GmPT7 was originally identified as an arbuscular mycorrhiza-inducible gene of soybean that encodes a member of subfamily I in the PHOSPHATE TRANSPORTER 1 family. In the present study, we established conditions under which a number of dwarf soybean plants complete their life cycles in a growth chamber. Using this system, we grew transgenic soybean with a GmPT7 promoter–β-glucuronidase fusion gene and evaluated GmPT7 expression in detail. GmPT7 was highly expressed in mature, but not in collapsed, arbuscule-containing cortical cells, suggesting its importance in the absorption of fungus-derived phosphate and/or arbuscule development. GmPT7 was also expressed in the columella cells of root cap and in the lateral root primordia of non-mycorrhizal roots. The expression of GmPT7 occurred only in the late stage of phosphorus translocation from leaves to seeds, after water evaporation from the leaves ceased, and later than the expression of GmUPS1-2, GmNRT1.7a and GmNRT1.7b, which are possibly involved in nitrogen export. GmPT7 expression was localized in a pair of tracheid elements at the tips of vein endings of senescent leaves. Transmission electron microscopy revealed that the tip tracheid elements in yellow leaves were still viable and had intact plasma membranes. Thus, we think that GmPT7 on the plasma membranes transports phosphate from the apoplast into the tip elements. GmPT7 knockdown resulted in no significant effects, the function of GmPT7 remaining to be clarified. We propose a working model in which phosphate incorporated in vein endings moves to seeds via xylem to phloem transfer.

**Keywords:** Arbuscular mycorrhiza • Dwarfing • Glycine max L. Merrill • Leaf senescence • Phosphate transporter • Vein ending.

**Abbreviations:** AM, arbuscular mycorrhiza(1); GUS, β-glucuronidase; PT, phosphate transporter; RT–PCR, reverse transcription–PCR; VIGS, virus-induced gene silencing.

**Introduction**

Soybean is an important crop as a source of vegetable oil, meal, feedstock, soy ink, biodiesel and other products. Its production, currently at 305 Mt year⁻¹ worldwide, (http://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf), is continuously increasing. Leguminous crops, including soybean, employ plant–microbe symbioses for nutrient acquisition, e.g. nitrogen fixation with rhizobia (Kouchi 2011) and arbuscular mycorrhizal (AM) symbiosis (Hata et al. 2010, Kobae et al. 2010). Furthermore, in order to obtain high seed yields, it is important to achieve efficient nutrient translocation in the crop body.

Leaf senescence is a type of programmed cell death that begins in mesophyll cells and subsequently proceeds to other cells. It is accompanied by a decreased expression of genes for photosynthesis and protein synthesis, and by an increased expression of senescence-associated genes (Lim et al. 2007). Possible involvement of autophagy in this process has been reported (Nang et al. 2011, Pottier et al. 2014). Leaf senescence is not simply a decline or decay but a strategy of effective nutrient utilization. In soybean, the dry matter of mature seeds corresponds to 29% of that of the total above-ground portions, including fallen leaves and petioles (Hanway and Weber 1971a). On the other hand, mature seeds contain 68, 73, and 56% of N, P and K, respectively (Hanway and Weber 1971b). Most of these nutrients are mobilized from leaves, petioles, stems and pods to seeds during maturation. Leaves are the largest source for this nutrient redistribution (Hanway and Weber 1971b).

Phosphate transporters (PTs) play a pivotal role in phosphorus uptake from soil and the mobilization of this essential nutrient within the plant body. Among them, transporters encoded by the PHOSPHATE TRANSPORTER 1 (Pht1) gene family have 12 transmembrane domains and reside on the plasma membrane (Rausch and Bucher 2002). The expression pattern of a family member of Arabidopsis, ARATH:Pht1;5,
suggested its involvement in remobilization of phosphate from germinating seeds to cotyledons, and from source old leaves to the sink such as floral tissues (Mudge et al. 2002). Two other members were also suggested to be involved in phosphate redistribution in barley and rice plants (Rae et al. 2003, Ai et al. 2009). As shown in Supplementary Table S1, the nomenclature of these genes differs among research groups (Tamura et al. 2012). Among them, GmPT1–GmPT14 of Tamura et al. (2012) and Xie et al. (2013) for simplification. Their formal nomenclatures are GLyMa;Pht1;1–GLyMa;Pht1;14.

Arbuscules are special nutrient exchange organs of AM fungi (Smith and Read 2008). The plant membrane surrounding an arbuscule and the thin space between the arbuscule and the membrane are called the periarbuscular membrane and peri-arbuscular space, respectively. The periarbuscular membrane is connected to the plant plasma membrane (Smith and Read 2008). In a previous study, we reported that three PT genes of the 14 Pht1 genes in the soybean genome were induced specifically in AM roots (Tamura et al. 2012). Among them, GmPT10 and GmPT11 encode major and typical AM-inducible PTs localized on the branch domains of periarbuscular membranes. The third gene, GmPT7, seemed to encode a minor AM-inducible PT, transcripts of which appeared later than those of GmPT10 and GmPT11 (Tamura et al. 2012). Although Fan et al. (2013) systematically examined the divergent expression of the 14 Pht1 genes and characteristics of their protein products, the detailed functions of the protein products remain unclear, except that for GmPT5 in root nodules (Qin et al. 2012). Xie et al. (2013) recently reported that an ortholog of GmPT7 in Chinese milk vetch (Astragalus sinicus), AsPT1, is expressed in arbusculated cells and is essential for mature arbuscule formation.

In the present study, we investigated GmPT7 expression in the whole plant body using β-glucuronidase (GUS) as a reporter. GmPT7 was expressed not only in AM roots but also in columella cells of root caps and in vein endings of senescent leaves. We discuss the involvement of GmPT7 in the translocation of phosphorus from senescent leaves to seeds. We also describe the conditions under which a number of dwarf soybean plants can be grown in a chamber.

Results

Establishment of growth conditions

We previously grew soybean plants in a growth chamber (inner capacity, 294 liters) under a 16 h day/8 h night cycle at a constant temperature of 27°C (Tamura et al. 2012). Under these conditions, cv. Jack required approximately 60 d from sowing to the beginning of leaf yellowing and 20 d from leaf yellowing to harvesting. The plants became dwarf, allowing us to grow as many as 30 plants per chamber (Supplementary Fig. S1). The plants also showed leaf senescence and seed development within a short period (Supplementary Fig. S2), making analyses of gene expression and nutrient translocation considerably easier. Because we grew the soybean plants in a growth chamber using an artificial soil mixture without inoculation of rhizobia (see the Materials and Methods), the formation of root nodules was very rare. The results for cv. Fukuyutaka, a late-maturing cultivar, were similar to those for cv. Jack, although the plants were approximately 5 cm higher and required a couple of weeks more until flowering (data not shown).

GmPT7 expression in mycorrhizal or non-mycorrhizal roots of soybean

As reported previously (Tamura et al. 2012), GmPT7 was identified as one of three AM-inducible PT genes in soybean. Phylogenetic analysis showed that GmPT7 belongs to subfamily I, similar to the other AM-inducible PTs GmPT10 and GmPT11 (Sisaphaithong et al. 2012, Tamura et al. 2012, Xie et al. 2013). However, a time course experiment indicated that the transcript level of GmPT7 increased later than that of the other two AM-inducible genes (Tamura et al. 2012). In the present study, we generated stable transgenic soybean lines containing a GmPT7 promoter–GUS fusion gene using Agrobacterium tumefaciens and investigated the localization of GmPT7 expression in AM roots. As shown in Fig. 1A, GUS activity was detected in inner cortical cells colonized by Rhizobagus irregularis. Arbuscules are functional for only 2–3 d, after which they suddenly shrink and collapse (Kobae and Hata 2010). Close inspection revealed that much stronger signals were detected in highly branched (mature) arbuscule-containing cells than in collapsed arbuscule-containing cells (Fig. 1B). We also introduced the GmPT7 promoter–GUS construct into soybean plants via hairy root transformation using A. rhizogenes. Again, strong GUS activity was localized in mature arbuscule-containing cells (Supplementary Fig. S3A). Vector control roots (Supplementary Fig. S3B) and non-transgenic AM roots (not shown) showed no positive signals.

Fan et al. (2013) recently reported that GmPT7 is expressed in non-mycorrhizal roots at the flowering stage. Using the GmPT7 promoter–GUS line, we found a columella-localized expression of the gene at the flowering stage (Fig. 2A; Supplementary Fig. S6A). In contrast to their results, however, in our study, GmPT7 expression was not restricted to the flowering stage but was detected even immediately after rooting (Fig. 2C). GUS activity was also detected in lateral root primordia (Fig. 2D; Supplementary Fig. S6B).

GmPT7 expression in other organs

We roughly evaluated the expression patterns of 13 of 14 GmPT genes in various organs using semi-quantitative reverse-transcription PCR (RT–PCR). Unexpectedly, GmPT7 showed...
GmPT7 expression. The result for cv. Fukuyutaka (Supplementary Fig. S5) was less clear but largely similar to that for cv. Jack. Up to 80% of phosphorus was exported from the leaves of both cultivars.

In a separate experiment, we evaluated water evaporation rates along with leaf senescence. Given that GmPT7 is expressed only after the Chl content declines below 1 mg g\(^{-1}\) FW (Fig. 3A; Supplementary Fig. S5), Fig. 4 clearly indicates that transpiration ceases well before GmPT7 expression.

We next evaluated the expression of five genes putatively involved in nitrogen transport during leaf senescence and found that GmUPS1-2, a gene likely to encode ureide permease (Collier and Tegeder 2012), was induced in senescent leaves earlier than GmPT7 (Fig. 3B; Supplementary Fig. S5B). In contrast, the PCR product of GmUPS1-1 was not detected (data not shown), and the expression level of the third ureide permease-like gene, GmUPS1-3 (Glyma02g12980), was very low (data not shown). We also searched the Phytozome database for soybean orthologs of AtNRT1.7, which is responsible for source to sink remobilization of nitrate in Arabidopsis (Fan et al. 2009), finding GmNRT1.7a (Glyma01g04830) and GmNRT1.7b (Glyma18g16490). Both genes were highly expressed in cv. Jack (Fig. 3), whereas only GmNRT1.7a was selectively expressed in cv. Fukuyutaka (Supplementary Fig. S5), suggesting variation between the cultivars. Again, these putative nitrate transporter genes were expressed earlier than GmPT7 (Fig. 3; Supplementary Fig. S5).

GmPT7 expression in the vein endings of senescent leaves

Using the GmPT7 promoter–GUS line, we found that high gene expression was localized in the vein endings of yellow leaves (Fig. 5A; Supplementary Fig. S6C, D). A non-transgenic control leaf showed no GUS signal (Fig. 5B). Esaü (1965) reported that the vein endings of dicotyledonous plants frequently consist of tracheids alone. In the vein ending of soybean, a pair of tracheid elements lies parallel to each other. A longitudinal section of a paraffin-embedded vein ending showed that one of the tracheids with spiral thickening of the cell wall expressed the GUS signal, being enclosed by bundle sheath cells (Fig. 5C). A transverse section showed positive signals only in the two tracheids. Interestingly, the blue signals were hollow (Fig. 5D). Thus, the positive GUS signal strongly suggests that the expressing cells were viable.

To confirm that the tip tracheid elements of vein endings were viable, we used electron microscopy. A transmission electron micrograph of the tip of a vein ending of a senescent leaf showed that two tracheal elements retained living organelles including central vacuoles (Fig. 6A). The presence of central vacuoles explains why GUS signals in Fig. 5D were hollow. Another enlarged picture clearly shows the intact plasma membrane in the tracheal elements (Pm in Fig. 6B). Furthermore, mesophyll cells of the same yellow leaf became spherical, accumulating several starch granules (Supplementary Fig. S7). Taken together, these data indicate that tip tracheid elements were still viable in the senescent leaves.
Effects of GmPT7 knockdown on the export of phosphorus from senescing leaves

To clarify the role of GmPT7 in phosphorus translocation from leaves to seeds, knockdown experiments of GmPT7 were performed using virus-induced gene silencing (VIGS) (Senda et al. 2004, Nagamatsu et al. 2007). Either a short coding region that appeared specific to GmPT7 or a 5′-non-coding region just upstream of the initiation codon was targeted. Although the transcript levels of GmPT7 in the yellow leaves infected with CMV2-A1:GmPT7-coding or CMV2-A1:GmPT7-5′ were much lower than those in the control yellow leaves infected with CMV2-A1:empty (Supplementary Fig. S8A), the phosphorus content remaining in the leaves was similar (Supplementary Fig. S8B). To determine the reason for this result, we evaluated the mRNA levels of other PT genes and found GmPT2 induction in the yellow leaves infected with CMV2-A1:GmPT7-coding and CMV2-A1:GmPT7-5′ (Supplementary Fig. S8D).

Discussion

Growth of miniaturized soybean plants

Under our experimental conditions, the plants showed a sharp transition from the vegetative stage to the reproductive stage, remaining small in size. In addition, their leaf senescence and seed development lasted only for a short period (Supplementary Fig. S2). Thus, soybean can be grown as a model plant. These conditions will be useful not only for elucidating the mechanisms of nutrient translocation and control of monocarpic senescence but also for soybean breeding.

GmPT7 expression in soybean plants

Although almost 75% of soybean genes have multiple paralogs in the genome (Schmutz et al. 2010), GmPT7 is a unique single-copy gene in the PHT1 family (Fan et al. 2013), suggesting its importance in soybean plants. GmPT7 was first identified as one of the three AM-inducible PT genes of soybean, and its induction occurs later than that of the other two genes, GmPT10 and GmPT11 (Tamura et al. 2012). In the present study, using transgenic soybean lines with GmPT7 promoter–GUS, we demonstrated that GmPT7 is expressed specifically in cortical cells containing active arbuscules (Fig. 1; Supplementary Fig. S3), like GmPT10 and GmPT11 (Tamura et al. 2012). Recently, Xie et al. (2013) revealed the presence of two AM-inducible transporters in Chinese milk vetch. Only the major AM-inducible transporter, AsPT4, was required for phosphate uptake from the fungi, and the minor AM-inducible transporter, AsPT1, an ortholog of GmPT7, was reported to play an essential role in

![Columella cells](https://example.com/columella.png)

**Fig. 2** GUS activity in the columella of root caps. (A and B) Root caps at the flowering stage. Scale bar = 50 μm. (C) Just after rooting out of a seed. Scale bar = 1 mm. (D) Lateral root primordium in the non-flowering stage. Scale bar = 100 μm. (A, C and D) Non-mycorrhizal roots transformed with the GmPT7 promoter–GUS construct. Results with line 4-1-4 are presented. Similar expression patterns were also observed using line 1-2-2 (Supplementary Fig. S6A, B). (B) A non-transgenic control root.
AM development, probably as a ‘transceptor’. In analogy to AsPT1 and AsPT4, GmPT7 may play a role different from that of GmPT10 and GmPT11. This hypothesis is also supported by the following observations. In rice and *Lotus japonicus*, there are major AM-inducible PT genes, OsPT11 (Paszkowski et al. 2002) and LjPT4 (Guether et al. 2009, Takeda et al. 2009), and minor AM-inducible PT genes, OsPT13 (Gamal et al. 2005) and LjPT3 (Maeda et al. 2006), respectively. Although the spatial expression patterns of the minor AM-inducible PT genes are similar to those of the major ones, knockdown or mutation of the minor genes impedes AM development and function (Maeda et al. 2006, Yang et al. 2012).

Interestingly, Xie et al. (2013) also showed that AsPT1 overexpression promoted fungal development, increasing phosphate accumulation in AM roots. Thus, GmPT7 overexpression in soybean may result in the increase of nutrient absorption and the elevation of yields.

GmPT7 was expressed in columella cells of root caps, the physiological role remaining to be clarified (Fig. 2). This observation is in accord with the very low but detectable expression level of GmPT7 in non-mycorrhizal roots of soybean (Tamura et al. 2012; Supplementary Fig. S4). In columella, ‘statocytes’ containing dense amyloplasts function in gravity perception (Hawes et al. 2002). Given that we observed GmPT7 expression...
in the columella at both the young root stage and flowering stage (Fig. 2; Supplementary Fig. S6), the expression probably continues throughout the life of the soybean plant. As the cells redifferentiated to mucilage-secreting cells, GmPT7 expression ceased (Fig. 2A; Supplementary Fig. S6A).

Monitoring of Chl content, gene expression and phosphate content of senescent leaves revealed that GmPT7 expression occurs at the late stage of phosphorus translocation from leaves to seeds. According to McClure and Israel (1979), xylem sap of non-nodulated soybean plants contained 6 and 58% of total nitrogen as ureide and nitrate, respectively. Collier and Tegeder (2012) reported the importance of soybean ureide permease genes, GmUPS1-1 (Glyma01g07120) and GmUPS1-2 (Glyma02g12970), in nitrogen export from root nodules. In this work, we identified GmNRT1.7a (Glyma01g04830) and GmNRT1.7b (Glyma18g16490) as putative nitrate transporter genes responsible for nitrate translocation. As the first step toward clarifying the mechanism of nitrogen translocation from leaves to seeds, we evaluated the expression levels of these genes. GmPT7 expression appeared later than the expression of GmUPS1-2, GmNRT1.7a and GmNRT1.7b (Fig. 3; Supplementary Fig. S5). If these genes are involved in nitrogen translocation to seeds, the above results may suggest that nitrogen and phosphorus are exported from leaves more or less independently (Crafts-Brandner 1992). However, given that significant phosphorus export occurs from leaves before GmPT7 expression, as discussed below, the different timing of nitrogen and phosphorus translocation awaits further study.

Unexpectedly, again using transgenic soybean plants with the
GmPT7 promoter–GUS, we found a localized GUS signal in a pair of tracheid elements at the tips of vein endings (Fig. 5; Supplementary Fig. 56). Mature tracheid elements are known to be dead (Holbrook 2010), but those at the tips of vein endings were still viable and had intact plasma membranes (Fig. 6).

It is likely that GmPT7 on the plasma membranes transports phosphate from the apoplast of an ultimate areole to the tip tracheid elements. Given that the mesophyll cells of senescent leaves had already lost their original shape (Supplementary Fig. S7), phosphate may have leaked out of the cells. Another possibility is that there is an unknown mechanism for secreting phosphate from mesophyll cells to the apoplast.

**Mechanism of phosphorus translocation**

In nitrogen translocation from root nodules to developing seeds of soybean, large amounts of nitrogen are transported via xylem to phloem transfer (Layzell and LaRue 1982). As for phosphorus, AtPT5 was shown to be involved in phosphate mobilization between the source and sink organs of Arabidopsis (Nagarajan et al. 2011). AtPT5 encodes a subfamily III PT of the PHT1 family, and Arabidopsis has no members of subfamily I (Sisaphaithong et al. 2012, Tamura et al. 2012, Xie et al. 2013).

Based on these reports and the present results, we propose a model of phosphate translocation from leaves to developing seeds (Fig. 7). When leaves are green and active, water flows vigorously from roots through xylem to the venation of leaves (Fig. 4). Thus, typical and major translocation in phloem occurs (the blue arrows in Fig. 7A), including phosphate loading through companion cells (Dunford 2010). This process corresponds to the decrease in phosphate in leaves before GmPT7 induction (Fig. 3; Supplementary Fig. S5) and is in accord with the report of Neumann and Nooden (1984) that stem girdling prevented most transfer of phosphate from leaves to pods. GmPT2, GmPT4, GmPT6, GmPT8 and/or GmPT9 may be involved in this step because they are expressed in green leaves (Supplementary Fig. S4A). However, after the leaves turn yellow, GmPT7 is transiently expressed. Its protein product is likely to play a scavenging role and transport as much phosphate as is collectable in ultimate areoles into the tracheal elements at vein endings. Because water evaporation has already ceased at that stage (Fig. 4), we think that phosphate in the vein endings will move to the seeds by xylem to phloem transfer (dotted red arrows in Fig. 7B). However, it still remains to be clarified whether or not the expression of GmPT7 in the tracheal elements at the late stage of leaf senescence contributes to phosphorus translocation from leaves to seeds. Because Arabidopsis has no orthologs of GmPT7, this accessory mechanism may be peculiar to leguminous plants. In our working model, it is noteworthy that GmPT7 functions as a typical PT in yellow leaves, in contrast to the putative transceptor in AM roots. Interestingly, Neumann and Nooden (1984) also pointed out that direct phosphate transfer from xylem to phloem in soybean stem or petiole may be significant when transpiration rates are low. Our knockdown experiment of GmPT7 using VIGS was unsuccessful, probably because phosphate redistribution was compensated by other PT genes, such as GmPT2. In fact, GmPT2 is one of the four soybean orthologs of AtPT5 (Sisaphaithong et al. 2012, Tamura et al. 2012). This indicates that the typical phloem pathway remains functional after leaves turn yellow (blue arrows in Fig. 7B). Of note, the transcript level of GmPT2 was augmented only when GmPT7 was knocked down (Supplementary Fig. S8). This result suggests a hierarchical redundancy in which GmPT7 is more important than GmPT2 for phosphate translocation at the late stage, similar to superior Dicer-like 4 and inferior Dicer-like 2 for antiviral...
activity in Arabidopsis (Deleris et al. 2006). Thus, phosphate translocation mechanisms from senescent leaves to developing seeds appear very plastic.

Materials and Methods

Plant and fungal materials

Soybean [Glycine max (L.) Merrill] cvs. Jack (P1540556, maturity group II), Fukusuytaka (P1506675, maturity group VI) and Akiisengoku (P1416778, maturity group VII) were used as plant materials. Spores of the AM fungus Rhizopogon irregularis (formerly Glomus intraradices) DAOM197198 were obtained from Premier Tech.

Plant growth

Several holes were drilled at the bottom of a disposable plastic cup (500 ml capacity) and 100 g of coarse particles of Akadama soil were added. A mixture of 100 g of Kureha soil, 100 g of fine particles of Kanuma soil and 30 g of Ezo sand was added, and the cup was placed on a tray with approximately 5 mm depth of water. Akadama soil and Kanuma soil are volcanic ash subsoils and Kureha soil is a type of compost with nutrients. After the germination of soybean seedlings, the seedlings were transplanted onto the soil mixture. For preparation of mycorrhizal roots, the seedlings were inoculated with 2,000 spores per plant of R. irregularis and then grown in the above soil mixture supplemented with 0.5× modified Hoagland nutrient solution that contained 0.1 mM phosphate. In other experiments, the seedlings were grown on a 1:1 mixture of JA Nippi Horticultural Soil #1 (Nihon Hiryo Co.) and Kanuma soil. JA Nippi Horticultural Soil #1 is another sort of compost with nutrients. Unless otherwise stated, the plants were grown in a growth chamber (MLR-350H; Panasonic) under an 8 h day (27°C/14°C) and 16 h night (20°C/0°C) with nutrients. Unless otherwise stated, the plants were grown in a growth chamber (MLR-350H; Panasonic) under an 8 h day (27°C/14°C) and 16 h night (20°C/0°C) with nutrients.

Preparation of transgenic pGmPT7:GUS soybean

A genomic fragment of GmPT7 (Glyma14g36650) containing a promoter region (2,019 bp in size) and a short coding region (69 bp in size) was amplified from G. max (cv. Fukusuytaka) by PCR using a primer pair (5'-CACCTGGGACACAAAAGAGGCACAA-3' and 5'-TGGCTCTGAACTGAAGGTGTC-3'). The amplified DNA fragment (4.5 kb in size) was ligated into pCR-Blunt II-TOPO (Invitrogen), digested by KpnI/SpeI, and ligated into the same restriction site of the soybean expression vector pUHR (Nishizawa et al. 2006). Transformation of soybean cv. Jack was conducted by particle bombardment and subsequent plant regeneration as described previously (Khaleefalla et al. 2005), and T2 seedlings were obtained from independent T1 plants in the growth chamber. Fourteen independent lines were prepared in total. As their representatives, lines 1-4-1 and 1-2-2 were examined in this work.

GUS staining

Root segments of pGmPT7:GUS soybean were submerged in a GUS reaction mixture (Nakagawa et al. 2003) for 2 h, and 5% agar sections were prepared as described previously (Kobae et al. 2010). Yellow leaves collected at the R7 stage (http://extension.agron.iastate.edu/soybean/production_growthstages.html) were cut into 5 mm squares and submerged in the GUS reaction mixture under vacuum for 5 h. Ethanol washes were used to stop staining and remove pigments from the leaf segments. When necessary, the GUS-stained leaf segments were fixed with a 5:5:90 mixture of formalin:acet acid:ethanol, dehydrated through a graded butanol series, and embedded in Paraplast Plus (McCormick Scientific). Microtome sections (10 μm thick) were applied to the glass slides, treated with Lemosol (Wako), rehydrated through a graded ethanol series, and stained with 1% safranin in 50% ethanol. Finally, the sections were dehydrated through an ethanol series and mounted with Mount-Quick (Daido Sangyo).

RT–PCR and real-time RT–PCR

Total RNA was extracted from various plant organs using a Plant Total RNA Mini Kit (Viogene Bio Tek) and was reverse transcribed as reported previously (Kobae et al. 2010). The resulting cDNA was used as a template for PCR (pre-denaturation at 94°C for 2 min; 25–35 cycles of 98°C for 20 s and 68°C for 24 s; and additional extension at 68°C for 3 min) with KOD FX Neo DNA polymerase (Toyobo). The sequences of gene-specific primers are given in Supplementary Table S2. The PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. Real-time PCR was conducted using a LightCycler 1.5 system (Roche Applied Science).

Determination of Chl and phosphorus contents in the leaves

Green or yellow leaves were collected from soybean plants at the R7 stage and cut into two portions along the midrib. The initial halves containing the midrib were used for determination of Chl contents as described above. The second halves without the midrib were cut into small pieces, with approximately 20 mg FW...
being immersed in 1 ml of N,N-dimethylformamide overnight. The absorbance of each leaf extract was measured at 646.8 and 663.8 nm. The Chl concentration (μg ml⁻¹ dimethylformamide) was calculated as the sum of 17.67 × OD646.8 and 7.12 × OD663.8 (Porra et al. 1989). The remainder of the second halves of the leaves were dried at 80°C for 2 d and then converted to ashes at 550°C for 6 h to determine the phosphorus content. The ashes were mixed with 1 ml of 4 M HCl and made up to 50 ml with water. An appropriate amount of the resulting solution was mixed with 8 ml of 1.25 M sulfuric acid, 0.6% ammonium molybdate, 0.03 M ascorbic acid and 0.2 mM potassium antimony tartrate, and made up to 50 ml with 80 mM HCl. Subsequently, the OD₇₅₃ of molybdenum blue was measured (Clausen and Shroyer 1948). The standards of P₄O₁₀ were treated with the same reagent, and their OD₇₅₃ values were compared with those of the leaf samples.

**Determination of transpiration rates**

As an indicator of water flow through tracheids, transpiration rates were determined using a leaf porometer (OSC-1; Ollie).

**Transmission electron microscopy**

The yellow leaves of a soybean plant that had not been inoculated with *R. irregularis* were used for electron microscopy studies. Small segments (approximately 1 × 2 mm) of leaf tissue were fixed in 3% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) for 3 h at 25°C and post-fixed in 1% osmium tetroxide in the same buffer for 12 h at 4°C. The specimens were dehydrated in a graded acetone series (30, 50, 70, 90, 99 and 100%) and propylene oxide, and were embedded in Spurr’s resin. The embedded samples were cut (90–100 nm) using a diamond knife on an ultramicrotome. The sections were mounted on a 200-mesh copper grid and double-stained with 2% uranyl acetate (Sigma-Aldrich) for 20 min, followed by lead citrate solution (Sigma-Aldrich) for 4 min. The sections were examined using an H-7500 transmission electron microscope (Hitachi) at 100 kV.

**VIGS of GmPT7**

A short coding region (50 bp in size) or 5'-untranslated region next to the initiation codon (102 bp in size) of GmPT7 was amplified from the genomic DNA of soybean (cv. Fukuyutaka) by PCR using primer pairs Mlul-GmPT7_Fw and GmPT7_Stul_Rv or Mlul-GmPT7_SUTR_Fw and GmPT7_SUTR-Stul_Rv. Each amplified fragment was digested with Stul and Mlul, and ligated into a Stul/Mlul-digested virus vector CMSV-A1 (Otagaki et al. 2006) in the antisense orientation. The resulting vectors were designated as CMSV-A1:GmPT7-coding or CMSV-A1:GmPT7-5′. In vitro transcription of virus RNA and inoculation steps were performed as described previously (Nagamatsu et al. 2007). Yellow leaves infected with the virus were collected from soybean plants (cv. Jack). One half of each leaf was used for RNA extraction, and the other half was used for Chl and phosphorus content analyses as described above. Virus infection in the *N. benthamiana* and soybean plants from soybean plants (cv. Jack). One half of each leaf was used for RNA extraction, and the other half was used for Chl and phosphorus content analyses as described above. Virus infection in the *N. benthamiana* and soybean plants

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**Disclosures**

The authors have no conflicts of interest to declare.

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