with AM symbiosis, and it has been widely investigated (Branscheid et al. 2010; Diao et al. 2011; Gianinazzi-Pearson et al. 2000; Liu and Wang 2003; Muller and Dulieu 1998; Viswanathan et al. 2011). In tobacco, neither Pht2 nor Pht3 genes are expressed in the roots (Chen et al. 2007). Five phosphate transporter genes belonging to the Pht1 family (also named as *NtPht1;1–5*, Chen et al. 2007) have already been cloned: *NtPT1* (GenBank accession number AB020061) and *NtPT2* (AB042950 or AF156696) (Baek et al. 2001), *NtPT3* (AB042951 or TEF091669) and *NtPT4* (AB042956 or TEF091672) (Kai et al. 2002), and *NtPT5* (EF091675) (Chen et al. 2007). All of the transcripts of *NtPT1–5* have been detected in tobacco roots (Chen et al. 2007; Kai et al. 2002). They are highly similar to known high-affinity phosphate transporters in higher plants. There is a 99.8% identity of mRNA sequences between *NtPT1* and *NtPT2* and 99.6% identity of amino acid sequences between NtPT1 and NtPT2, which differ in only two amino acids. The expression of *NtPT1* increases in both the leaves and roots under P-starvation conditions. The expression of *NtPT2* decreases in roots under P-depletion conditions, expression of *NtPT3* is enhanced with mycorrhiza, and expressions of *NtPT4* and *NtPT5* are induced by mycorrhiza (Chen et al. 2007). However, the roles of these genes in the development of AM remain unknown, and the expression pattern of each *NtPT* corresponding to the development of AM in roots is unclear.

It takes several days for AM to develop from no colonization to high colonization (Wenkart et al. 2001). However, to our best knowledge, there is no report about the pattern of *PT* expression levels in time course of AM development. The aim of this work is to reveal the pattern of *NtPT1–5* transcript expression throughout the process of colonization in tobacco roots. In addition, we try to reveal the evolutionary pattern of Pht1 family members in six solanaceous plants.

Materials and Methods

Plant Material and Cultivation Conditions

Tobacco (*N. tabacum* L. cv. Wisconsin 38, W38) seeds were washed with sterile water and immersed in 0.1% mercuric chloride for 5 min, followed by four washes with sterile water. These seeds were germinated in a sterilized mixture of vermiculite/nutritive soil in a ratio of 3:1. The P concentration of the mixture was 2.75 mg L^{-1}. The seeds or plantlets were watered every 3 days with sterile water. After 20 days, the plantlets were split and the strong and uniform seedlings were selected to be cultured in 18 cm pots filled with sterilized soil (vermiculite/nutritive soil ratio of 3:1). The plants were grown in a greenhouse (light/dark rhythm of 14:10 h and 28:22°C).

AM Fungal Inoculum

Fifteen days after splitting, the uniform plantlets were transplanted to sterilized soil and inoculated with *Glomus etunicatum* (BEG 168). The control plants were transplanted to sterilized soil without inoculation. The inoculum consisted of soil, spores (the spore density was 10,000 per 10 g inoculum), hyphae, and infected root fragments from a stock culture of *G. etunicatum*. Every 1 kg of sterilized soil was inoculated with 20 g inoculum for mycorrhizal treatments or 20 g sterilized (autoclaved) inoculum for the non-mycorrhizal control treatment. The mycorrhizal and sterilized inocula were mixed with sterilized soil.

Detection of AMF Colonization

The root samples were carefully washed under running water to remove soil. Roots were immersed in 10% KOH at 100°C for 15 min and then washed with water three times. The samples were stained with 0.05% (*w*/*v*) trypan blue for 5 h, rinsed several times, and destained overnight in 50% lactic acid (Phillips and Hayman 1970; Vierheilig et al. 2005). They were then stored in lactic acid, glycerol, and water (1:1:1, by volume). We looked for AM colonization under a microscope (Olympus BX40, Japan), using the magnified line-intersect method (McGonigle et al. 1990), counting at least 150 intersect points of presence or absence of colonization in each sample. Pictures were captured by microscope (Axioskop 2 plus, Zeiss, Germany). A rate of colonization of 100% would indicate that the root was colonized by the fungus throughout its length as shown by arbuscules or intraradical hyphae (Biermann and Linderman 1981).

P Content Measurement

Leaf P concentration was determined by the molybdenum blue method (Fogg and Wilkinson 1958) after the plant material was digested by H_2SO_4. Briefly, dry leaf amounts corresponding to 5 g of fresh weight were used for each extraction. Leaves were oven dried to constant weight at 80°C for approximately 40 h. Dry tobacco leaf (0.5 g) was digested with 4 ml of 98% H_2SO_4 and 20 μl H_2O_2

for 10 h. The digested sample was then diluted to 20 ml with distilled water. The samples were filtrated with quantitative filter paper (ashless grade 40 filter papers for medium speed and retention). The P concentration in the solution was measured with the absorbance read at 450 nm on a 722 UV–visible spectrophotometer (UNICO UV-2100, Shanghai, China).

RNA Extraction and RT-PCR

Approximately 0.5 g fresh root of tobacco was gathered, and total RNA was isolated using TRIzol reagent (Invitrogen, USA). After the extraction, total RNA was digested by DNase I (TaKaRa, Japan) in 37°C for 2 h. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, USA) in a reaction volume of 25 μl containing 1× PCR buffer, 1 mM dNTPs, 0.5 mM oligo(dT)B_{18B}, and 1 U RNase inhibitor (TaKaRa). The reactions were bathed for 2 h in water at 37°C. The reverse transcription production was checked by PCR with primers of *NtActin* (Chen et al. 2007) to ensure the production was successful. Amplified *NtActin* fragments were detected by electrophoresis on 1% (*W*/*V*) agarose gel and visualized by ethidium bromide staining. PCR reactions were also performed with non-reverse transcribed total RNA to exclude fragment amplification because of the presence of genomic DNA in the samples.

Quantitative Real-Time RT-PCR

To detect the expressions of *NtPT1–5*, we used real-time RT-PCR due to its accuracy, sensitivity, and reproducibility (Li et al. 2010; Qi et al. 2010). The primer design for real-time PCR was performed with the software Beacon Designer 7.0 (PREMIER Biosoft International, Palo Alto, CA, USA). All primers were designed according to same criteria, including a nearly equal annealing temperature (Table 1). All primers were tested by real-time PCR, and amplification was sequenced to ensure accuracy. Each primer pair produced a single product and amplified the target transcript with equal efficiency over a 1,000-fold range of cDNA templates diluted to a ratio of 1:10. Real-time PCR was performed on the Chromos 4 (Bio-Rad) using the SYBR Green PCR Master Mix (TOYOBO, Japan). The PCR reaction consisted of 10.0 μl of the Mix, 300 nM of forward and reverse primers, and 6.0 μl of 1:10-diluted template cDNA, added to deionized water for a total volume of 20.0 μl. Cycling was performed using the conditions specified by Opticon Monitor Software 3.0 and programmed with the following sequence of parameters: 1 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C, an incubation at 72°C for 5 min with a melting curve from 55°C to 95°C, readings every 1.0°C, and a hold for 2 s (Andersen et al. 2004; Gupta et al. 2011).

Table 2 Dry weight and P concentration of tobacco inoculated and not inoculated with AMF

Time (DAI)	Dry weight of shoot (g)		Leaf P concentration (mg g^{-1})	
	W38−AM	W38+AM	W38−AM	W38+AM
0	0.56±0.09b	0.56±0.08b	2.27±0.14b	2.27±0.16b
5	0.59±0.08b	0.59±0.09b	2.29±0.15b	2.28±0.13b
10	0.63±0.10b	0.64±0.11b	2.28±0.15b	2.2 7±0.15b
15	0.80±0.11b	0.81±0.12b	2.30±0.18b	2.28±0.20b
20	0.95±0.15b	0.97±0.13b	2.28±0.15b	2.30±0.17b
40	2.25±0.15b	2.57±0.23a	2.29±0.20b	2.30±0.19b

Dry weight of shoot and leaf P concentration of tobacco colonized and not colonized by *G. etunicatum*. Values are mean ± SD (standard deviation); different letters indicate significant difference and same letters indicate no statistically significant difference ($P<0.05$) according to a *t* test. $n=6$

DAI days after inoculation, *W38* wild-type tobacco, *+AM* with inoculation, *−AM* without inoculation

Data Analysis

To analyze relative gene expression data, it is suggested to adopt $2^{-\Delta\Delta C(t)}$ method (Schmittgen and Livak 2008). The experimental data were subjected to correlation analysis and one-way analysis of variance (ANOVA). Differences between means of variables were examined by the standard *t* test for independent samples. Statistical significance for all analyses was accepted at the $P<0.05$ level of probability. Statistical analyses were performed using Excel 2003 software (Microsoft Office).

Table 1 Primers used in the real-time PCR experiment

Gene	Forward primer	Reverse primer
NtAct	GCCATTCAAGCCGTTCTATC	CAGTCAAGTCACGACCAGCT
NtPT1	CGACAAAATAGGTCGATTTGCAA	TGTTGACCTAAGCCTGGCTG
NtPT2	CGACAAAATAGGTCGATTTGCAT	TGTTGACCTAAGCCTGGCTG
NtPT3	TCAACGTGCCACGGAATATC	GGATTCTGGCACCAAGAATG
NtPT4	GGCCAGACTTCGATCAACAT	TTGTGCAGCGTACAGGAATC
NtPT5	GTGCAGCAGTACACGCAAGA	TCTTCATCTGCGTCTCATCC

The melting temperature of all primers is 60°

Results

Shoot Biomass Increase and P Concentration Stabilization

The plants were harvested from 0 to 40 days after inoculation (DAI). We measured the weight of shoots (stalk plus the leaves) and the P concentration of leaves (Table 2). At 40 DAI, shoot dry weight was statistically significantly greater in AM plants than that in non-mycorrhizal (NM) plants ($P<0.05$) (Table 2). In contrast, there was no significant difference in shoot biomass from 0 to 30 DAI, but suggesting that AM could have effect after a period of time to accumulate and eventually appear significant difference.

Plants colonized by *G. etunicatum* had similar leaf phosphorus concentrations compared with NM plants. There was no significant difference in leaf P concentration from 0 to 40 DAI between NM and AM plants (one-way ANOVA, $P<0.05$). Leaf phosphorus concentrations consistently ranged from 0 to 40 DAI in both AM tobacco and non-AM tobacco.

AM Root Colonization Increased Continuously

The plants were harvested at 0, 5, 10, 15, 20, and 40 DAI (Fig. 1). Few intracellular hyphae were seen in cortical cells at 5 DAI (Fig. 1b). Intracellular hyphae increased noticeably from 5 to 20 DAI and completely covered the surface of tobacco root cells at 40 DAI (Fig. 1f). To evaluate the state of root colonization, we calculated the percentage of mycorrhizal coverage at each post-harvest interval (Fig. 2). The percent colonization (mean±SD) increased rapidly from 5 to 40 DAI. The percent colonizations were 0%, 0.5%, 15%, 45%, 57%, and 73% for each of the six post-

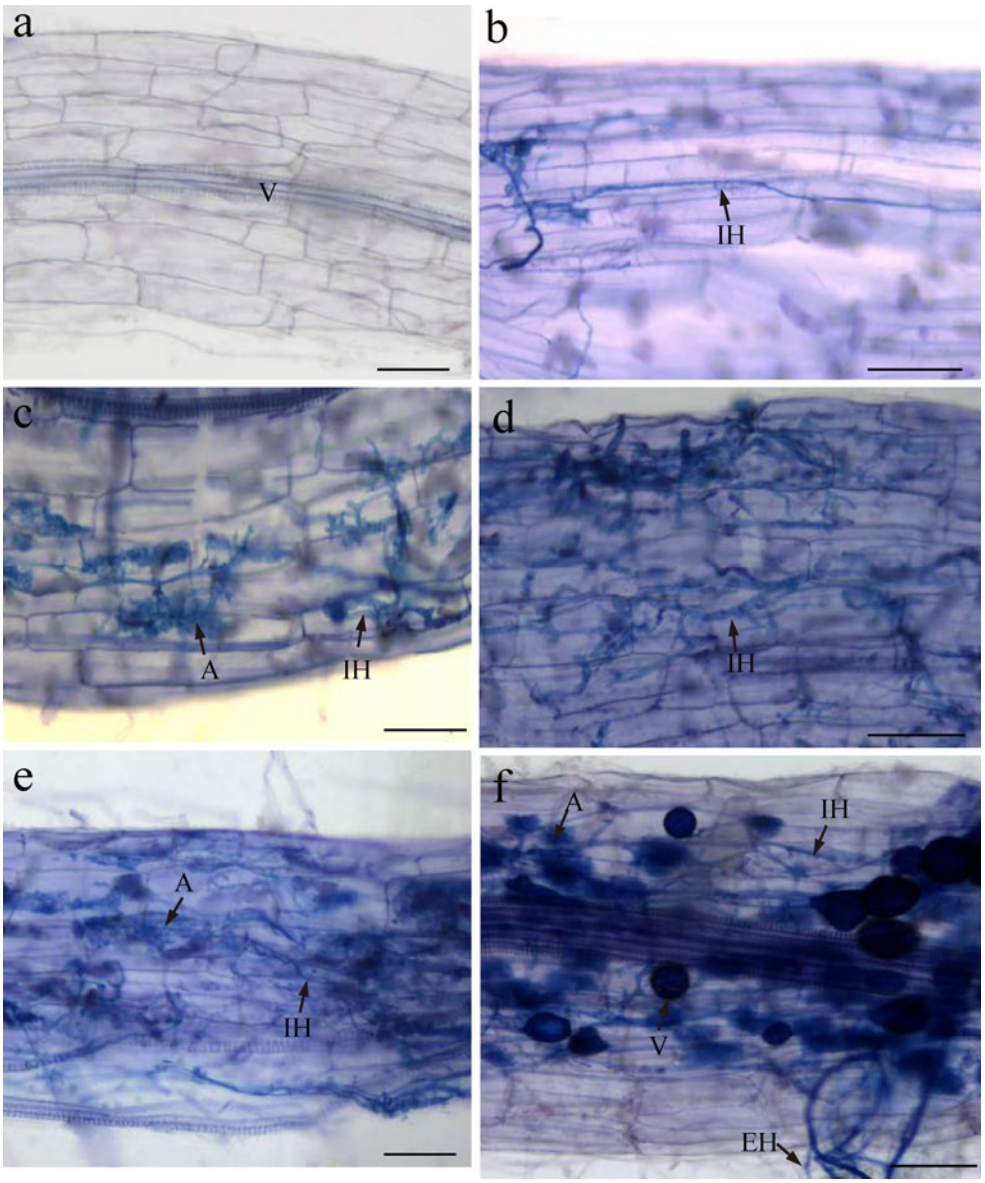

Fig. 1 AM development after inoculation: **a** 0 DAI; **b** 5 DAI; **c** 10 DAI; **d** 15 DAI; **e** 20 DAI; **f** 40 DAI. *A* arbuscule, *EH* extraradical hypha, *IH* intraradical hypha, *V* vesicule, *DAI* days after inoculation; *bars*, 50 μm

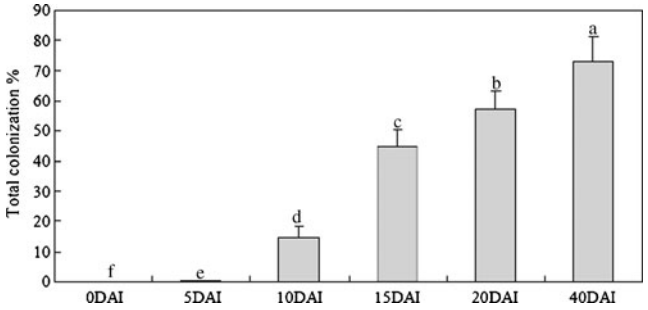

Fig. 2 Percentage of total colonization by *G. etunicatum*. *Values followed by different letters* in each group of data are significantly different ($P<0.05$). *DAI* days after inoculation

harvest intervals (Fig. 2). At 40 DAI, plants inoculated by *G. etunicatum* appeared to have the highest percent colonization.

Relative Expressions of *NtPT1–5* Show Different Changes

Real-time RT-PCR results showed the relative expression levels of *NtPT1* changing little from 0 to 40 DAI. At 10, 15, and 40 DAI, the relative expression of *NtPT1* increased slightly. At other time intervals, it hardly changed (Fig. 3a). Relative expression levels of *NtPT2* were significantly higher in AM plants than in NM plants at 10 and 15 DAI. However, its expression changed only slightly at other time intervals (Fig. 3b). Relative expression levels of *NtPT3* did not change from 0 to 10 DAI but increased at each interval from 15 to 40 DAI. They enhanced 4-, 3-, and 6-fold at 15, 20, and 40 DAI, respectively (Fig. 3c). Relative expression levels of *NtPT4* increased 5- and 3-fold at 10 and 40 DAI, respectively, but showed almost no change at the other intervals (Fig. 3d). It was special for the pattern of relative

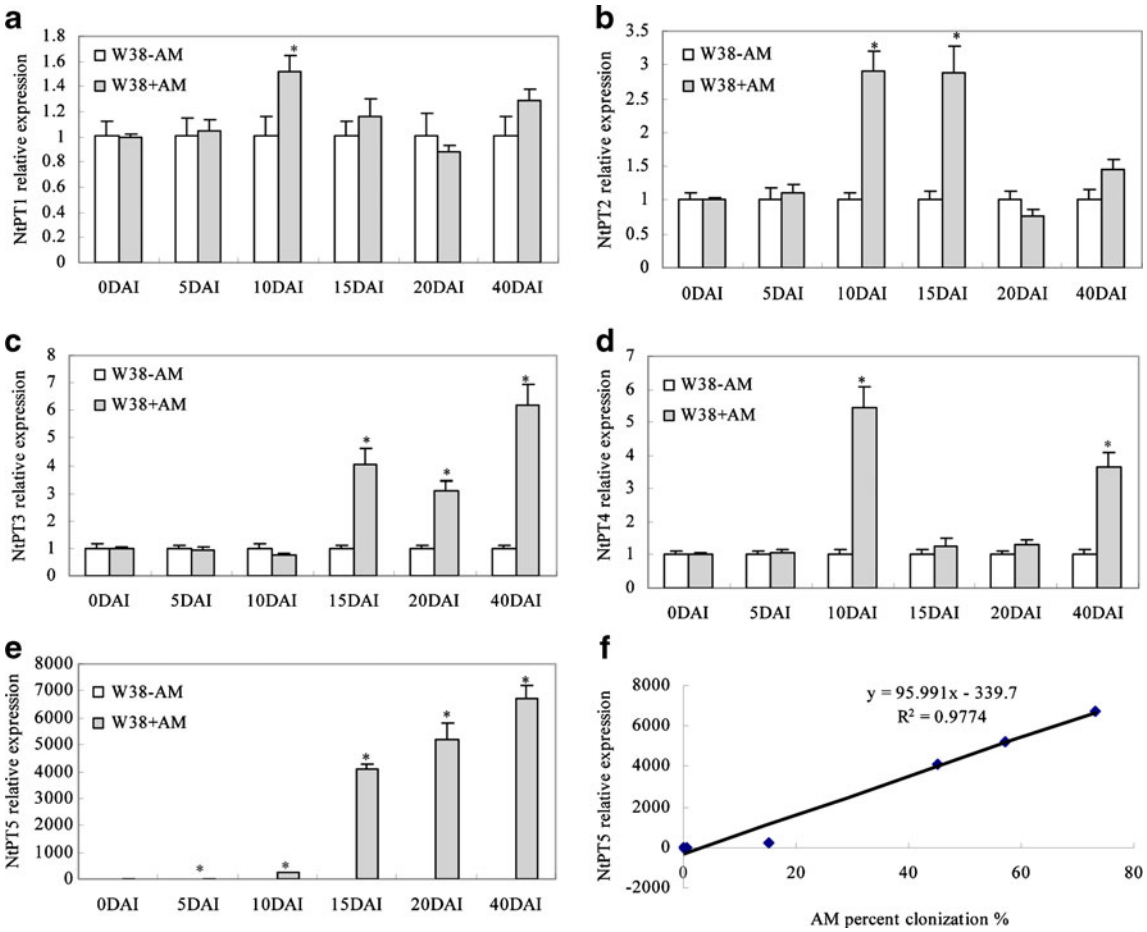

Fig. 3 Relative expressions of *NtPT1–5* in non-mycorrhizal roots (*white bars*) and mycorrhizal roots (*AM*; *gray bars*) from 0 to 40 days after inoculation with *G. etunicatum*. **a**–**e** represent the relative expressions of *NtPT1-5*, respectively. **f** The relationship between tobacco root colonization and the relative expression of *NtPT5*. The C_t values (threshold cycles) of the samples were corrected against the C_t values of the housekeeping gene *NtAct*. Data for each condition are presented as the mean ± SD and were obtained from three biological and three technical replicates. The analysis of the relative gene expression data used the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen 2001). For each condition, all gene transcript levels were first normalized against *NtActin* and then expressed relative to the genes' expression in W38 tobacco without inoculation. *$P<0.05$ indicate statistically significant differences; *DAI* days after inoculation

expression of *NtPT5*. Its relative expression changed from 0 to 40 DAI, with consistent, acute increases at every interval. It experienced more than a tenfold increase from 0 to 15 DAI, and a relatively slower increase from 15 to 40 DAI. It did, however, increase at each interval from 0 to 40 DAI: 0-, 23-, 215-, 4,081-, 5,183-, and 6,745-fold (Fig. 3e). Relative expressions of *NtPT1–5* showed different changes. A positive correlation has been shown between the increase in percent colonization of tobacco by AMF and the increased level of *NtPT5* expression (Fig. 3f).

The Phylogenetic Tree of Pht1 in Solanaceous Plants

To review the Pht1 family members associated with *Solanum*, we clustered all PT of *Solanum* to produce the solanaceous phylogenetic tree (Fig. 4). At least five differently expressed Pht1 genes are present in six solanaceous plants: potato (*Solanum tuberosum*), tobacco, tomato (*Lycopersicon eseulentum*), eggplant (*Solanum melongena*), pepper (*Capsicum frutescens*), and petunia (*Petunia hybrida*) (Chen et al. 2007; Kai et al. 2002; Wegmueller et al. 2008). Based on the solanaceous phylogenetic tree, PT4-5 was a main cluster, and PT1–3 was another main cluster. The PT4–5 cluster divided into PT4 and PT5. PT1–3 divided into the PT2 sub-cluster and the PT1–PT3 sub-cluster. PT1–PT3 sub-cluster eventually branched off to PT1 and PT3. However, NtPT2 was highly homologous with solanaceous PT1. Our data are consistent with the hypothesis that different PT are induced by AM in different plant species and that the Pht1 family of solanaceous plants has undergone consistent evolution.

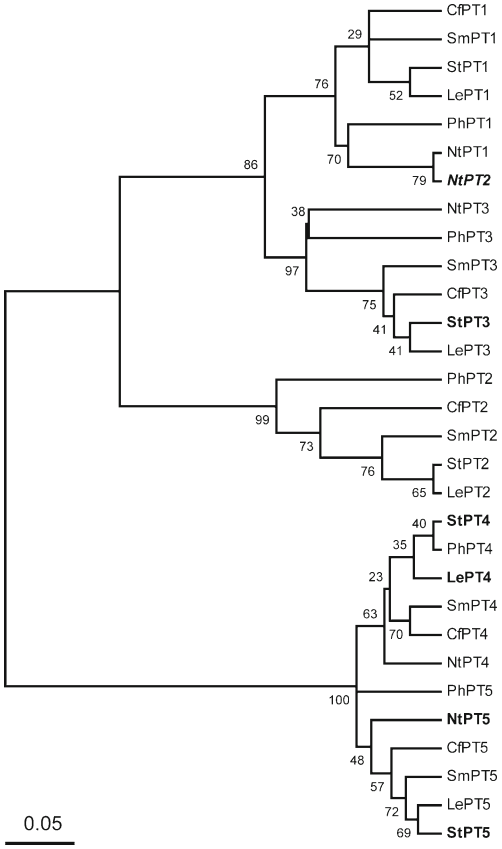

Fig. 4 Phylogenetic tree for the amino acid sequences of Pht1 family members in six solanaceous plants. The dendrogram was generated by MEGA 4.0 based on a ClustalX alignment and the neighbor-joining method for the construction of the phylogeny (Tamura et al. 2007; Zhang et al. 2011). Bootstrap tests were performed using 1,000 replicates. The *numbers in parentheses* refer to bootstrap values. The branch lengths are proportional to the phylogenetic distances. *Lycopersicon esculentum*: *LePT1*(AAB82146), *LePT2* (AAB82147), *LePT3*(AAV97729), *LePT4* (AAX85193), and *LePT5*(AAX85194) (Nagy et al. 2005); *Nicotiana tobacco*: *NtPT1* (BAA86070), *NtPT2* (BAB21545), *NTPT3* (BAB21562), *NtPT4* (BAB21563), and *NtPT5* (ABK63970) (Chen et al. 2007; Kai et al. 2002); *S. tuberosum*: *StPT1* (CAA67395), *StPT2* (CAA67396), *StPT3* (CAC87043), *StPT4* (AAW51149), and *StPT5* (AAX85195) (Nagy et al. 2005); *S. melongena*: *SmPT1* (ABK63959), *SmPT2*(ABK63961), *SmPT3* (ABK63963), *SmPT4*(ABK63966), and *SmPT5* (ABK63969) (Chen et al. 2007); *Petunia hvbrida*: *PhPT1* (ABS12068), *PhPT2* (ACB37439EU532761), *PhPT3* (ACB37440), *PhPT4* (ACB37441), and *PhPT5* (ACB37442) (Wegmueller et al. 2008); *C. frutescens*: *CfPT1*(ABK63958), *CfPT2* (ABK63960), *CfPT3* (ABK63962), *CfPT4* (ABK63965), and *CfPT5*(ABK63968) (Chen et al. 2007). Plant phosphate transporters shown to be induced upon formation of mycorrhiza are shown in *boldface* (*not italic*). Gene names are given using the accepted nomenclature or as published (Bucher et al. 2001; Karandashov and Bucher 2005)

Discussion

P Uptake Is Complicated in Plant Roots

In this article, we investigated tobacco root colonization, shoot biomass, and transcriptional activities of genes *NtPT1–5* after inoculation with *G. etunicatum*. It was clear that colonization of the roots increased continuously from 0 to 40 DAI. AM developed in the roots of tobacco normally and had nearly saturated colonization at 40 DAI. Mycorrhizal tobacco had more biomass compared to non-mycorrhizal tobacco at 40 DAI (Table 2). Most previous studies have supported the findings that AM can increase host biomass (Hartwig et al. 2002; Zhu et al. 2010). However, tobacco leaf P concentration was not significantly different between the AM plants and NM plants.

Based on our results, P concentration in the soil may influence plant P uptake. If tobacco is cultured in soil with lower P concentrations, the plant P concentration may be significantly different between NM plants and AM plants. We conjectured that the contribution of the direct uptake pathway and the mycorrhizal uptake pathway to total P uptake would vary dramatically in response to different stages of tobacco development. Mycorrhizal roots

had a wider available P zone than NM tobacco, allowing for a higher P uptake efficiency (Seufferheld and Curzi 2010). Finally, mycorrhizal tobacco was able to more quickly access nutrients, including P, in response to plant growth. This ability gave mycorrhizal tobacco a significantly higher biomass with no difference in P concentration compared to non-mycorrhizal tobacco. We presume that difference in P concentration will be magnified if we measure it in the later. Similar conclusion was drew in *Lolium perenne* that AMF increased plant biomass significantly but did not increase P concentration (Hartwig et al. 2002).

NtPT5 Was Mycorrhiza-Specific Gene

During the course of AM development in tobacco roots, we detected a change in the expressions of *NtPT1–5* according to the progress of AMF colonization. In our study, *NtPT5* did not express in tobacco roots at 0 DAI, and it increased from 5 to 40 DAI in parallel with the development of AM. Previous similar studies have showed that the *PT* induced by AM was expressed only in the root cortical cells containing arbuscules (Siciliano et al. 2007; Wegmueller et al. 2008). The expressions of *LePT3* and *LePT4* have been used as molecular markers for the transfer of P from AMF to tomato plants, irrespective of the magnitude of plant response (Poulsen et al. 2005). Based on our results, we concluded that *NtPT5* was mycorrhiza-specific phosphate transporter. However, our results differed in some ways from other previous works (Chen et al. 2007, 2011). Chen et al. (2007) analyzed the expression patterns of phosphate transporters in solanaceous species, including tobacco. This research demonstrated that mycorrhiza-enhanced expression was increased 6- to 21-fold for *NtPT3*, 153- to 1,250-fold for *NtPT4*, and 50- to 325-fold for *NtPT5* 2 months after colonization under reduced-phosphate conditions. The difference could be explained by the use of different AMF inoculants. In our experiment, we used *G. etunicatum*, whereas Chen et al. (2007, 2011) used *G. intraradices*.

Different species may show different influences in the expression of genes in tobacco roots. Similarly, it has been shown that different AMF have functional diversity not only at the level of mycorrhiza formation, plant nutrient uptake, and plant growth but also at the molecular level in the expression of plant genes in roots involved in plant P starvation response (Lopez-Raez et al. 2010). Under the same experimental conditions, *G. mosseae* promoted the expression of *MtPT2* and *MtPT4* in *Medicago truncatula*, while *Gigaspora rosea* depressed the expression of both (Burleigh et al. 2002). Plants can respond differently to AMF, not only at the level of colonization, nutrient uptake, and growth but also at the level of gene expression (Branscheid et al. 2010; Feddermann et al. 2008; Grunwald et al. 2009).

Through continuous detection of the spreading colonization of tobacco roots and the expression of the phosphate transfer genes involved in the development of AM, our results revealed that *NtPT5* may be the only mycorrhiza-specific phosphate transfer gene. The expression of *NtPT5* was enhanced in conjunction with the development of AM. We propose that plants may balance the expression level among all *PT* genes in response to internal and external conditions.

The Evolutional Mechanism of Pht1 Family Members in Solanaceous Plants

Based on the solanaceous phylogenetic tree, Pht1 family members in solanaceous plants showed some regular evolutional mechanisms (Fig. 4). PT4–5 is a main cluster and PT1–3 is another main cluster. The PT4–5 cluster divides into PT4 and PT5, while PT1–3 divides into the PT2, PT1, and PT3 sub-clusters. The PT1 and PT3 sub-clusters divide into PT1 and PT3, respectively. Our data are consistent with previous research on LePT and StPT (Nagy et al. 2005). Interestingly, NtPT2 was highly homologous with six solanaceous plants PT1. Analysis of the amino acid sequences of NtPT1 and NtPT2 demonstrated only two different amino acids between them. The pattern of Pht1 in solanaceous plants could reflect a long period of evolution.

Mycorrhiza was an ancient symbiosis dating to 400 Ma ago, as shown in the fossil record (Stubblefield et al. 1987). Plants and mycorrhizal fungi have undergone co-evolution with corresponding adaptations. Changes in plant root structure and function, including *PT* gene activity, facilitated P transfer in the newly established plant–fungus interaction (Bonfante and Genre 2008; Glassop et al. 2005; Paszkowski et al. 2002). New genes were generated via gene duplication events during evolution (Hurles 2004). The subsequent changes in regulatory and coding sequences of new genes brought new characters and novel subfunctions (Force et al. 1999). Based on this theory, Nagy et al. (2005) set up the proposed series of gene duplications that led to the formation of potato and tomato Pht1 gene families and postulated that the *LePT4/StPT4* group ultimately evolved to be strictly expressed in AM. Our work verifies this hypothesis for the solanaceous plant Pht1 gene family phylogenetic tree. Five of the six *PT* genes in solanaceous plants had similar evolutionary mechanisms, with the exception of *NtPT1*. However, previous research as well as our study results disagree with the hypothesis that the expressions of solanaceous *PT4* genes were induced by AM (Drissner et al. 2007; Karandashov and Bucher 2005; Nagy et al. 2005; Rausch et al. 2001). The difference may result from different study conditions, such as different plant varieties or AMF (Burleigh et al. 2002).

In summary, a time-course experiment was conducted to characterize the kinetic changes of *NtPT1–5* expressions parallel to colonization from 0 to 40 DAI in wild-type tobacco roots. Our results show that *NtPT5* may be the only mycorrhiza-specific *PT* gene and its induction is tightly correlated with the degree of root colonization by *G. etunicatum*. Additionally, we revealed the evolutionary pattern of the Pht1 family in six solanaceous plants. Our results support the theory on the proposed series of gene duplications that led to the formation of the solanaceous plants' Pht1 family (Nagy et al. 2005). However, further investigation needs to carry out not only at plant nutrient uptake and plant growth but also the expression of *NtPT1–5* genes in tobacco roots involved in plant P starvation response.

Acknowledgments We thank Dr. Xinglong Gu at Cold Spring Harbor Laboratory, NY, USA, for helpful comments on the text and English revision. This work was supported by the National High-Tech Research Project of China (863 plan) under grant nos. 2007AA021403 and the National Transgenic Crop Research Project of China under grant nos. 2008ZX0811-005 and 2009ZX08004-003B.

Conflict of Interest We have no conflict of interest.

References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250. doi:10.1158/0008-5472.CAN-04-0496

Baek SH, Chung IM, Yun SJ (2001) Molecular cloning and characterization of a tobacco leaf cDNA encoding a phosphate transporter. Mol Cells 11:1–6

Biermann B, Linderman RG (1981) Quantifying vesicular-arbuscular mycorrhizae—a proposed method towards standardization. New Phytol 87:63–67. doi:10.1111/j.1469-8137.1981.tb01690.x

Bonfante P, Genre A (2008) Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends Plant Sci 13:492–498. doi:10.1016/j.tplants.2008.07.001

Branscheid A, Sieh D, Pant BD, May P, Devers EA, Elkrog A, Schauser L, Scheible W-R, Krajinski F (2010) Expression pattern suggests a role of MiR399 in the regulation of the cellular response to local Pi increase during arbuscular mycorrhizal symbiosis. Mol Plant Microbe Interact 23:915–926. doi:10.1094/MPMI-23-7-0915

Bucher M (2007) Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol 173:11–26. doi:10.1111/j.1469-8137.2006.09135.x

Bucher M, Rausch C, Daram P (2001) Molecular and biochemical mechanisms of phosphorus uptake into plants. J Plant Nutri Soil Sci 164:209–217. doi:10.1002/1522-2624(200104)164:2<209::AID-JPLN209>3.0.CO;2-F

Burleigh SH, Cavagnaro T, Jakobsen I (2002) Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J Exp Bot 53:1593–1601. doi:10.1093/Jxb/Erf013

Chen AQ, Hu J, Sun SB, Xu GH (2007) Conservation and divergence of both phosphate- and mycorrhiza-regulated physiological responses and expression patterns of phosphate transporters in solanaceous species. New Phytol 173:817–831. doi:10.1111/j.1469-8137.2006.01962.x

Chen AQ, Gu MA, Sun SB, Zhu LL, Hong SA, Xu GH (2011) Identification of two conserved *cis*-acting elements, MYCS and P1BS, involved in the regulation of mycorrhiza-activated phosphate transporters in eudicot species. New Phytol 189:1157–1169. doi:10.1111/j.1469-8137.2010.03556.x

Coelho GTCP, Carneiro NP et al (2010) A phosphate transporter promoter from *Arabidopsis thaliana AtPHT1;4* gene drives preferential gene expression in transgenic maize roots under phosphorus starvation. Plant Mol Biol Rep 28:717–723. doi:10.1007/s11105-010-0199-8

Diao GP, Wang YC, Wang C, Yang CP (2011) Cloning and functional characterization of a novel glutathione *S*-transferase gene from *Limonium bicolor*. Plant Mol Biol Rep 29:77–87. doi:10.1007/s11105-010-0212-2

Drissner D, Kunze G, Callewaert N, Gehrig P, Tamasloukht M, Boller T, Felix G, Amrhein N, Bucher M (2007) Lyso-phosphatidylcholine is a signal in the arbuscular mycorrhizal symbiosis. Science 318:265–268. doi:10.1126/science.1146487

Feddermann N, Boller T, Salzer P, Elfstrand S, Wiemken A, Elfstrand M (2008) *Medicago truncatula* shows distinct patterns of mycorrhiza-related gene expression after inoculation with three different arbuscular mycorrhizal fungi. Planta 227:671–680. doi:10.1007/s00425-007-0649-1

Fogg DN, Wilkinson NT (1958) The colorimetric determination of phosphorus. Analyst 83:406–414. doi:10.1039/an9588300406

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151:1531–1545

Gianinazzi-Pearson V, Arnould C, Oufattole M, Arango M, Gianinazzi S (2000) Differential activation of H^+-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. Planta 211:609–613. doi:10.1007/s004250000323

Glassop D, Smith SE, Smith FW (2005) Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots. Planta 222:688–698. doi:10.1007/s00425-005-0015-0

Grunwald U, Guo WB, Fischer K, Isayenkov S, Ludwig-Muller J, Hause B, Yan XL, Kuster H, Franken P (2009) Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, Pi-fertilised and phytohormone-treated *Medicago truncatula* roots. Planta 229:1023–1034. doi:10.1007/s00425-008-0877-z

Gupta N, Gupta AK, Singh NK, Kumar A (2011) Differential expression of *PBF Dof* transcription factor in different tissues of three finger millet genotypes differing in seed protein content and color. Plant Mol Biol Rep 29:69–76. doi:10.1007/s11105-010-0208-y

Harrison MJ, Vanbuuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:626–629. doi:10.1038/378626a0

Harrison MJ, Dewbre GR, Liu J (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14:2413–2429.doi:10.1105/tpc.004861

Hartwig UA, Wittmann P et al (2002) Arbuscular mycorrhiza infection enhances the growth response of *Lolium perenne* to elevated atmospheric pCO_2. J Expl Bot 53:1207–1213. doi:10.1093/jexbot/53.371.1207

Hurles M (2004) Gene duplication: the genomic trade in spare parts. PLoS Biol 2:900–904. doi:10.1371/journal.pbio.0020206

Kai M, Takazumi K, Adachi H, Wasaki J, Shinano T, Osaki M (2002) Cloning and characterization of four phosphate transporter cDNAs in tobacco. Plant Sci 163:837–846. doi:10.1016/S0168-9452(02)00233-9

Karandashov V, Bucher M (2005) Symbiotic phosphate transport in arbuscular mycorrhizas. Trends Plant Sci 10:22–29. doi:10.1016/j.tplants.2004.12.003

Li QF, Sun SSM, Yuan DY, Yu HX, Gu MH, Liu QQ (2010) Validation of candidate reference genes for the accurate normalization of real-time quantitative RT-PCR data in rice during seed development. Plant Mol Biol Rep 28:49–57. doi:10.1007/s11105-009-0124-1

Liu RJ, Wang FY (2003) Selection of appropriate host plants used in trap culture of arbuscular mycorrhizal fungi. Mycorrhiza 13:123–127. doi:10.1007/s00572-002-0207-4

Liu H, Trieu AT, Blaylock LA, Harrison MJ (1998) Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi. Mol Plant Microbe Interact 11:14–22. doi:10.1094/MPMI.1998.11.1.14

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(t)}$ method. Methods 25:402–408. doi:10.1006/meth.2001.1262

Lopez-Raez JA, Verhage A, Fernandez I, Garcia JM, Azcon-Aguilar C, Flors V, Pozo MJ (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. J Exp Bot 61:2589–2601. doi:10.1093/jxb/erq089

Maldonado-Mendoza IE, Dewbre GR, Harrison MJ (2001) A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. Mol Plant Microbe Interact 14:1140–1148. doi:10.1094/MPMI.2001.14.10.1140

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective-measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. New Phytol 115:495–501. doi:10.1111/j.1469-8137.1990.tb00476.x

Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. Plant J 31:341–353. doi:10.1046/j.1365-313X.2002.01356.x

Muller J, Dulieu H (1998) Enhanced growth of non-photosynthesizing tobacco mutants in the presence of a mycorrhizal inoculum. J Exp Bot 49:707–711. doi:10.1093/jxb/49.321.707

Nagy R, Karandashov V, Chague W, Kalinkevich K, Tamasloukht M, Xu GH, Jakobsen I, Levy AA, Amrhein N, Bucher M (2005) The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. Plant J 42:236–250. doi:10.1111/j.1365-313X.2005.02364.x

Paszkowski U, Kroken S, Roux C, Briggs SP (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 99:13324–13329. doi:10.1073/pnas.202474599

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. T Brit Mycol Soc 55:158–161

Poulsen KH, Nagy R, Gao LL, Smith SE, Bucher M, Smith FA, Jakobsen I (2005) Physiological and molecular evidence for Pi uptake via the symbiotic pathway in a reduced mycorrhizal colonization mutant in tomato associated with a compatible fungus. New Phytol 168:445–454. doi:10.1111/j.1469-8137,2005.01523.x

Qi JN, Yu SC, Zhang FL, Shen XQ, Zhao XY, Yu YJ, Zhang DS (2010) Reference gene selection for real-time quantitative polymerase chain reaction of mRNA transcript levels in Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*). Plant Mol Biol Rep 28:597–604. doi:10.1007/s11105-010-0185-1

Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. Nature 414:462–466. doi:10.1038/35106601

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol 116:447–453. doi:10.1104/pp.116.2.447

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108. doi:10.1038/nprot.2008.73

Seufferheld MJ, Curzi MJ (2010) Recent discoveries on the roles of polyphosphates in plants. Plant Mol Biol Rep 28:549–559. doi:10.1007/s11105-010-0187-z

Siciliano V, Genre A, Balestrini R, Cappellazzo G, deWit PJGM, Bonfante P (2007) Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. Plant Physiol 144:1455–1466. doi:10.1104/pp.107.097980

Stubblefield SP, Taylor TN, Trappe JM (1987) Fossil mycorrhizae: a case for symbiosis. Science 237:59–60. doi:10.1126/science.237.4810.59

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092

Vierheilig H, Schweiger P, Brundrett M (2005) An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. Physiol Plantarum 125:393–404. doi:10.1111/j.1399-3054.2005.00564.x

Viswanathan A, Kuriakose B, Bharadwaj S, Thomas G (2011) Expression of *Aprotinin* in anther causes male sterility in tobacco var *Petit havana*. Plant Mol Biol Rep 29:825–834. doi:10.1007/s11105-011-0288-3

Wang B, Qiu YL (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16:299–363. doi:10.1007/s00572-005-0033-6

Wegmueller S, Svistoonoff S, Reinhardt D, Stuurman J, Amrhein N, Bucher M (2008) A transgenic *dTph1* insertional mutagenesis system for forward genetics in mycorrhizal phosphate transport of *Petunia*. Plant J 54:1115–1127. doi:10.1111/j.1365-313X.2008.03474.x

Wenkart S, Roth-Bejerano N, Mills D, Kagan-Zur V (2001) Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. Plant Cell Rep 20:369–373. doi:10.1007/s002990100325

Zhang XG, Yin DM, Ma CZ, Fu TD (2011) Phylogenetic analysis of *S*-locus genes reveals the complicated evolution relationship of *S* haplotypes in *Brassica*. Plant Mol Biol Rep 29:481–488. doi:10.1007/s11105-010-0251-8

Zhao LM, Versaw WK, Liu JY, Harrison MJ (2003) A phosphate transporter from *Medicago truncatula* is expressed in the photosynthetic tissues of the plant and located in the chloroplast envelope. New Phytol 157:291–302. doi:10.1046/j.1469-8137.2003.00677.x

Zhu XC, Song FB, Xu HW (2010) Influence of arbuscular mycorrhiza on lipid peroxidation and antioxidant enzyme activity of maize plants under temperature stress. Mycorrhiza 20:325–332. doi:10.1007/s00572-009-0285-7