A transgenic *dTph1* insertional mutagenesis system for forward genetics in mycorrhizal phosphate transport of *Petunia*

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Summary

The active endogenous *dTph1* system of the *Petunia hybrida* mutator line W138 has been used in several forward-genetic mutant screens that were based on visible phenotypes such as flower morphology and color. In contrast, defective symbiotic phosphate (P_i) transport in mycorrhizal roots of *Petunia* is a hidden molecular phenotype as the symbiosis between plant roots and fungi takes place below ground, and, while fungal colonization can be visualized histochemically, P_i transport and the activity of P_i transporter proteins cannot be assessed visually. Here, we report on a molecular approach in which expression of a mycorrhiza-inducible bi-functional reporter transgene and insertional mutagenesis in *Petunia* are combined. Bi-directionalization of a mycorrhizal P_i transporter promoter controlling the expression of two reporter genes encoding firefly luciferase and GUS allows visualization of mycorrhiza-specific P_i transporter expression. A population of selectable transposon insertion mutants was established by crossing the transgenic reporter line with the mutator W138, from which the *P_i transporter downregulated* (*ptd1*) mutant was identified, which exhibits strongly reduced expression of mycorrhiza-inducible P_i transporters in mycorrhizal roots.

Keywords: *Petunia*, forward genetics, transposon mutagenesis, mycorrhiza, phosphate transporter, luciferase.

Introduction

Forward genetics seeks to find the genetic basis of a phenotype and thus has considerable potential to unveil new genes for distinct biological processes. A primary step in forward genetics comprises generation of a mutant population and subsequent screening of the population for a particular phenotype. Mutagenesis has been successfully achieved by use of chemical agents such as ethyl methanesulphonate (EMS), by radiation (fast neutrons, X-rays, accelerated ions) or by insertion of T-DNA or transposons (reviewed by Alonso and Ecker, 2006). Forward genetics combined with the first two methods is very powerful for isolation of mutants, but a labor-intensive map-based cloning strategy needs to be applied to identify the mutated gene that is responsible for the mutant phenotype. In comparison, insertional mutagenesis has an inherent lower efficiency but allows more direct identification and cloning of the respective genes by identifying the sequence tag and neighboring

sequences at the site of insertion (reviewed by Alonso and Ecker, 2006).

Arabidopsis thaliana (L.) Heynh. is one of the most powerful model plant species due to technological advances such as its fully sequenced genome and high-coverage genetic maps and databases, which considerably speed up the time from isolation of the mutant to identification of the gene (Alonso and Ecker, 2006). However, Arabidopsis does not form an arbuscular mycorrhizal (AM) symbiosis, and is therefore unsuitable for studies in this research area. Prompted by the fast-growing interest in this field, new model systems for fundamental AM symbiosis studies are required, enabling investigations that encompass the entire developmental program of AM symbiosis and its biological functions.

Intensive screening of EMS- and radiation-induced mutant populations of the model legumes *Lotus japonicus* (Regel) Larsen and *Medicago truncatula* Gaertn. yielded several mutants with disturbances in nodulation symbiosis between the plants and nitrogen-fixing bacteria belonging to the *Rhizobiaceae* family, and, in some cases, with disturbances in the AM symbiosis also (reviewed by Oldroyd, 2001). Map-based cloning of the mutated genes revealed the involvement of a common signaling pathway including a receptor-like kinase (Endre *et al.*, 2002; Stracke *et al.*, 2002), putative cation channels (Ane *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005), and a calcium- and calmodulin-dependent kinase (Levy *et al.*, 2004; Mitra *et al.*, 2004) in the early events of root colonization by both the AM and nodulation symbionts, respectively (Kistner and Parniske, 2002). Other plant mutants were found to exhibit mutations up- or downstream of this common pathway and were disturbed in nodule development, but mycorrhiza formation was not affected (Oldroyd and Downie, 2006; Paszkowski, 2006). Attempts to use T-DNA tagging in *L. japonicus* and *M. truncatula* are underway (Martirani *et al.*, 1999; Schauser, 1998; Scholte *et al.*, 2002), but so far they have not led to the cloning of genes involved in mycorrhizal symbiosis. Several mutants perturbed in mycorrhization have been isolated from a *Mutator* transposon-tagged population of the monocot maize in a visual, macroscopic screen of mycorrhizal roots (Paszkowski *et al.*, 2006), and it will be interesting to see which genes are involved.

Among the Solanaceae, *Petunia hybrida* stands out as a model plant for use in forward and reverse genetics due to its endogenous transposable elements. *P. hybrida* W138, for which no transformation protocol exists, is renowned for its active endogenous *dTph1* transposable element system, which has allowed the generation of many interesting flower development (Vandenbussche *et al.*, 2003), flower pigmentation (van Houwelingen *et al.*, 1998) and floral pollination mutants (Stuurman *et al.*, 2004). Other qualities of the *P. hybrida* model system, such as (1) easy growth habit and relatively short life cycle of about four months from seed to seed, (2) easy asexual propagation from cuttings, and (3) easy transformation procedures, are found in the white-flowered 'Mitchell' (W115) variety, which exhibits superior fertility and growth (Gerats and Vandenbussche, 2005; Gerats *et al.*, 1990). A mutant with disturbed mycorrhization has been identified in *Petunia*: this has been named *penetration and arbuscule morphogenesis* (*pam)1*, and lacks arbuscules after cortical penetration of the hyphae (Sekhara Reddy *et al.*, 2007). With the exception of the study by Sekhara Reddy *et al.* (2007), forward-genetic approaches have not yielded 'late mycorrhizal mutants' that are affected with respect to events occurring at advanced stages during establishment of a functional symbiosis, such as the regulation of symbiotic nutrient transport.

In arbuscular mycorrhizae, host plant roots form an intimate symbiosis with fungi of the phylum Glomeromycota. In these interactions, it is mainly inorganic phosphate (P_i) that is taken up by extra-radical fungal hyphae and ultimately delivered to root cells colonized by the fungus, in exchange for plant-derived photosynthates (Marschner, 1995; Smith and Read, 1997). There is increasing evidence that P_i transporter proteins are essential for operation of the mycorrhizal P_i uptake pathway, which constitute the predominant route of P_i uptake in plants (Javot *et al.*, 2007; Maeda *et al.*, 2006; Smith *et al.*, 2003) and thus be a major driving force of global plant productivity. To understand the fine tuning of this uptake pathway, it is essential to elucidate the regulatory mechanisms underlying expression of mycorrhiza-inducible P_i transporter genes. In recent years, several mycorrhiza-inducible P_i transporters belonging to the Pht1 family of proton/P_i co-transporters have been characterized, mostly in species of the Solanaceae, legumes and cereals (reviewed by Karandashov *et al.*, 2004). The promoter of one such P_i transporter, *StPT3*, of potato (Rausch *et al.*, 2001), fused to the GUS gene, has recently been used to identify the early mycorrhizal signaling factor lyso-phosphatidylcholine in potato (Drissner *et al.*, 2007).

AM fungi can provide most of the P absorbed by the host plant, even when overall plant growth or P uptake remains unaffected (Smith *et al.*, 2003). Thus, physiological parameters such as the shoot P content are unreliable markers in forward-genetic approaches to identify regulatory components of the mycorrhizal P_i uptake pathway.

Here, a *StPT3* bi-functional promoter fused to two reporter genes encoding GUS and luciferase, respectively, was used to establish a transgenic *dTph1* transposon insertional mutant population in *Petunia hybrida* W115. Forward screening of this mutant population was found to be useful for the identification of solanaceous mutants defective in the regulation of mycorrhiza-specific P_i transport.

Results

Synopsis of the screening to identify mutants affecting the expression of mycorrhiza-specific genes

In order to screen for mutants perturbed in mycorrhiza-specific P_i transporter activity, we first generated a homozygous transgenic *Petunia* line using a construct containing the *Escherichia coli* GUS and firefly luciferase (Luc) reporter genes under the control of the bi-directionalized *StPT3* promoter (*StPT3::GUS/Luc*). Homozygous offspring were then crossed with the mutator strain W138 to establish a population of transposon insertions in the transgenic line. The M_2 generation of this cross was subsequently screened for the absence of Luc (luc−) and GUS activity (GUS−), the presence of mycorrhizal colonization (myc+), the presence of the transgene (tr+) in the genome, and repression of endogenous mycorrhiza-specific P_i transporters, to identify putative mutants. The individual steps of the screen are described in more detail below.

*Cloning of p*StPT3::GUS/Luc *and transformation of* Petunia hybrida *W115*

As mutants in the regulation of mycorrhiza-induced P_i transport may not exhibit a visible phenotype, a reporter gene approach was chosen to facilitate screening. As a marker gene, we chose the previously identified mycorrhiza-inducible P_i transporter *StPT3* (Rausch *et al.*, 2001). In previous work, we demonstrated that a fragment of this gene retains its responsiveness to mycorrhizal activation when introduced in *P. hybrida* (Karandashov *et al.*, 2004). Luc was chosen as a reporter gene as it allows a non-destructive, rapid and sensitive assay with little or no background luminescence in plants (Xiong *et al.*, 1999). Expression of the Luc gene in mycorrhizal roots allows screening of a large number of transgenic plants of the mutant population in a relatively short time, allowing a first selection of inoculated plants exhibiting a reduced level of bioluminescence. To assess whether reporter gene activity co-localizes in roots with mycorrhization, a histochemical assay involving GUS activity in combination with trypan blue or ink staining of fungal structures in the roots was chosen (Nagy *et al.*, 2005; Rausch *et al.*, 2001).

In order to avoid transcriptional gene silencing, which may occur when a single promoter is used in multiple copies (De Wilde *et al.*, 2000), we decided to bi-directionalize the *StPT3* promoter (p*StPT3*; Xie *et al.*, 2001). Typical eukaryotic promoters consist of a minimal promoter, including the TATA box that determines the direction of gene transcription, and upstream *cis*-acting elements defining the spatio-temporal expression pattern of the respective gene. In the bi-directional p*StPT3*, the CaMV 35S minimal promoter (p*35Smin*) was fused in the opposite direction ('back-to-back') to the 5′ end of p*StPT3*. This allows simultaneous mycorrhiza-specific transcription of the Luc and GUS coding regions situated downstream of p*35Smin* and p*StPT3*, respectively (Figure 1). Leaf discs of *P. hybrida* W115 were subsequently transformed with this p*StPT3::GUS/Luc* construct using *Agrobacterium tumefaciens*-mediated gene transfer. Ten independent transgenic lines were selected based on their resistance to kanamycin, and were transferred to the greenhouse for mycorrhization with *Glomus intraradices* and eventual production of T_1 seeds.

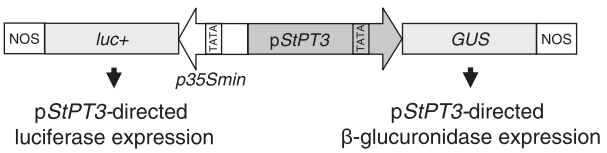

Figure 1. The pBINdual *StPT3* bi-directional promoter construct.
The *StPT3* promoter (p*StPT3*) directing GUS expression is bi-directionalized by fusion of the 35S minimal promoter (p35Smin) to its 5′ end in the opposite orientation, enabling it to also direct luciferase (luc+) expression. TATA, TATA box.

*Selection of a p*StPT3::GUS/Luc *T_1 line and physiological comparison with W115*

T_1 *StPT3::GUS/Luc* plants of each of the 10 lines were tested for GUS staining in mycorrhized roots. In eight lines, GUS staining was easily detectable 1 h after incubation in GUS staining solution. Southern blot analysis identified one line (Pet1) carrying a single insertion in its haploid genome (Figure 3d). Five plants per line of the progeny were to form mycorrhizas with *G. intraradices* in the greenhouse and were subsequently tested for Luc activity. Three lines, among them Pet1, showed strong Luc-mediated bioluminescence in roots, while Luc activity was absent from W115 control roots and non-mycorrhizal roots (Figure 3a), as well as from leaves (data not shown). The same mycorrhizal roots exhibited GUS staining co-localized with trypan blue-stained fungal structures (Figure 3c), but W115 showed no GUS staining (Figure 3b). Subsequently, Pet1 plants were selfed, and segregation of the *StPT3* promoter and the GUS gene was checked by GUS staining of mycorrhizal plants and molecular confirmation of the presence of the transgene.

To examine whether insertion of the transgene affects the physiology of Pet1, three homozygous Pet1 and W115 control plants, respectively, were grown in the greenhouse with and without inoculation with *G. intraradices*. After 5 weeks, the plants were harvested, and shoot and root fresh weight were determined. Dried shoot material was used for determination of shoot P content. In parallel, the roots were stained for succinate dehydrogenase (SDH) activity to quantify biologically active colonization units,

and with trypan blue to assess total root colonization. Overall, the physiological traits of transgenic Pet1 and wild-type W115 were comparable (Figure S1).

*p*StPT3::GUS *as a marker for functional symbiosis*

To estimate whether *StPT3* can be used as a marker gene for symbiotically active mycorrhizae (i.e. in which symbiotic P_i transport occurs) as opposed to inactive fungal structures, p*StPT3*-dependent GUS activity was analyzed and compared with various markers for mycorrhiza development. In three independent experiments, the roots of three mycorrhizal Pet1 plants were each divided into three parts (see Experimental procedures). GUS staining followed by trypan blue staining for mycorrhization (Brundrett *et al.*, 1984) was performed using the first part. The second part was used for SDH staining (Pearse, 1968), and the third part was stained for alkaline phosphatase (ALP) activity (Tisserant *et al.*, 1993). While trypan blue staining reveals all, including dead, fungal structures in mycorrhized roots, SDH and ALP staining occur in metabolically active or actively transporting symbiotic structures, respectively (Tisserant *et al.*, 1993). Root sectors containing only hyphae and/or vesicles (−arbs) were compared with root sectors containing arbuscules alone or in combination with hyphae and/or vesicles (+arbs). The percentage of roots showing p*StPT3*-directed GUS staining correlated closely with the level of SDH vital staining in root cells colonized by metabolically active arbuscules, suggesting that active arbuscules are the major site of symbiotic P_i transport in *Petunia* roots (Figures 4 and S2). The proportion of ALP-stained mycorrhizal root sectors was similar to that of root sectors exhibiting GUS staining, demonstrating that both ALP and GUS activity are good markers for symbiotically active arbuscules. We infer that metabolically active fungal structures in root cells trigger *StPT3* promoter activation and hence symbiotic P_i transport (Figure S2). Thus, integration of the *StPT3* bi-directional promoter–reporter gene construct into the *Petunia* genome and reporter gene expression analysis facilitate rapid visualization of cells competent for symbiotic P_i transport.

Crossing of Pet1 with the P. hybrida *mutator strain W138 and assessment of mutagenicity*

Mycorrhizal development in Pet1 was found to be comparable to that of the wild-type. Subsequently, the Pet1 line was crossed with the mutator strain W138, which is a *P. hybrida* strain containing 150–200 *dTph1* transposon insertions (De Keukeleire, 2001), one of which is in the *AN1* gene for anthocyanin biosynthesis (Spelt *et al.*, 2002), leading to white flowers with pink and red revertant spots when the transposon is somatically excised. The *dTph1* element is a non-autonomous, mobile transposon that is *trans*-activated by the *Act1* locus (De Keukeleire, 2001; Gerats *et al.*, 1990). Several flowers per plant from 19 W138 plants originating from a heterogenous population were crossed to homozygous Pet1, and approximately 10 seeds per capsule were used to generate a segregating mutant population of 891 M_1 families. Contrary to our expectations, more than 70% of the plants had an altered phenotype. The flowers of about 53% of the progeny had petaloid stamens (Figure S3a), 9.5% of the progeny had extensions growing out of the petals (Figure S3b), 5% were dwarfed (Figure S3c), another 5% were sterile, and 1.5% had other phenotypes. Subsequent to cumbersome selfing of these flowers with aberrant phenotypes, seeds could be harvested and used for the M_2 mutant screen. Ten plants per family, i.e. plants derived from seeds of a single capsule, were inoculated with *G. intraradices* in the greenhouse. Some of the flowers had colored spots and sectors, demonstrating an active transposon system (Figure S3e–g). The existence of plants showing visible phenotypes, such as anthocyanin accumulation in leaves, also indicated that mutations had taken place (Figure S3h). Thus, a cross of transgenic Pet1 with W138 is a promising source of insertional mutants suitable for forward genetics of symbiotic P_i transport regulation, or of any regulatory pathway, depending on the transgenic line used.

M_2 screen for mutants in the regulation of mycorrhizal root P_i transporter gene expression

Five weeks after inoculation with *G. intraradices*, root samples of M_2 family members were harvested and directly screened for luciferase activity (Figure 2a). Roots lacking luciferase (luc−) activity were stained overnight for GUS activity (Figure 2b). Roots not exhibiting GUS staining (GUS−) were further examined for AM fungal colonization by staining the fungal structures with either trypan blue or ink (Figure 2c).

Subsequently, a PCR approach was employed to demonstrate the presence of the transgene in the genome of mycorrhizal plants selected for the absence of reporter gene activity. This step was necessary as there is not only a segregation of the transposon insertions but also of the transgene itself in the M_2 generation, i.e. a quarter of the plants per family lack the transgene and thus cannot show transporter gene activity independently of transposon insertion. Thus the PCR step allows us to distinguish between lack of reporter gene activity due to lack of the transgene and lack of reporter gene activity due to transposon insertion leading to a mutant. To this end, two sets of primers were used, the first amplifying approximately 500 bp of an endogenous P_i transporter gene to indicate sufficient DNA quality, the second amplifying an approximately 300 bp fragment of the luciferase coding region to confirm the presence of the transgene in the genome (Figure 2d).

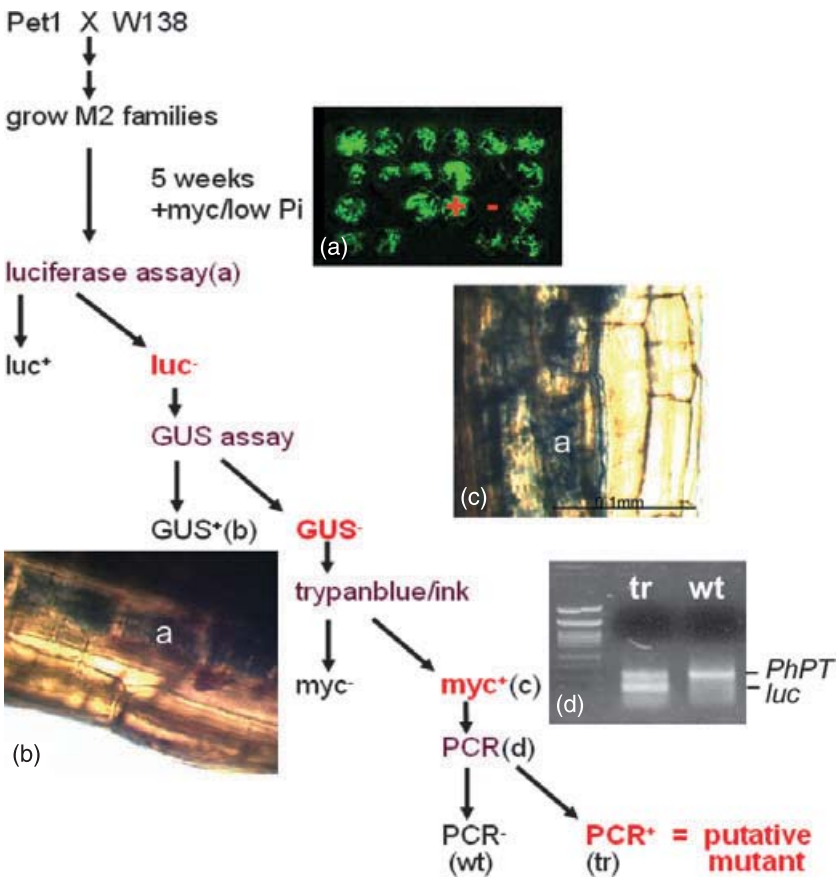

Figure 2. Flow chart for screening of the M_2 transgenic mutant population.
(a) Luciferase (luc) assay in 24-well plate. +: luc+, –: luc–.
(b, c) Roots assayed for GUS activity (magenta) and mycorrhizal structures (myc) co-stained with trypan blue. a, arbuscule. (b) Root showing GUS+ phenotype (c) Root showing GUS– phenotype.
(d) PCR showing two bands for transgenic (tr) plants and one band for (wt) plants.

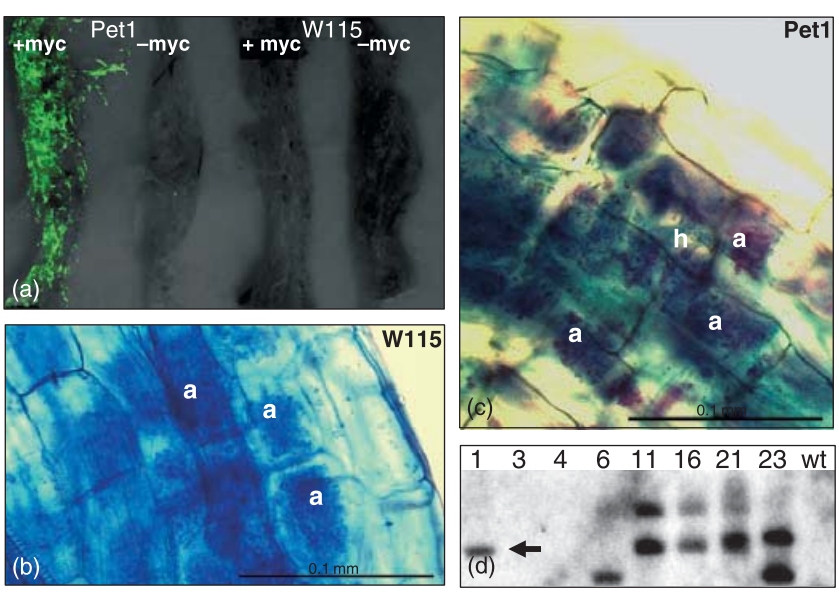

Figure 3. Selection of transgenic Pet1 line showing strong luciferase and GUS activity.
(a) Overlay of luminescence (green) and bright field picture of mycorrhized (+myc) and non-mycorrhized (–myc) transgenic (Pet1) and wt (W115) roots.
(b, c) Co-staining of GUS activity (magenta) and mycorrhizal structures stained by trypan blue (blue) in mycorrhized roots of W115 (b) and Pet1 (c). a, arbuscules; h, hyphae.
(d) Southern blot of several transgenic lines tested using a luciferase probe. The arrow shows the single insertion in the Pet1 line.

Using this approach, 1300 plants of 130 M_2 families have been screened for transgene activity upon mycorrhization and 45 families have been tested by PCR. Altogether, six putative mutants in four M_2 families have been identified (Table 3).

An alternative to screening the M_2 generation would have been to self individual lines homozygous for the transgene and screen only the M_3. This approach was not chosen, due to the fact that kanamycin selection at the seedling stage could not be applied reliably (see Discussion). The advan-

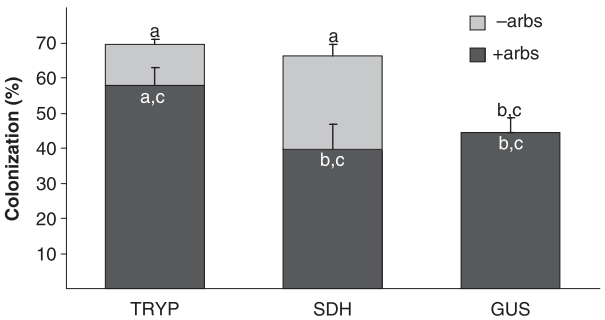

Figure 4. p*StPT3:GUS* as a marker of functional symbiosis in Pet1 plants. The percentage total colonization stained by trypan blue (TRYP) and the percentage vital colonization stained for succinate dehydrogenase (SDH) activity is compared with GUS staining co-localized with trypan blue-stained fungal structures. Root sectors containing arbuscules alone or in combination with hyphae and/or vesicles (+arbs, dark gray) are compared with root sectors containing vesicles and/or hyphae (–arbs, light gray). Values are means and SE of three independent experiments with three plants per treatment. Bars with the same letters are not significantly different between the treatments, $P < 0.05$.

tage of such a screen would have been that segregation of putative mutants could have been determined directly by the absence of GUS expression.

Cloning of Petunia *P_i transporters and their expression in mycorrhizae*

To exclude the possibility that the absence of GUS and Luc activity, respectively, in the putative mutant lines was caused by an epigenetic effect or a transposon insertion in the reporter genes including the bi-functional promoter, it was essential to show that expression of endogenous P_i transporters was also repressed. To identify the *Petunia* genes coding for Pht1 P_i transporters, PCR reactions were performed on genomic DNA using DNA primers based on multiple sequence alignments with the solanaceous *Pht1* gene family members available in the GenBank database (Table 1). Five genomic DNA fragments were identified that shared strong similarity with the corresponding potato and tomato genes *StPT1–5* and *LePT1–5*, respectively. The corresponding *Petunia* genes were named *PEThy;Pht1;1–PEThy;Pht1;5*, in accordance with the published Pht1 nomenclature (Bucher *et al.*, 2001). For simplicity, here they are called *PhPT1–5*.

The entire coding regions of *PhPT3*, *PhPT4* and *PhPT5*, respectively, corresponding to the mycorrhiza-inducible P_i transporter genes in tomato and potato, were subsequently cloned using genome walking. Sequences corresponding to open reading frames containing 538, 529 and 530 amino acids, respectively, were identified, sharing 93, 97 and 96% similarity with previously described genes from the Solanaceae (Table 2 and Figure S4). Similar to their potato and tomato orthologs, the three genes were found to lack introns and to code for proteins of 58 kDa with 12 transmembrane domains as predicted using the TMPRED software (http://www.ch.embnet.org/software/TMPRED_form.html).

Gene-specific primers were designed to amplify cDNAs of *PhPT1–5* (Table 1) and to analyze respective gene expression patterns in *Petunia*. RNA was extracted from roots of plants inoculated for 4 weeks and roots of non-inoculated control plants. Quantitative RT-PCR was performed using primer pairs specific for the *PhPT*s, with the constitutively expressed *PhGAPDH* as a reference (Figure 5a). *PhPT3*, *PhPT4* and *PhPT5* expression was found to be clearly mycorrhiza-inducible. While *PhPT4* expression was mycorrhiza-specific, *PhPT3* and *PhPT5* were expressed at low levels in non-mycorrhized roots. There was no significant difference in *PhPT1* expression levels in the presence or absence of the AM fungus, and *PhPT2* expression was slightly repressed upon mycorrhization. In summary, the expression patterns of the *Petunia* P_i transporters were very similar to those of their orthologs in other solanaceous species such as tomato and potato (Nagy *et al.*, 2005). Moreover, *PhPT* gene expression was comparable in Pet1 and wild-type W115 (data not shown).

Unlike mycorrhiza-specific p*StPT3::GUS* activity in Pet1 (Figure 3) and potato (Rausch *et al.*, 2001), *PhPT3* expression in *Petunia* was detectable at low levels in non-mycorrhized roots also (Figure 5a). This observation

Table 1 Primers used for quantitative PCR of the *PhPT* genes and the *PhGAPDH* control

Gene	Primer name	Primer sequence (5′ → 3′)
PhGAPDH	PhGAPDHF3	GGAATCAACGGTTTTGGAAGAATTGGGCG
	PhGAPDHR4	GGCCGTGGACACTGTCATACTTGAACA
PhPT1	PhPT1 RTF	GAAATTTCTCAAGGCAATGAC
	PhPT1RTR2	GATTCTGTTGTCCTTGTGAGT
PhPT2	PhPT2F2	GCAAAGGTGGAACGAATGGCTA
	PhPT2R2	ATGACGAACCCAATTCGGTTCTCCTGTAA
PhPT3	Ph-PT3-R7	GCGTTCATAGTTTCTGGAGGAGGAATCCA
	Ph-PT3-F7	GCTTACCCTGCACCAACTTATTCAGCTC
PhPT4	Ph-PT4-F7	TTGATGAATTTTGAAGGTAAACCATTTAACGTG
	Ph-PT4-R7	AGTGTTGGCTTTGCTAGTAAGTCCCATAAC
PhPT5	Ph-PT5-F7	TAGTACCACAAAACACAGCCTAAATTTGC
	Ph-PT5-R7	GGTATGTGGCAGCTTTCCAGGAGCATGAGTATG

Table 2 BLASTP search for homologies to other P_i transporters and predicted protein properties determined by analysis of the P_i transporters of *Petunia* using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and PEPSTATS (http://bioweb.pasteur.fr/seqanal/interfaces/pepstats.html)

Protein[a]	Amino acid identity	Score/E Value	Number of amino acids	Charge	Mass (kDa)	IP	TMD
PhPT1[b] (NtPT1)	252/274 (91%)	503/6e-141	274[b]				
PhPT2[b] (SmPT2)	172/188 (91%)	360/3e-98	188[b]				
PhPT3 (SmPT3)	480/538 (89%)	958/0.0	538	8.5	58.72	8.58	12
PhPT4 (LePT4)	493/529 (93%)	1003/0.0	529	7.5	58.874	8.41	12
PhPT5 (NtPT5)	493/530 (93%)	990/0.0	529	11.5	58.913	8.82	12

IP, isoelectric point; TMD, transmembrane domains; aa, amino acids.
[a]Text in parentheses indicates P_i transporters with the highest scores in the BLASTP search compared to the PhPT. The accession numbers of the genes are AB020061 (NtPT1); EF091666 (SmPT2); EF091668 (SmPT3); AY885652 (LePT4) and EF091675 (NtPT5).
[b]Partial sequences.

corresponded to those regarding *StPT3* expression in potato (Nagy *et al.*, 2005; Rausch *et al.*, 2001). Thus, p*StPT3::GUS* activity reflects mycorrhiza-specific induction of endogenous P_i transporter genes in *P. hybrida*.

Repression of mycorrhizal P_i transporter gene expression in ptd1

A candidate mutant line was selected from the six putative mutants and was designated *phosphate transporter down-regulated 1* (*ptd1*). *PhPT* gene-specific quantitative RT-PCR was performed using mycorrhizal *ptd1* and W115, which exhibited 49 and 56% AM fungal colonization, respectively. In both lines, the pattern of mycorrhization and the frequency of different types of fungal structures found in the mycorrhizae were comparable (data not shown). While expression of *PhPT1* and *PhPT2* was not significantly altered, expression of the mycorrhiza-inducible P_i transporters was strongly repressed in *ptd1* compared to mycorrhized W115, i.e. the expression levels of *PhPT3*, *PhPT4* and *PhPT5* were reduced 18-, 11- and 8-fold, respectively, compared to the corresponding expression level in W115 (Figure 5b,c). This molecular phenotype arose a new in cuttings of *ptd1* and in progeny of selfed M_3 plants exhibiting the luc−, GUS−, myc+ and PCR+ phenotype, respectively. Crossing with the *act1*^{S6} introgression line *P. hybrida* W5, which is deficient in *dTph1* transposition at the *an1*^{W138} locus (Stuurman and Kuhlemeier, 2005), is currently being undertaken to fix the *dTph1* insertion in the *ptd1* allele for subsequent molecular/physiological analysis of mycorrhized *ptd1*.

Discussion

Molecular phenotyping using a mycorrhiza-specific bi-directional promoter

Establishment of symbiotic P_i transport is a late developmental step in AM formation, and the regulatory mechanisms for mycorrhizal P_i uptake are almost entirely unknown. Mycorrhiza-specific P_i transport cannot easily be visualized and requires monitoring of the transfer of radioactive ^{32}P from the mycorrhizal fungus to the plant symbiont (see Pearson and Jakobsen, 1993; for example). In addition, the symbiosis usually takes place below ground, and roots of inoculated plants need to be stained in order to check for colonization by the symbiotic fungus. P_i transporters are indispensable for functional AM symbiosis (Javot *et al.*, 2007; Maeda *et al.*, 2006), but their activities may be redundant to some extent in both the direct (Misson *et al.*, 2004; Shin *et al.*, 2004) and mycorrhizal P_i transport pathways (Nagy *et al.*, 2005; Xu *et al.*, 2007). To fully understand the inter-dependence of the two uptake pathways with respect to plant growth (Poulsen *et al.*, 2005; Smith *et al.*, 2003), it is essential to understand the regulation of the two pathways. Here we show how a molecular-genetics approach including transposon insertional mutagenesis and invasive and non-invasive analyses of reporter gene expression (Figure 2) can be useful in the dissection of the regulatory mechanisms underlying mycorrhizal P_i transport in *Petunia*. Bi-directionalization of a plant promoter was demonstrated for the first time in Arabidopsis using the two reporter genes GUS and GFP (Xie *et al.*, 2001). To visualize P_i transporter expression in mycorrhizal plants, we chose a reporter gene approach using bi-directional p*StPT3* (Figures S1 and S2). The *Petunia* ortholog to *StPT3*, i.e. *PhPT3*, was cloned, and its expression shown to be similar to that of *StPT3* from potato, thus validating the use of p*StPT3* in *Petunia* (Figure 5, and data not shown). In addition, the *StPT3* promoter has previously been shown to be activated in a mycorrhiza-specific way in several plant species including *Petunia* and legume species (Karandashov *et al.*, 2004; Rausch *et al.*, 2001).

Fusion of the bi-directional *StPT3* promoter to GUS and Luc (Figure 1) and stable insertion in the genome of a mutator strain of *Petunia* allowed us to establish and efficiently screen a *dTph1* insertional mutant population (Figure 2). Detection of Luc activity (Millar *et al.*, 1992) prior to the more time-consuming GUS staining assay allowed a

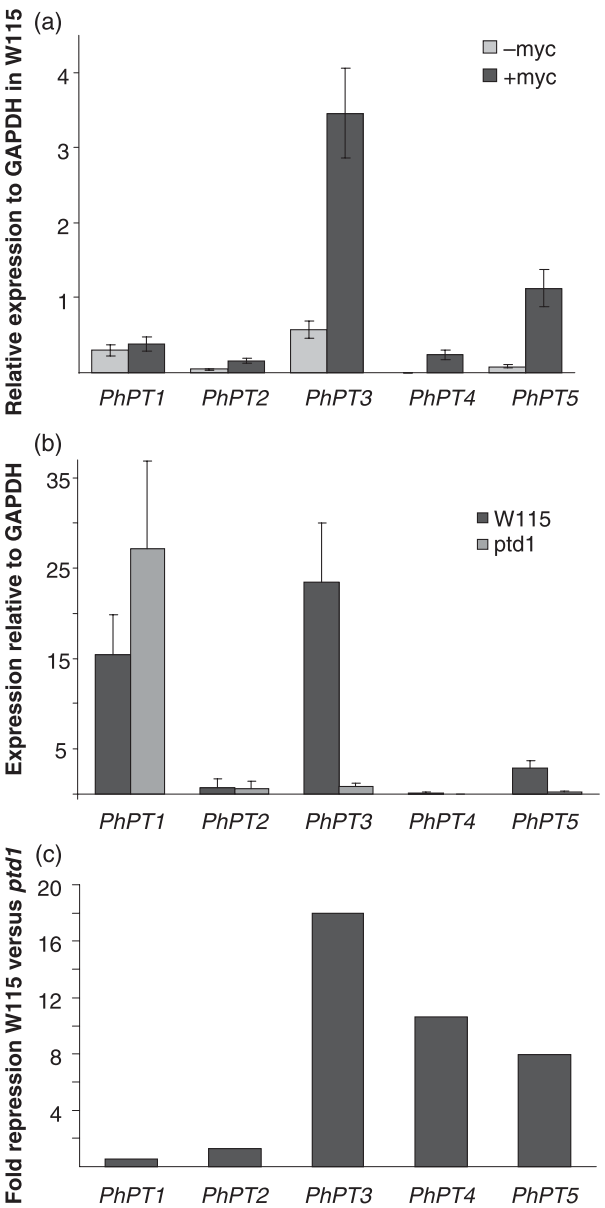

Figure 5. Expression of endogenous *Petunia* P_i transporter genes and comparison to expression levels in *ptd1*.
Quantative RT-PCR with primers for *PhPT1*, *PhPT2*, *PhPT3*, *PhPT4* and *PHPT5*.
(a) Expression of *PhPT*s in W115 in non-mycorrhizal (light gray) and mycorrhizal roots (dark gray), relative to *GAPDH*. Mean values of triplicate measurements ± SE are shown.
(b) Expression of *PhPT*s relative to *GAPDH* in mycorrhizal W115 (dark gray) compared with their expression in *ptd1* (light gray). Mean values of duplicate measurements ± SE are shown.
(c) Expression of *PhPT*s in mycorrhizal mutant roots relative to GAPDH (49% colonization) compared to mycorrhizal W115 control roots (56% colonization), calculated as fold repression in the *ptd1* compared to W115. Mean values of duplicate measurements are shown.

first rapid screen to eliminate all those transgenic plants within the population that exhibited p*StPT3*-directed gene expression in mycorrhizal roots. As analysis of Luc activity is non-destructive, GUS staining could subsequently be performed with the same root material. This could also be advantageous for plant species with small root systems, such as the legumes *Lotus japonicus* and *Medicago truncatula*, which provide only limited amounts of root material for subsequent experimental studies. Histochemical analysis of GUS expression is useful to determine co-localization of p*StPT3* activity and stained fungal material in the root tissue and thus to demonstrate correct spatial and temporal expression of the reporter gene (Karandashov *et al.*, 2004; Rausch *et al.*, 2001). Finally, PCR on genomic DNA of selected plants defective in marker gene expression was required to prove the presence of the transgene, because the *nptII* gene has been shown to be an unreliable marker for kanamycin selection in *Petunia* (Deroles and Gardner, 1988; Ulian *et al.*, 1994). In summary, this transgenic approach allows screening of an otherwise invisible molecular phenotype and involves step-by-step reduction of sample numbers, which is of great advantage as the screening methods become more time-consuming.

Forward screening of an insertional mutant population based on a W138 × Pet1 cross

The mutator *Petunia* strain W138, with its active endogenous *dTph1* transposon system, has been successfully used in forward and reverse mutagenesis (see Introduction). However, a transgenic approach cannot be used in this strain because it has so far proved recalcitrant to transformation. We chose to transform *Petunia* Mitchell (W115) with the *pStPT3*–reporter gene construct and then cross it with W138 for mutagenesis. *Petunia* Mitchell is a highly fertile double haploid line originating from an anther culture of a hybrid between *Petunia hybrida* var. Rose of Heaven and *Petunia axillaris* (Ausubel *et al.*, 1980).

The unexpected flower phenotype in the M_1 generation (Figure S3a) could not be suppressed by modification of the culture conditions, including water and fertilizer application, and at present must remain unexplained. However, it has been observed that the unstable flower pigmentation *shriveled-up* phenotype, which was not expressed in the W138 background, became visible when crossed into the W62 line, indicating that an as yet unknown factor in W62 is required for the expression of the phenotype (van Houwelingen *et al.*, 1998). Similar phenotypes have been described in tobacco plants exhibiting reduced levels of nicotianamine leading to disturbance of internal metal transport (Takahashi *et al.*, 2003). Thus, unknown endogenous factors could have influenced flower development in our M_2 mutant population.

The flavonoid pathway in *Petunia* petals is under transcriptional control. Line W138 was selected for based on a transposon insertion in *AN1* (allele *an1-W138*) (Doodeman *et al.*, 1984; Spelt *et al.*, 2000), a transcription factor that

Table 3 List of putative mutants

	StPT3::luc activity	StPT3::GUS activity	Mycorrhization	StPT3::GUS/Luc transgene	*PhPT1* and *2* expression	*PhPT3–5* expression
*ptd1**	–	–	+	+	+	–
*ptd2**	–	–	+	+	NA	NA
ptd3	–	–	+	+	NA	NA
ptd4	–	–	+	+	NA	NA

NA, not yet determined.
*The mutant phenotype, based on lack of transgene activity but a positive PCR result for the transgene (luc–, GUS–, tr+), was found in two out of ten plants tested of the *ptd1* and *ptd2* M_2 families, as well as in one plant of a sibling capsule of *ptd1* (M_2, different capsule but from the same M_1 plant).

regulates petal vacuolar pH and thus influences coloration. Multiple flavonoid biosynthesis and regulatory genes, respectively, determine petal color and color shade. The pale magenta color of the M_1 and M_2 flowers shown here is thought to be determined by the two regulatory genes AN1 and AN2, and by the two biosynthesis genes FL (flavonol synthase) and RT (rhamnosyltransferase) (Brugliera *et al.*, 1994; Holton *et al.*, 1993; Kroon *et al.*, 1994; Quattrocchio *et al.*, 1999; Spelt *et al.*, 2000). Colored sectors in the corolla of progeny plants were a reliable marker for somatic transposition events in the Pet1 × W138 cross (Figure S3d–g). Similarly, mutator activity presumably caused mutations in regulation of the bi-functional *StPT3* promoter that negatively affected mycorrhiza-dependent Luc and GUS activity and was transmitted to the offspring M_2 generation. This forward-genetics approach has so far revealed six putative mutants in *StPT3* promoter regulation from four M_2 families, which now need to be characterized in detail and tested for allelism. It is concluded that the *Petunia* system described here represents a novel alternative to forward-genetics approaches for the study of mycorrhiza development.

Reduced mycorrhizal P_i transporter expression in ptd1

Only few mycorrhiza mutants have been characterized in solanaceous species: *rmc* (Barker *et al.*, 1998), *pmi1* (David-Schwartz *et al.*, 2001) and *pmi2* (David-Schwartz *et al.*, 2003) of tomato all share a phenotype of suppressed mycorrhization. The *Petunia* mutant *pam1* originated from a transposon insertional mutant population derived from the mutator line W138 and exhibits frequent abortion of penetrating hyphae in epidermal cells (Sekhara Reddy *et al.*, 2007). Fungal hyphae that progress to the cortex fail to develop arbuscules. The *Petunia* mutant *ptd1* identified in the present work is a regulatory mutant affected in P_i transporter gene expression but exhibiting normal AM fungal colonization. Further analysis of *ptd1* will aid in understanding the apparent phenotypical differences between mycorrhizal P_i transporter loss-of-function mutants from legume (Javot *et al.*, 2007; Maeda *et al.*, 2006) and solanaceous species (Nagy *et al.*, 2005; Xu *et al.*, 2007).

The five *Petunia* P_i transporters identified (Table 2 and Figure S4) share high similarity with five Pht1 proteins described earlier in tomato and potato (Nagy *et al.*, 2005). Orthologs to the mycorrhiza-inducible P_i transporter genes *PhPT3*, *PhPT4* and *PhPT5* have been described in detail in potato and tomato (Karandashov *et al.*, 2004; Nagy *et al.*, 2005; Rausch *et al.*, 2001; Xu *et al.*, 2007) and have also been identified in pepper, egg-plant (aubergine) and tobacco (Chen *et al.*, 2007). This indicates a high degree of conservation of P_i transport regulation within the Solanaceae. It is therefore anticipated that detailed analysis of the *pdt1* mutant will provide more insight into the regulation of the solanaceous mycorrhizal P_i transport pathway.

All of the cloned mycorrhiza-inducible P_i transporters, i.e. PhPT3–5, were repressed in *ptd1*, although *PhPT3* is non-orthologous to *PhPT4* and *PhPT5*. This suggests that a common mechanism is involved in regulation of the mycorrhizal P_i uptake pathway. Interestingly, the expression levels of *PhPT1* and *PhPT2* remain largely unchanged in *ptd1*, suggesting that two independent pathways for P_i transport regulation exist in *Petunia* and most likely in other solanaceous species such as tomato (Poulsen *et al.*, 2005).

Pet1 as a system to study the physiology of mycorrhiza-induced P_i transport

In addition to their utility in the mutant screen, Pet1 plants served as a system to study mycorrhizal P_i transport physiology in *Petunia*. Our detailed analysis comprising intracellular symbiotic structures (arbuscules, hyphal coils, branched hyphae), vital staining of colonization units and reporter gene expression led us to conclude that *pStPT3*–GUS is a suitable marker gene for symbiotic P_i transport (Figure 4 and S2), and, moreover, that *pStPT3*-driven GUS and luciferase activity can be used as a promising marker for active P_i transport at symbiotic interfaces that is superior to the fungal alkaline phosphatase (ALP), a previously proposed marker for symbiotic efficiency of mycorrhizae (Tiss-

erant *et al.*, 1993). ALP activity was demonstrated in fungal arbuscule branches and hyphae, and has been suggested to be involved in the fungal transfer of P_i from the soil to the roots (Tisserant *et al.*, 1993). It has been proposed that quantification of ALP activity may underestimate the activity of symbiotic interfaces and that acid phosphatases may be more important for fungal metabolism than ALP (van Aarle *et al.*, 2005). The *StPT3* promoter, combined with specific reporter genes such as the Fluorescent Timer (Ds RED-E5), GUS or luciferase, has been shown in this and related work to be useful for visualization of active symbiotic interfaces with spatial and temporal resolution, including non-destructive assays (Karandashov *et al.*, 2004; Rausch *et al.*, 2001).

We conclude that *dTph1* transposon-based insertional mutagenesis of transgenic *Petunia* is suitable for forward-genetics approaches to the study of mycorrhiza-inducible P_i transporter expression, and could be applied generally for selection of other molecular phenotypes.

Experimental procedures

Plant and fungal material

The plant material used was *P. hybrida* W115 (Mitchell), W5 (Stuurman and Kuhlemeier, 2005) and W138 (Doodeman *et al.*, 1984), which a standard laboratory line that is maintained by inbreeding. The fungal isolate of *Glomus intraradices* was kindly provided by Dr Jan Jansa (ETH Zurich, Eschikon, Switzerland).

Bi-directional promoter generation and transformation of Petunia *W115*

The 35S minimal promoter comprising 90 bp upstream of the start ATG in the CaMV 35S gene and the mycorrhiza-specific fragment of the *StPT3* gene promoter (Rausch *et al.*, 2001) were cloned back to back into the binary vector BIN19 (Bevan, 1984) using standard procedures. The genes encoding firefly luciferase (luc^+; Promega, http://www.promega.com) and β-glucuronidase (Jefferson *et al.*, 1987) were flanked by the nopaline synthase (NOS) transcriptional terminator (Depicker *et al.*, 1982), and integrated into the BIN19 construct as shown in Figure 1. *P. hybrida* W115 (Mitchell) was transformed following the transformation protocol described by Fillatti *et al.* (1987).

Plant growth

Petunia seeds were germinated under sterile conditions on MS medium (Murashige and Skoog, 1962) containing 2% sucrose and 2 g l^{-1} SEQUESTRENE Rapid (Syngenta AG, http://www.syngenta-agro.ch) at 25°C. Two- to three-week-old *Petunia* seedlings were transplanted into pots (6 × 6 × 5 cm). The substrate used for mycorrhization was a sterilized soil/quartz sand mixture (1:10) containing *G. intraradices* dry inoculum. For flowering, plants were grown in soil. Fertilization of the mycorrhizal plants was carried out using half-strength Hoagland medium (Hoagland and Broyer, 1936) containing 5 μM $NH_4H_2PO_4$. SEQUESTRENE Rapid (2 g l^{-1}) and $CuSO_4$ (1 μM) were added when leaves were light green. Plants were tested 4–7 weeks after inoculation. For the *PhPT* expression studies, W115 plants were grown on a sand/soil mixture (2:1) and inoculated with *G. intraradices* (MUCL 43204). Root samples were harvested 4 weeks after inoculation.

Extraction of genomic DNA and Southern blotting

Genomic DNA for Southern blotting was isolated from *Petunia* leaves as described previously (Dellaporta *et al.*, 1983). Genomic DNA was digested overnight with *Not*I and *Nco*I and was subsequently separated electrophoretically on a 0.8% agarose gel. Nucleic acids were blotted onto Hybond NX nylon membrane (Amersham Biosciences, http://www.amersham.com). Hybridization was carried out using 5 × SSC, 5% Denhardt's solution and 0.5% SDS (w/v) at 65°C, with a final wash using 0.1 × SSC, 0.1% SDS at 50°C (Sambrook *et al.*, 1989). The radioactively labeled luciferase gene from plasmid $pSPluc^+$ (Promega) was used as the probe.

Reporter gene assays and histochemical staining procedures

Roots were sampled, thoroughly washed with tap water and directly sprayed with 1 mM D-luciferin potassium salt (MP Biomedicals Inc., http://www.mpbio.com) and 0.01% Triton-X (Xiong *et al.*, 1999). After 5 min incubation in the dark, an image was taken with the Sensicam QE (type 672KS) CCD camera (Gloor Instruments, http://www.gloorinstruments.ch) using CAMWARE version 2.10 software (PCO AG, http://www.pco.de) with the settings: 10 min exposure, 2 × 2 binning, low light mode, and subtraction of reference picture. Using the same roots, β-glucuronidase staining was performed according to the method described by Karandashov *et al.* (2004), with incubation overnight at room temperature. To visualize the fungal structures, the roots were stained with either ink (Sheaffer Scrip black ink, Sheaffer, http://www.sheaffer.com) (Vieweg *et al.*, 2004) or trypan blue (Brundrett *et al.*, 1984). A separate sample of the same roots was stained for SDH (Pearse, 1968) or ALP (Tisserant *et al.*, 1993) activity. The percentage colonization, as well as the percentage of GUS staining, was calculated using the gridline intersect method (Brundrett *et al.*, 1984). Total percentage colonization comprised root intercepts containing hyphae, vesicles/spores, arbuscules or combinations of these fungal structures. In addition, the percentage of root intercepts containing arbuscules alone or in combination with other structures was also calculated. Differences between means of variables were examined by the standard *t*-test for independent samples and ANOVA. Statistical significance for all analyses was accepted at the $P \leq 0.05$ level of probability. Statistical analyses were performed using SPSS 10.1 (http://www.spss.com).

Determination of shoot P content

Shoot P content was determined using dried shoot material that was homogenized with mortar and pestle, and subsequently incinerated for 8 h at 550°C. Total P concentration was measured using the malachite green colorimetric method as described by Ohno and Zibilske (1991).

PCR to test for transgenic plants

To test for homozygosity, PCR was performed with an annealing temperature of 58°C for 35 cycles, using the primers 5′-GAGA-

GAGCTCGGTCTTGAAAAAAAGCCATTAG-3′ (position −1051 to −1030 in the *StPT3* promoter, containing a *Sac*I restriction site) and 5′-CTTCGCGCTGATACC-3′ (position 203 to 217 in the GUS gene) to amplify the *StPT3* promoter–GUS junction of the transgene.

Genomic DNA for PCR screening was isolated from leaves or flower buds essentially as described previously (Dellaporta *et al.*, 1983) following the protocol until the first DNA precipitation step. PCR was performed with an annealing temperature of 54°C for 40 cycles using two sets of primers simultaneously: 5′-TCCAGATCCACAACCTTCGCTTCA-3′ (lucs F) and 5′-CAAGATTCAAAGTGCGCTGCTGGT-3′ (lucs R) amplifying a 291 bp fragment of the luciferase reporter gene, and 5′-GGACCAAAATTCGCGAAGAAAAAGG-3′ (cons F) and 5′-GGCGTATGAAGATGCCTGA-3′ (StPT3R3), amplifying a constitutively expressed fragment of approximately 500 bp to check the quality of the DNA.

Cloning of Petunia *P_i transporters*

Petunia plants of line W5 (Stuurman and Kuhlemeier, 2005) were grown in a conventional greenhouse. Genomic DNA was extracted from leaves and used as a template in a PCR reaction with primers based on multiple sequence alignments of all the available solanaceous *Pht1* P_i transporters (Table 1). PCR fragments were cloned in the pGEM-T vector (Promega) and sequenced. Whole-length sequences were identified using the GenomeWalker kit (Clontech, http://www.clontech.com) using the primers listed in Table 1 according to the manufacturer's instructions. Multiple sequence alignment was performed using CLUSTAL W. Transmembrane structure prediction and protein properties were analyzed using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and PEPSTATS (http://softwarebioweb.pasteur.fr/seqanal/interfaces/pepstats.html) software.

RNA extraction and quantitative PCR

For the *PhPT* expression studies, RNA was extracted according to the hot phenol protocol. One microgram of total RNA was reverse-transcribed using the Omniscript RT kit (Qiagen, http://www.qiagen.com). For analysis of the *ptd1* and W115 control, total RNA was isolated using the RNeasy plant mini kit (Qiagen) and treated with RQ1 RNase-free DNase (Promega). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen, http://www.invitrogen.com). Quantitative PCR was performed using ABsolute QPCR SYBR Green mix (ABgene, http://www.abgene.com) on a Rotor-Gene 2000 cycler (Corbett Research, http://www.corbettlifescience.com).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Physiology of Pet1 compared to W115.

Figure S2. p*StPT3:GUS* as a marker for functional symbiosis in Pet1 plants.

Figure S3. Phenotypes in the M_1 and M_2 generations showing active transposition.

Figure S4. Phylogenetic tree and alignment of the *Petunia* phosphate transporters and selected homologs.

This material is available as part of the online article from http://www.blackwell-synergy.com

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