Long-Distance Transport of Thiamine (Vitamin B_1) Is Concomitant with That of Polyamines[1][OPEN]

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Thiamine (vitamin B_1) is ubiquitous and essential for cell energy supply in all organisms as a vital metabolic cofactor, known for over a century. In plants, it is established that biosynthesis de novo is taking place predominantly in green tissues and is furthermore limited to plastids. Therefore, transport mechanisms are required to mediate the movement of this polar metabolite from source to sink tissue to activate key enzymes in cellular energy generating pathways but are currently unknown. Similar to thiamine, polyamines are an essential set of charged molecules required for diverse aspects of growth and development, the homeostasis of which necessitates long-distance transport processes that have remained elusive. Here, a yeast-based screen allowed us to identify Arabidopsis (*Arabidopsis thaliana*) PUT3 as a thiamine transporter. A combination of biochemical, physiological, and genetic approaches permitted us to show that PUT3 mediates phloem transport of both thiamine and polyamines. Loss of function of *PUT3* demonstrated that the tissue distribution of these metabolites is altered with growth and developmental consequences. The pivotal role of PUT3 mediated thiamine and polyamine homeostasis in plants, and its importance for plant fitness is revealed through these findings.

Thiamine (vitamin B_1) is essential for all organisms being well recognized in its diphosphorylated form, thiamine diphosphate (TDP; Supplemental Fig. S1), as a necessary cofactor for key metabolic enzymes involved in glycolysis and the citric acid cycle (Fitzpatrick and Thore, 2014). In plants, it is also necessary for the Calvin cycle, the biochemical route of carbon fixation (Khozaei et al., 2015). Therefore, thiamine is vital for cell energy supply in all organisms. Interestingly, some additional roles for thiamine have been proposed that include involvement in responses to DNA damage (Machado et al., 1996), as well as the activation of plant defenses, resistance to pathogen attack, and attenuation of environmental stress responses (Ahn et al., 2005; Rapala-Kozik et al., 2012). Humans cannot produce thiamine de novo with plants representing one of the most important sources of the compound in the human diet. However, deficiency remains a critical global problem causing detrimental neurological effects and cardiovascular problems particularly among populations that rely on a single crop for sustenance, e.g. polished rice (Fitzpatrick et al., 2012). Thiamine is formed by the condensation of the two separately biosynthesized heterocycle moieties (hydroxyethylthiazole [HET] and hydroxymethylpyrimidine [HMP], respectively; Supplemental Fig. S1A) and is later diphosphorylated to the cofactor form, TDP (for review, see Fitzpatrick and Thore, 2014). Several previously elusive steps of thiamine biosynthesis de novo have been resolved recently, one of which involves an unexpected suicide mechanism, where the catalytic protein donates sulfur to the molecule in a single turnover reaction (Chatterjee et al., 2011; Supplemental Fig. S1A). Remarkably, the majority of the enzymes involved in biosynthesis de novo are exclusively found in the chloroplast, the exception being the kinase that diphosphorylates thiamine to TDP, which is in the cytosol (Ajjawi et al., 2007a). Accordingly, the thiamine biosynthetic genes are strongly expressed in green tissues, but only at a very low level (if at all) in other nonphotosynthetic tissues such as roots (Colinas and Fitzpatrick, 2015). However, thiamine is essential in all actively dividing meristematic stem cells and organ initial cells (Wightman and Brown, 1953) and thus in nonphotosynthetic organs such as the roots. It is therefore relevant that a physiological study in 1974

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T.B.F. conceived the study; all authors designed and performed various aspects of the research, as well as analyzed and interpreted the data; T.B.F. wrote the article with assistance from J.M., E.G.-P., and N.S.

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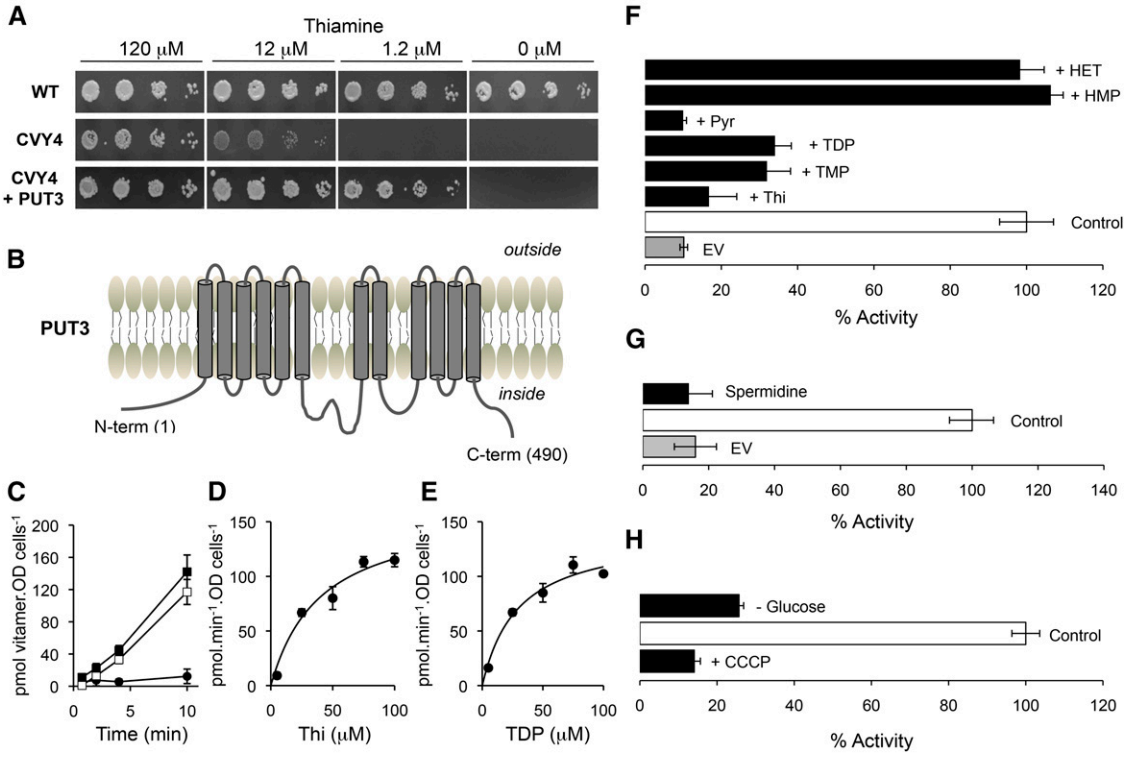

Figure 1. Identification of an Arabidopsis locus that mediates transport of thiamine in yeast. A, Complementation of *Saccharomyces cerevisiae* CVY4 that is unable to biosynthesize or transport thiamine with the Arabidopsis locus At5g05630 (*PUT3*). Pictures were captured after 3 d of growth on synthetic dextrose medium supplemented with the indicated thiamine concentrations. The individual rows of yeast growth represent 10-fold serial dilutions of the wild type (WT; BY4742), CVY4, or CVY4 transformed with *PUT3*. B, Cartoon of the predicted topology of PUT3 as determined by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). C, Time course of thiamine (black squares) or TDP (white squares) transport (5 μM each) by *S. cerevisiae* CVY3 harboring PUT3 in citrate-phosphate buffer, pH 5.5. *S. cerevisiae* CVY3 transformed with the empty vector serves as control (black circles). D, Representative Michaelis-Menten plot, $f(x) = V_{max} \times X/(K_M + X)$, of the rates of thiamine (Thi) uptake as a function of the indicated concentrations of substrate. E, As in D but rates are as a function of the indicated concentrations of TDP. F, Competition studies with the thiamine precursor HET or HMP, the antivitamin pyrithiamine (Pyr), or B_1 vitamer thiamine, TMP, or TDP. TDP uptake activity was determined at a concentration of 5 μM in the presence of a 20-fold excess of competitor. Activities are expressed as a percentage (% Activity) compared with the activity measured with TDP alone (Control). EV, Empty vector. G, As in F but measured with Spd as the competitor. H, Energy requirement of PUT3 uptake activity in yeast. The assays were performed as described in C in the absence or presence of 1% (w/v) D-Glc (Control). The protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to a final concentration of 100 μM 2 min before the labeled substrate. The data suggest that PUT3 is a proton symporter. For C to H, values shown are the mean ± SD from ≥ three independent experiments.

(Kruszewski and Jacobs, 1974) showed that thiamine moves through petiolar sections of tomato with strong basipetal polarity and with kinetics similar to auxin and GA (at a velocity of 3–5 mm per hour). Indeed, in the 1940s, thiamine was considered to fulfill the requirements of a hormone in higher plants (Bonner, 1940, 1942). This was due to the observation that to grow aseptically, excised roots of many species require thiamine in small amounts. Moreover, such observations were considered as confirmation of a hypothesis that in the intact plant, thiamine is biosynthesized in the leaves and moves from there to the roots, which cannot biosynthesize it sufficiently but need it for development. As thiamine and its phosphorylated derivatives (commonly referred to as vitamin B_1) are charged molecules at physiological pH, the existence of transporters that can mediate their trafficking therefore become apparent. Although the characterization of a homolog of the yeast mitochondrial transporter was reported recently (Frelin et al., 2012), it remains uncharacterized in planta, while the proteins participating in long-distance transport of thiamine in plants have remained elusive.

Analogous to thiamine, polyamines are charged molecules at physiological pH essential for the growth and survival of all organisms. The diverse range of activities include regulation of cell division, developmental processes such as those of root formation and flowering initiation, as well as environmental stress responses (Alcázar et al., 2010; Kumar et al., 1997; Kusano et al., 2008). Key representative molecules include putrescine

(Put), spermidine (Spd), and spermine (Spm), all three of which are derived from the amino acid Arg via the intermediate agmatine (Agm), as well as cadaverine (Cad) that is derived from Lys (Supplemental Fig. S1B). Akin to other essential multifunctional plant metabolites, polyamine contents must be carefully controlled to maintain cellular homeostasis. This is achieved through biosynthesis, degradation, conjugation (to phenolic acids, proteins, nucleic acids, and membrane structures), and transport (Martin-Tanguy, 2001). Although polyamine metabolism has been studied extensively, little is known of its transport in plants with the exception of the recently identified PUT family (Fujita et al., 2012; Mulangi et al., 2012) and the OCT1 transporter from Arabidopsis (*Arabidopsis thaliana*) that is implicated in Cad transport (Strohm et al., 2015). The PUT family of proteins is localized at the cellular level to various compartments, including the plasma membrane, Golgi apparatus, and endoplasmic reticulum (Fujita et al., 2012; Li et al., 2013). However, polyamine homeostasis is also regulated by long-distance transport, and indeed their presence in the vascular system of plants has been established in several species (Friedman et al., 1986; Pommerrenig et al., 2011; Sood and Nagar, 2005; Yokota et al., 1994). To date nothing is known on the components of intercellular or long-distance transport of polyamines.

Here, a yeast-based screen allowed us to identify Arabidopsis PUT3 as a thiamine transporter. Expression studies showed that PUT3 is localized to the phloem tissue in Arabidopsis, and loss-of-function studies showed that *PUT3* is required for growth and development. A series of micrografting and biochemical experiments demonstrated that *PUT3* is important for long-distance transport of both thiamine and polyamines and thereby revealed its importance for homeostasis of these essential molecules and maintenance of plant fitness.

RESULTS

Identification of an Arabidopsis Thiamine Transporter

This study was initiated by performing a complementation screen employing a cDNA library from Arabidopsis (Minet et al., 1992) with the yeast mutant CVY4, deficient in both the biosynthesis and the transport of thiamine (Vogl et al., 2008). This strain grows either in the presence of strong thiamine supplementation (120 μM) or can be complemented with the yeast thiamine biosynthesis gene *THI4* (Vogl et al., 2008). In line with this, several clones harboring the ortholog of the latter from Arabidopsis (THI1) were found to complement the growth phenotype even in the absence of thiamine supplementation in our screen. On the other hand, a single Arabidopsis locus (At5g05630) encoding a protein belonging to the amino acid permease family was found to restore growth in the presence of low levels of thiamine (1.2 μM), thus complementing the impairment in thiamine transport in this yeast strain (Fig. 1A). This protein has been previously reported to facilitate the uptake of polyamines in a yeast-based study and was accordingly named POLYAMINE UPTAKE TRANSPORTER3 (PUT3; Mulangi et al., 2012), and mediated paraquat uptake in Arabidopsis for which it was given the name RESISTANT TO METHYL VIOLOGEN1 (RMV1; Fujita et al., 2012). The *PUT3/RMV1* locus, which we will refer to as *PUT3* from here on, encodes a protein of 490 amino acid residues and is predicted to have 12 transmembrane domains with the N- and C-terminal hydrophilic domains residing on the inside of the membrane (Fig. 1B). It has four paralogs in Arabidopsis, At1g31820 (68% identity), At1g31830 (63% identity), At3g19553 (52% identity), and At3g13620 (43% identity), respectively. None of the latter paralogs were found in our screen and presumably cannot transport thiamine (at least in yeast) under the conditions used. Additional close homologs of *PUT3* were identified in several plants, including *Vitis vinifera* (grapevine), *Populus trichocarpa* (poplar), *Oryza sativa* (rice), *Zea mays* (corn), *Sorghum bicolor* (*Sorghum*), as well as *Physcomitrella patens* (moss), while weaker homologs could be identified in *Chlorella variabilis* (green algae). Notably, an extensive recent phylogenetic study hypothesized that this family of transporters was acquired by horizontal transfer from green algae (Mulangi et al., 2012).

Kinetics of PUT3-Facilitated Thiamine Transport

The kinetics of thiamine transport by PUT3 in yeast was characterized using the tritiated vitamers [^{3}H]thiamine and [^{3}H]TDP. Note, vitamers are related chemical substances that fulfill the same specific vitamin function. Uptake occurred at an optimal pH of 5.5 (Supplemental Fig. S2A). Both vitamers could be transported at similar rates (Fig. 1C). In addition, the vitamers displayed similar Michaelis-Menten parameters (V_{max} 169.3 $\pm$ 12.3 pmol min^{-1} OD cells^{-1}, K_M 43.3 $\pm$ 7.7 μM for thiamine; and V_{max} 146.5 $\pm$ 13.7 pmol min^{-1} OD cells^{-1}, K_M 34.2 $\pm$ 8.6 μM for TDP, respectively; Fig. 1, D and E). Competition experiments showed that B_1 vitamers TDP, thiamine monophosphate (TMP), and thiamine were able to compete for TDP transport, whereas HET and HMP (the thiamine biosynthesis precursors) were not (Fig. 1F). Interestingly, the thiamine antagonist pyrithiamine also competed with TDP uptake (Fig. 1F). As anticipated, the polyamine Spd, shown previously to be transported by PUT3 in a yeast based assay (Mulangi et al., 2012), competed strongly with TDP transport (Fig. 1G), indicating that thiamine uses the same transport pathway as polyamines. The withdrawal of Glc or addition of the protonophore carbonylcyanide-3-chlorophenylhydrazone abolishes the transport activity, suggesting that transport occurs via H^+ symport (Fig. 1H). Since PUT3 was originally annotated as a putative amino acid transporter (Jack et al., 2000), we also tested for competition of TDP uptake in the presence of unlabeled cyclic amino acids, but none showed significant modulation of transport (Supplemental Fig. S2B). In the same context and as the human thiamine transporter belongs to the folate transporter family (Zhao and

Goldman, 2013), we also tested folate, but it did not compete for TDP transport (Supplemental Fig. S2B). Similar results were obtained when the competition studies were performed with thiamine instead of TDP. It can therefore be concluded that PUT3 is a polyspecific transporter than mediates thiamine as well as polyamine transport, at least in yeast.

PUT3 Is Localized to the Phloem and Mediates Transport of B_1 Vitamers

Given the demonstrated ability of PUT3 to import thiamine/TDP in yeast, we wanted to establish its localization in planta, to complement the previous cellular localization studies of this protein (Fujita et al., 2012). To this end, we generated plants expressing the *GUS* reporter gene under control of the 850-bp region immediately upstream of the transcriptional start site of *PUT3* (*pPUT3-GUS*). Representative pictures of 20 independent transgenic lines analyzed are shown in Figure 2A. In all lines examined, *PUT3* promoter-driven GUS activity was predominant in the vascular tissue of leaves and the central cylinder of roots. In particular, two strands of GUS activity could be observed in the central stele of the differentiation region of the root. Moreover, transverse sections of the root tissue established localization to the phloem tissue (Fig. 2B). Weak GUS activity was also observed in the anthers of flowers, as well as the base and tip of mature siliques (Supplemental Fig. S3). At the subcellular level, fusion of the full-length PUT3 protein sequence to YFP and examination of the corresponding fluorescence upon transient expression in Arabidopsis mesophyll protoplasts confirmed the exclusive location of this protein to the plasma membrane (Supplemental Fig. S4), in agreement with a previous report (Fujita et al., 2012).

Since B_1 vitamers need to be translocated from sites of biosynthesis (source) to sites where they cannot be biosynthesized but are required (sink), we were prompted to investigate if they are found in the phloem sap. Indeed, the three B_1 vitamers could be detected in the phloem sap of Arabidopsis (Fig. 3A) and thereby imply that a transport system is in place for translocation into this tissue. It is worth noting that the ratio between TDP and either TMP or thiamine in the phloem sap is drastically reduced when compared with extracts from whole rosette leaves (Supplemental Fig. S5). To test the contribution of PUT3 to the vitamin B_1 transport process, the B_1 vitamer profiles of phloem exudates from wild-type plants were compared with that of the previously characterized T-DNA insertion mutant line *rmv1* (GT_3_3436; Fujita et al., 2012), which is equivalent to a *put3* mutant. Down-regulation of *PUT3* expression was confirmed by real-time quantitative PCR (Supplemental Fig. S6). Indeed, the total vitamin B_1 content was decreased in the *rmv1* mutant phloem exudates compared with that of the wild type (Fig. 3B). Importantly, B_1 vitamer contents were restored to wild-type levels in phloem exudates of *rmv1* carrying the *PUT3* transgene under control of its own promoter (Fig. 3B, *rmv1/pPUT3:PUT3*). These observations suggest that *PUT3* is involved in transport of vitamin B_1 in the phloem.

PUT3 Also Mediates Phloem Transport of Polyamines

The localization of PUT3 to the phloem tissue and its previous reported ability to facilitate polyamine transport, at least in yeast (Mulangi et al., 2012), prompted us to next investigate the polyamine profiles of phloem exudates from Arabidopsis. In particular, we adapted a protocol (Fontaniella et al., 2001) to profile the diamines Cad and Put, the triamine Spd, the tetramine Spm, as well as the Put/Spd/Spm precursor Agm. All of these compounds could be detected in phloem exudates from Arabidopsis (Fig. 4A). Put was the most abundant polyamine detected followed by Spd and Cad, while Spm accumulated at much lower levels (Fig. 4B). Significantly, there was a considerable decrease in the levels of Put, in particular, in phloem exudates of *rmv1* (Fig. 4B). Importantly, the polyamine profile was restored to that of the wild type in phloem exudates of *rmv1* carrying the *PUT3* transgene under control of its own promoter (Fig. 4B, *rmv1/pPUT3:PUT3*), validating

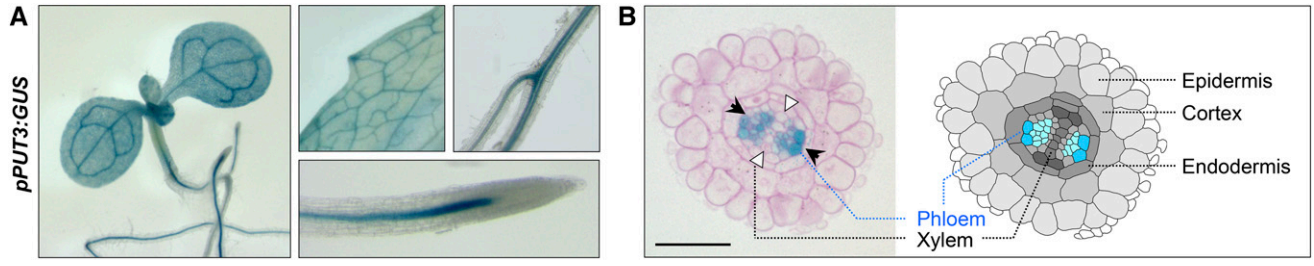

Figure 2. PUT3 is localized to the phloem. A, Histochemical localization of GUS activity in Arabidopsis. Representative images of Arabidopsis expressing *GUS* under the control of the promoter of *PUT3*: 7-d-old whole seedling (left), enlarged image of a mature rosette leaf margin (top middle), differentiation zone of a mature root (top right), and 7-d-old seedling root tip (bottom right). B, Transverse section of the root of a *GUS*-stained transgenic seedling (left). Arrows point to the position of the phloem, whereas arrowheads indicate the position of the xylem axis. The scale bar represents 50 μm. A scheme of the cross-sectional layers observed is illustrated on the right.

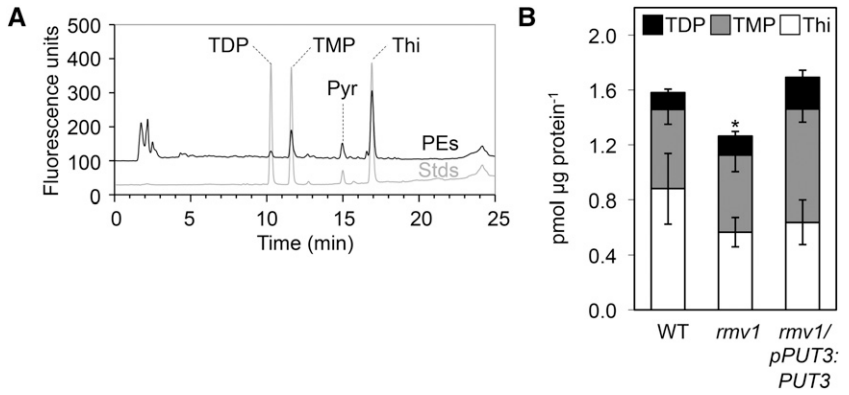

Figure 3. Transport of B_1 vitamers in the phloem requires PUT3. A, Representative HPLC profile of B_1 vitamers in phloem exudates (PEs) collected from Arabidopsis leaf petioles. The commercially available B_1 standards (Stds) are TDP, TMP, and thiamine (Thi). The chemical derivative pyrithiamine (Pyr) was used as an internal standard. Two pmol of each compound was injected. B, Quantification of the B_1 vitamer content of phloem exudates of *rmv1* and the complementation line *rmv1/pPUT3:PUT3* versus the wild type (WT). Values are the average of four biological replicates with error bars representing SD. Statistically significant changes for total B_1 vitamers compared with the wild type were calculated by a two-tailed Student's *t* test for $P < 0.05$ and are indicated by an asterisk.

the role of *PUT3* in this molecular phenotype. Taken together, these results imply that *PUT3* is required for transport of both polyamines and vitamin B_1 in phloem tissue.

Alterations in Growth and Developmental Processes Are Associated with *PUT3*

As B_1 vitamers and polyamines are essential for development in plants, we next examined the phenotypic consequences of loss of function of *PUT3* on plant growth. Notably, previous studies on *PUT3* in Arabidopsis were restricted to quantifying its association with paraquat resistance (Fujita et al., 2012). Interestingly, here we observed that the *rmv1* mutant showed stunted growth under standard growth conditions that was not reported on in previous studies (Fig. 5A) and was corroborated by a slower rate of root growth compared with the wild type (Fig. 5B). Furthermore, *rmv1* entered the reproductive phase later than the wild type (Fig. 5C), bolting at day 67 instead of day 62 (Fig. 5D), concomitant with an increase in the number of rosette leaves at this developmental stage (Fig. 5, E and F). The observed phenotypes were most pronounced under an 8-h photoperiod and were significantly attenuated under longer photoperiods (data not shown). All of the growth and developmental phenotypes were abrogated in *rmv1* upon introduction of the *PUT3* transgene (Fig. 5, A–F, *rmv1/pPUT3:PUT3*). It must therefore be concluded that *PUT3* is required for proper growth and development in Arabidopsis.

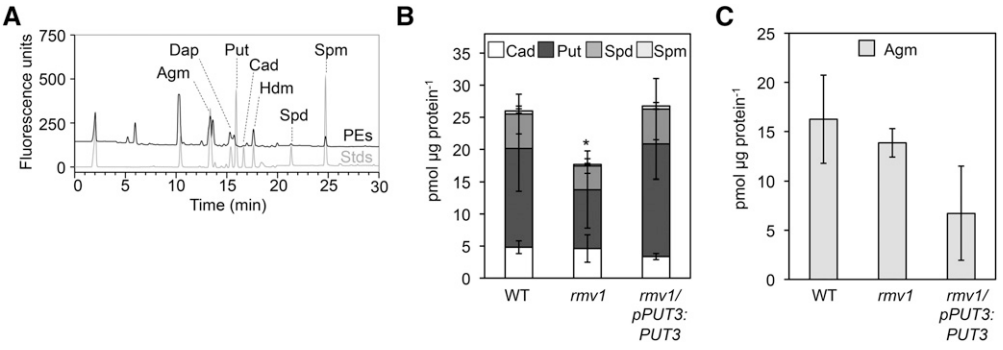

Figure 4. Transport of polyamines in the phloem requires PUT3. A, Representative HPLC profile of polyamines in phloem exudates (PEs) collected from Arabidopsis leaf petioles. The commercially available standards (Stds) used were Put (8 pmol), Cad (2 pmol), Spd (40 pmol), Spm (4 pmol), the polyamine precursor Agm (20 pmol), and the degradation product 1,3-diaminopropane (Dap; 8 pmol). Hexamethylenediamine (Hdm; 2 pmol) was used as an internal standard. B, Quantification of the polyamine contents of phloem exudates from *rmv1* and the complementation line *rmv1/pPUT3:PUT3* versus the wild type (WT). C, Quantification of the precursor Agm. The degradation product 1,3-diaminopropane could not be detected. The values for B and C are the average of four biological replicates with errors bars representing SD. Statistically significant changes for total polyamines compared with the wild type were calculated by a two-tailed Student's *t* test for $P < 0.05$ and are indicated by an asterisk.

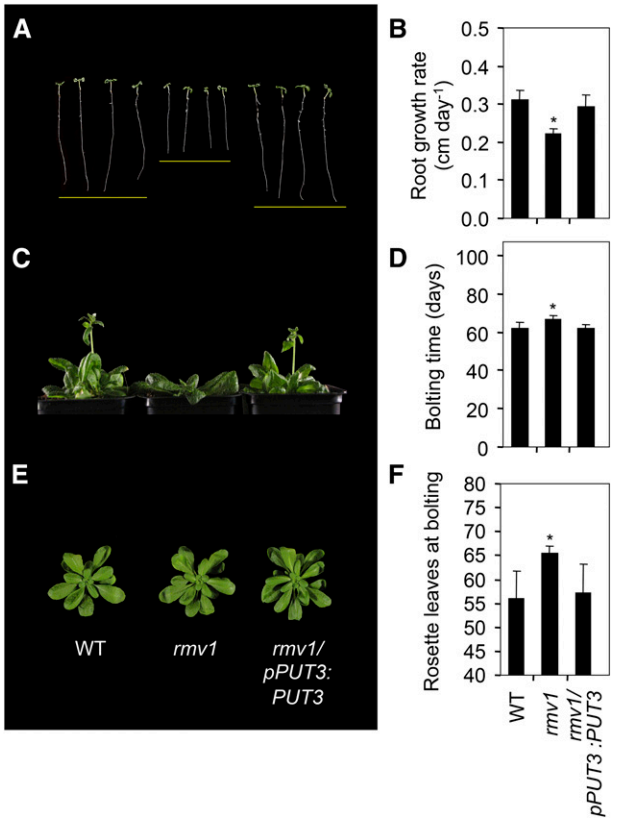

Figure 5. *PUT3* is required for proper root and timely reproductive development in Arabidopsis. A, Representative photographs of 12-d-old seedlings illustrating root growth impairment of *rmv1* compared with the respective wild type (WT) growing vertically in culture. Complementation of the phenotype is observed in transgenic line *rmv1/pPUT3*:*PUT3*. B, Root growth rate of the same lines shown in A. C, Photographs of the plant lines as in A grown on soil 65 d after germination, illustrating the late bolting phenotype of *rmv1* compared with the wild type but recovers in *rmv1/pPUT3*:*PUT3*. D, Bolting time of soil grown lines as indicated. E, Representative photographs of the lines as indicated during the late vegetative stage. Pictures were captured 55 d after germination. F, Number of rosette leaves at the time of bolting, illustrating the increased number of leaves in *rmv1* but returns to that of the wild type in *rmv1/pPUT3*:*PUT3*. In the case of B, D, and F, values shown are the mean ± SD from ≥ three independent experiments. Statistically significant changes compared with the wild type were calculated by a two-tailed Student's *t* test for $P < 0.05$ and are indicated by an asterisk. In all cases, plants were grown under an 8-h photoperiod (150 μmol photons m^{-2} s^{-1} at 22°C) and 16 h of darkness at 18°C.

Long-Distance Transport of a Shoot-Derived Metabolite by PUT3 Is Responsible for the Root Development Phenotype in *rmv1*

To further probe the growth and development phenotype of *rmv1* and the possible physiological relevance of *PUT3* in long-distance transport, we performed a series of grafting experiments. Notably, grafted plants with the same genetic combinations exhibited the same phenotypes as nongrafted plants, i.e. grafting of an *rmv1* scion onto its corresponding rootstock (*rmv1*/*rmv1*) led to retention of the stunted growth phenotype compared with grafting of the wild-type scion onto its corresponding rootstock (wild type/wild type; Fig. 6). Remarkably, grafting of the wild-type scion onto the *rmv1* rootstock (wild type/*rmv1*) led to recovery of the stunted root growth phenotype to that of the corresponding grafted wild-type plants (wild type/wild type; Fig. 6). On the other hand, the reciprocal graft of the *rmv1* scion onto the wild-type rootstock (*rmv1*/wild type) did not lead to recovery of root growth impairment (Fig. 6). Indeed, root growth was even further impaired than *rmv1*/*rmv1*. These data support the notion that *PUT3* is required for long-distance transport of a shoot-derived metabolite that is essential for proper root growth.

Shoot-to-Root Partitioning of Vitamin B_1 as a Function of *PUT3*

Given the implication of PUT3 in long-distance transport, we next sought to obtain a molecular explanation for the growth impairment phenotypes observed. In the first instance, we therefore examined shoot and root partitioning of B_1 vitamers in seedlings. Intriguingly, B_1 vitamer levels in shoots and roots (albeit to a much lesser extent) of *rmv1* were lower than those of the wild type (Fig. 7A). While the slight depletion in root tissue (sink tissue) is consistent with the role of *PUT3* in long-distance transport of B_1 vitamers from source to sink, we expected a corresponding accumulation of B_1 vitamers in shoots (source tissue) rather than a depletion. Interestingly, *rmv1* does not show the well-characterized pale shoot phenotype associated with a deficiency in B_1 vitamers (Ajjawi et al., 2007a, 2007b; Raschke et al., 2007). Nonetheless, levels of B_1 vitamers in shoots and roots of *rmv1* recovered upon reintroduction of the *PUT3* transgene (Fig. 7A). To provide evidence that shoot-derived vitamin B_1 is important for root growth in particular, another series of grafting experiments was performed with the Arabidopsis mutant line *th1-201* (Ajjawi et al., 2007b). In this line, the THIAMIN SYNTHASE enzyme TH1 (encoded at locus At1g22940), which condenses the

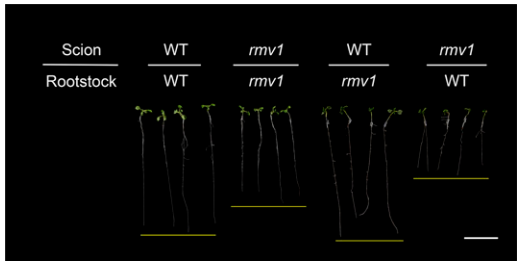

Figure 6. Micrografting illustrates the importance of long-distance transport mediated by PUT3. Representative pictures of micrografts of scion and rootstock of the wild type (WT) and *rmv1* genetic combinations as indicated. Pictures were captured 7 d after the procedure from micrografts grown vertically under 150 μmol photons m^{-2} s^{-1} for 8 h at 22°C and 16 h dark at 18°C. The scale bar represents 1 cm.

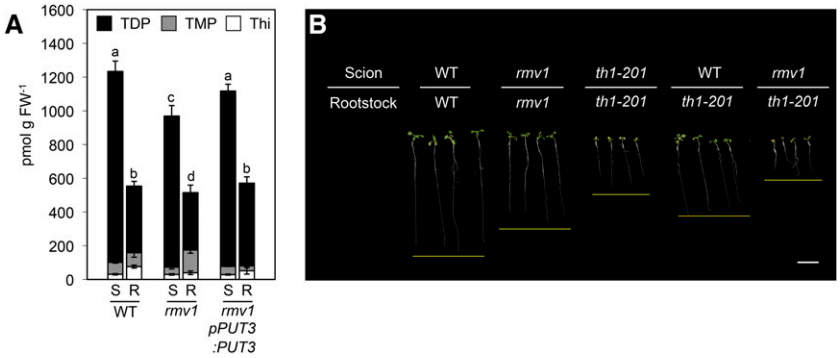

Figure 7. PUT3 is required for long-distance transport of vitamin B_1. A, B_1 vitamer levels in shoots (S) and roots (R) of 14-d-old seedlings of *rmv1* compared with the wild type (WT) and the transgenic line *rmv1/pPUT3:PUT3*. The vitamers measured are TDP, TMP, and thiamine (Thi). Values shown are the mean ± SD from ≥ four independent experiments. Significant differences of total B_1 vitamer contents were determined by one-way ANOVA ($P < 0.05$). B, Micrografting of the wild type (WT), *rmv1*, and the thiamine biosynthesis mutant *th1-201* as indicated. Pictures were captured 7 d after the procedure from micrografts grown vertically under 150 μmol photons m^{-2} s^{-1} for 8 h at 22°C and 16 h dark at 18°C. The scale bar represents 1 cm.

thiazole and pyrimidine heterocycles to form TMP, is impaired, preventing thiamine biosynthesis de novo and resulting in overall stunted shoot and root growth and pale leaves. Remarkably, grafting of the wild-type scion onto the *th1-201* rootstock (wild type/*th1-201*) led to recovery of the stunted root growth phenotype that is observed with *th1-201* (Fig. 7B). However, grafting of the *rmv1* scion onto the *th1-201* rootstock (*rmv1*/*th1-201*) did not lead to recovery of the short-root phenotype in *th1-201*. Indeed, root growth was even more impaired than for the *th1-201*/*th1-201* genetic combination, corroborating the notion that loss of function of *PUT3* in the shoot, in particular, has a negative impact on root growth (Fig. 7B). Taken together, the data demonstrate that long-distance transport of B_1 vitamers from the shoot is necessary for root growth and that impairment of *PUT3* in the shoot has a negative impact on root growth.

Shoot-to-Root Partitioning of Polyamines as a Function of *PUT3*

Given that PUT3 is also implicated in polyamine transport, we next looked at shoot-to-root partitioning of polyamine contents. In the first instance, we noted that the total polyamine content is very similar between shoots and roots in wild-type plants (Fig. 8A). A significant reduction of total polyamine levels was observed in the roots of *rmv1*, whereas shoot content remained similar to that of the wild type (Fig. 8A). Notably, levels of root polyamines recovered upon reintroduction of the *PUT3* transgene in *rmv1* (Fig. 8A). Intriguingly, a closer look at the polyamines measured indicates that the predominant polyamine in shoots and roots is Spd and that it is mainly this polyamine that is depleted in roots of *rmv1* (Fig. 8, B–E). This is in contrast to the polyamine profile of phloem exudates, where Put was the predominant polyamine detected and the one most depleted in *rmv1* (Fig. 4B).

DISCUSSION

A fundamental understanding of phloem transport with its important role in plant development, reproduction, signaling, and growth is central to plant biology. This is further emphasized by the core set of compounds, such as biological cofactors and growth regulators, required in all living cells to drive key metabolic processes, the biosynthesis of which may be restricted to certain organs and therefore must be transported to the site of requirement. As central components of metabolic function, the supply of many of these molecules must be strictly regulated, such that provision is coordinated with metabolic needs. However, despite their critical roles, surprisingly little is known on the intercellular transport of such molecules in plants. In this study, we demonstrated the importance of long-distance transport of vitamin B_1 and polyamines as mediated by the phloem-localized transporter PUT3. Disruption of PUT3 led to a deficit of B_1 vitamers and polyamines in phloem exudates that perturbed the homeostasis of these compounds in the organs where they are required, resulting in a negative impact on growth and development in Arabidopsis.

Long-distance transporters of the vital cofactor vitamin B_1 have been supposed for almost a century (Bonner, 1940, 1942), yet the proteins carrying out these processes and evidence for the presence of this family of compounds in the vascular system have remained elusive. Only a single transporter has been reported for vitamin B_1 in plants and operates at the subcellular level in the mitochondrial membrane (Frelin et al., 2012). Polyamines, on the other hand, were detected in the phloem sap several decades ago (Friedman et al., 1986). While they can be biosynthesized to a certain extent in the phloem (Pommerrenig et al., 2011), the factors facilitating transport of these important charged molecules in the vascular system to maintain homeostasis were unknown. Here, we have demonstrated the involvement of *PUT3* in these transport processes in plants. Although *PUT3* was previously implicated in subcellular polyamine transport

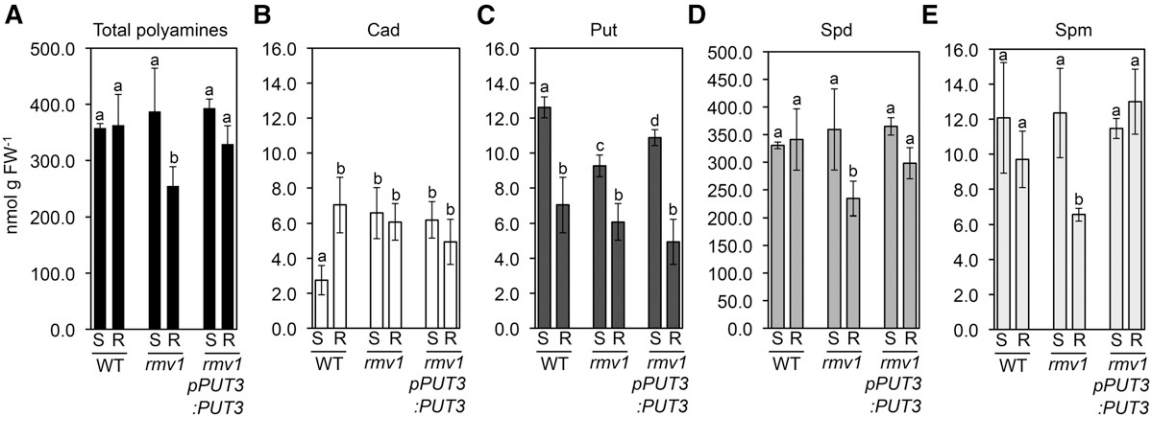

Figure 8. PUT3 is required for long-distance transport of polyamines. A, Total polyamine levels in shoots (S) and roots (R) of 14-d-old seedlings of *rmv1* compared with the wild type (WT) and the transgenic line *rmv1/pPUT3:PUT3*. B to E, Levels of the individual polyamines Cad, Put, Spd, and Spm of the same samples shown in A. The values shown are the mean ± SD from four biological replicates. Significant differences for each polyamine were determined by a one-way ANOVA ($P < 0.05$). In all cases, plants were grown under an 8-h photoperiod (150 μmol photons m^{-2} s^{-1} at 22°C) and 16 h of darkness at 18°C.

based on a yeast complementation assay (Mulangi et al., 2012) as well as paraquat transport in Arabidopsis (Fujita et al., 2012), it went unnoted that it is localized to the phloem and mediates long-distance transport. Here, we have shown that the affinity of PUT3 for B_1 vitamers is similar to that reported for polyamines and paraquat, i.e. low μM range (Fujita et al., 2012), classifying it as a polyspecific high-affinity cationic transporter. Indeed, exogenous thiamine reportedly enhances tolerance to paraquat (Tunc-Ozdemir et al., 2009), supporting the notion that all of these compounds use the same trafficking pathway. The functional significance of *PUT3* for phloem transport of B_1 vitamers and polyamines became evident from the deficit of these compounds in the phloem exudates of the *PUT3* mutant line *rmv1*, previously characterized because of its resistance to paraquat (Fujita et al., 2012). The implication of *PUT3* in these transport processes is validated upon restoration of the B_1 vitamer and polyamine contents in phloem exudates of the transgenic *rmv1* line expressing *PUT3* under the control of its own promoter sequence (*rmv1/pPUT3:PUT3*). We acknowledge that the EDTA-based method employed here for the collection of phloem sap has well-documented pitfalls (Turgeon and Wolf, 2009), including that the procedure requires the cutting of a surface and extended exposure of cells to EDTA causes damage, leakage from which may contaminate the sap. Therefore, we chose the EDTA-facilitated phloem exudation method described by Tetyuk et al. that has been optimized for Arabidopsis (Tetyuk et al., 2013), in which the samples are exposed to EDTA for 1 h and washed with water, followed by collection of the phloem exudate into water. This strives to minimize cell damage and downstream interference from EDTA, while removing compounds that have accumulated from damaged or cut cells. Indeed, assuming no metabolite degradation occurs during the procedure, the divergent B_1 vitamer and polyamine profiles of phloem exudates versus tissue extracts could serve to support the predominant recovery of metabolites being transported in the phloem with this method rather than that from damaged cells. Moreover, the collection of material directly into water without prior EDTA treatment was free of B_1 vitamers and polyamines and used as a negative control. Nonetheless, the use of another method designed to exclusively measure mobile elements in the phloem sap could serve to validate the findings.

As vitamin B_1 biosynthesis de novo is predominantly limited to photosynthesizing tissue (for review, see Colinas and Fitzpatrick, 2015), it can be suggested that PUT3 activity in its capacity as long-distance B_1 vitamer transporter is mainly restricted to shoot tissue. On the one hand, the requirement for shoot-to-root long-distance transport of vitamin B_1 is supported by the recovery of root growth in the biosynthesis mutant *th1-201* upon grafting of a wild-type scion. On the other hand, the failure to restore root growth upon grafting a *PUT3* mutant scion (*rmv1*) onto *th1-201* rootstock demonstrates the important role of *PUT3* in this process. In line with this, we predict that *PUT3* is involved in phloem loading of B_1 vitamers and is supported first by the decrease in B_1 vitamer contents in phloem exudates and second by the decrease (albeit slight) in the root tissue of *rmv1*. We noted above that the ratio between TDP and either TMP or thiamine in the phloem sap is drastically reduced when compared with extracts from whole rosette leaves. Therefore, either a selective transport system takes place in the leaves for phloem loading, and/or vitamers can be interconverted within the phloem tissues by phosphatases that may be nonspecific. The deficit in shoot tissue B_1 vitamer contents of *rmv1* cannot be explained at present and requires further investigation. Nonetheless, recovery toward wild-type shoot-to-root partitioning of B_1 vitamers is observed in the transgenic *rmv1/pPUT3:PUT3* line, implicating *PUT3* in the process. The retention of

considerable levels of vitamin B_1 in the roots even in the absence of *PUT3* suggests that more components of long-distance transport or perhaps salvaging of the vitamin in the root remain to be discovered in future studies. As noted previously, *PUT3* has four additional homologs in Arabidopsis, which although not found in our yeast screen may add a level of redundancy to this transporter family and should be assessed for their contribution to the transport of vitamin B_1 in planta. Interestingly, the Arabidopsis *PUT* family appears to have a divergent pattern of tissue expression as assessed by reverse transcription PCR (Mulangi et al., 2012), as well as divergent intracellular localization patterns (Li et al., 2013), which may suggest specialization in a spatial manner. Notwithstanding, the small deficit of B_1 vitamers observed in *rmv1* may contribute to the stunted root phenotype but is likely to have a stronger contribution from the disruption in polyamine homeostasis (see below).

In contrast to vitamin B_1, polyamines can be biosynthesized and interconverted ubiquitously in tissues (Urano et al., 2003), including that of the phloem (Pommerrenig et al., 2011). Nonetheless, their involvement in diverse cellular activities warrants that their content is carefully regulated to maintain cellular homeostasis. Intercellular transport is an important part of this process and is validated here through the characterization of the role of PUT3 in phloem transport of polyamines. As for the B_1 vitamers, *PUT3* can be implicated in phloem loading of polyamines based on the observation that the total polyamine content decreased in phloem exudates of the *PUT3* mutant *rmv1* and were restored to wild-type levels in the transgenic line *rmv1/pPUT3:PUT3*. Notably, a closer look at the individual polyamines revealed that Put is the most predominant polyamine in phloem exudates and was considerably decreased along with its polyamine derivatives, Spd and Spm, in the *rmv1* mutant line. Therefore, PUT3 may either load all three polyamines into the phloem or it may have a preference for Put, which can be metabolized to Spd and Spm by the corresponding synthases in the phloem. On the other hand, there is no change in the levels of Cad in phloem exudates of *rmv1* compared with the wild type, implying that PUT3 does not transport this polyamine. This would be consistent with the recent implication of OCT1 in the transport of Cad (Strohm et al., 2015) but remains to be verified in planta.

The striking reduction in total polyamine content in the root tissue of *rmv1* can be used to provide a molecular explanation for the stunted root growth observed. It has long been demonstrated that polyamines play a major role in cell proliferation and cell division (Fariduddin et al., 2013; Galston and Kaur-Sawhney, 1987; Jang et al., 2002; Kaur-Sawhney et al., 2003). Moreover, alterations in polyamine levels can be correlated with modifications in root development. In particular, increased and decreased levels of the polyamine Spd translate to enhanced and stunted root growth, respectively (Hummel et al., 2002; Jang et al., 2002; Watson et al., 1998). Spd was by far the most abundant polyamine found in roots of Arabidopsis in this study, having been found at approximately 20-fold higher levels than Put, Spm, or Cad. Notably, this is in contrast to phloem exudates, where Put was the most prevalent polyamine and is significantly reduced in *rmv1* but levels were restored to that of the wild type in *rmv1/pPUT3:PUT3*. Spd levels were strongly reduced in roots of *rmv1*, thereby contributing the most to the overall deficit in polyamines seen in the roots of this mutant. Spm levels are also reduced in root tissue of *rmv1*, which may be due either to reduced transport into the root or a consequence of the deficit in Spd as it is the precursor to Spm. Both Spd and Spm levels are restored to wild-type levels in the transgenic line *rmv1/pPUT3:PUT3*, as is the stunted growth phenotype. Taken together, we hypothesize from this study that Put is the predominant polyamine carried in the phloem by PUT3 and as the direct precursor to Spd and Spm may contribute to the pool of the latter polyamines in the root. Notably, there is no significant change in the levels of Put or Cad in root tissue of *rmv1* compared with the wild type.

It is striking on the other hand that Put levels decreased in shoots of *rmv1* and may account for the delayed bolting phenotype, as elevated Put levels serve as a marker for the transition to flowering (Applewhite et al., 2000; Havelange et al., 1996). Put levels recovered toward that of the wild type in the transgenic line *rmv1/pPUT3:PUT3* concomitant with the restoration of the bolting time. Cad levels were also increased in shoots of *rmv1* but appeared to be independent of *PUT3*, as homeostasis was not restored in *rmv1/pPUT3:PUT3*. It is unlikely that the reduced B_1 vitamer levels in the shoots of *rmv1* contribute to the delayed bolting time, as foliar application of thiamine had no effect on bolting time (data not shown). Taken together, the changes in polyamine homeostasis upon disruption of *PUT3* may account for the growth and developmental phenotypes observed in *rmv1*.

In summary, this study identifies a long-sought component of vitamin B_1 shoot-to-root translocation and highlights the necessity of maintaining the balance of two sets of vital metabolites, vitamin B_1 and polyamines as a function of *PUT3*, and the importance of their appropriate tissue distribution for proper growth and development in plants.

MATERIALS AND METHODS

Yeast Studies

The complementation screen was performed employing the *Saccharomyces cerevisiae* knockout mutant *CVY4* (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 thi7Δ::kanMX4 thi71Δ::LEU2 thi72Δ::LYS2 thi4Δ::his5*$^+$) deficient in the biosynthesis and transport of vitamin B_1 (Vogl et al., 2008) and that only grows in the presence of high concentrations of thiamine (≥120 μM). The strain was transformed with an Arabidopsis cDNA expression library (Minet et al., 1992), and transformants were selected on media containing 1.2 μM thiamine. *S. cerevisiae* BY4742 (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used as the wild-type strain. Synthetic dextrose medium lacking vitamin B_1 and uracil (Formedium) and containing 2% (w/v) Glc was used as the basic growth medium and supplemented with the indicated concentrations of thiamine. The cDNA corresponding to Arabidopsis *PUT3* was amplified using

the primer pairs listed in Supplemental Table S1 and after cloning by homologous recombination into *pGREG506-TEF* (Jansen et al., 2005) to generate *pGREG506-TEF-PUT3* was used for the analysis shown in Figure 1. Yeast uptake assays were carried out with strain *CVY3* (*thi10-1Δ*::*KanMX thi71Δ*::*LEU2 thi72Δ*::*LYS2*; Vogl et al., 2008) transformed with *pGREG506-TEF-PUT3* or *pGREG506-TEF* as control, essentially as described by Stolz and Vielreicher (2003). Notably, in contrast to strain *CVY4*, the *THI4* gene is not disrupted in strain *CVY3*, which can therefore grow in the absence of thiamine supplementation making it suitable for uptake studies. Briefly, cells were grown to midlogarithmic phase in SD-URA medium (Formedium) containing 2% (w/v) Glc, 0.67% (w/v) yeast nitrogen base and amino acids, washed twice in water, and resuspended in citrate-phosphate buffer at pH 5.5. Five hundred microliters of cells (at 0.5 OD_{600} units/mL) were incubated at 30°C and energized with 1% (w/v) Glc 1 min prior to starting the experiment. Then, 5 μL of a mixture of tritiated thiamine or TDP (both at 20 Ci $mmol^{-1}$; American Radiolabeled Chemicals) and unlabeled thiamine or TDP at 500 μM were added to give a final concentration of 5 μM (specific activity 0.1 nCi μL^{-1}). At given times, 90-μL aliquots were withdrawn, diluted with 5 mL of water, and filtered through GN-6 metricel membrane filters (0.45-μm pore size; Pall Corporation). After washing with excess water, the radioactivity in the filters was quantified in 4 mL of Emulsifier Safe scintillation cocktail (PerkinElmer) in a Beckman LS6500 liquid scintillation counter for 5 min. Uptake velocities were determined from the slope of the linear fit of time points covering a period of 10 min after substrate addition. The optimum pH of PUT3 was determined with citrate-phosphate buffers adjusted to the indicated values. Competitors or inhibitors were all used at 20-fold excess (100 μM final concentration), tested at pH 5.5, and added 30 s before starting the experiment. The K_M value was determined with substrate solutions containing a constant amount of labeled thiamine or TDP and various concentrations of unlabeled thiamine or TDP. The K_M value was calculated from a Michaelis-Menten plot. The energy requirement of thiamine uptake was determined in the absence or presence of 1% (w/v) Glc. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone was used at a final concentration of 100 μM. It was added 2 min before the labeled substrate and tested in assays that also contained 1% (w/v) D-Glc.

Generation of Plant Material and Growth Conditions

The mutant line used was GT_3.3436, recently annotated as *rmv1* (Fujita et al., 2012), obtained from the European Arabidopsis Stock Centre and isolated to homozygosity (primer pairs used are given in Supplemental Table S1), as well as its corresponding wild type (L*er*-1). The cloning of *PUT3* for plant transformation was carried out by amplification of the full-length sequence from cDNA using the primer pairs *PUT3.attB1.F/PUT3.attB2.R* (Supplemental Table S1). The resulting PCR product was used to generate the *pENTR221-PUT3* Gateway-compatible vector (Life Technologies). *PUT3* was recombined into the *pUBC-YFP-DEST* vector (Grefen et al., 2010) to generate *UBQ:PUT3-YFP*. The *UBQ10* promoter was replaced by an *Eco*RI/*Nco*I restriction/ligation with the 850-bp intergenic region upstream of the *PUT3* start codon, amplified from genomic DNA using the primer pairs *promPUT3:PUT3.EcoR1.F* and *promPUT3:PUT3.R*, indicated in Supplemental Table S1, to generate *promPUT3:PUT3-YFP* for complementation of *rmv1*. For *GUS* expression, the 850-bp region upstream of the *PUT3* start codon was amplified by PCR from genomic DNA using primer pairs *promPUT3.SalI.F* and *promPUT3.NcoI.R* inserted into the *pENTR/D-TOPO* vector (Life Technologies), followed by recombination upstream of the *GUS* coding sequence of *pMDC162* (Curtis and Grossniklaus, 2003), to generate *pPUT3:GUS*. *Agrobacterium*-mediated transformation of Arabidopsis by the floral dip method was used (Clough and Bent, 1998). For PUT3 subcellular localization, *UBQ:PUT3-YFP* was used. Transient expression in Arabidopsis mesophyll protoplasts was performed essentially as described by Szydlowski et al. (2013) using 3- to 4-week-old plants maintained on half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium (Duchefa) containing 1% (w/v) Suc grown under 100 μmol photons m^{-2} s^{-1} for 16 h at 22°C and 8 h dark at 18°C with some modifications: incubation in the enzyme solution was performed overnight, in the dark, and without agitation. Cells were allowed to recover 24 to 48 h before analysis by confocal laser scanning microscopy (Leica Microsystems) using an ArKr laser at 488 nm. YFP fluorescence was recorded between 503 and 550 nm. In all cases, transformants were selected on the appropriate antibiotic, either kanamycin or hygromycin (50 mg L^{-1}), followed by segregation analysis to isolate lines with a single insertion, and were confirmed by PCR analysis and sequencing (Microsynth). Unless stated otherwise, plants were grown either on soil or in sterile culture plates (after surface sterilization) on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) under standard growth conditions (150 μmol photons m^{-2} s^{-1} for 8 h at 22°C and 16 h dark at 18°C).

Gene Expression Analyses

Histochemical *GUS* expression analyses was carried out essentially as described by Boycheva et al. (2015). Images were captured with a Leica MZ16 stereomicroscope equipped with an Infinity 2 digital camera (Leica Microsystems). To visualize cross-sections of *GUS*-stained seedlings, they were fixed for 5 h at room temperature in 100 mM phosphate buffer (pH 7.2) supplemented with 4% (v/v) formaldehyde and 1% (v/v) glutaraldehyde, followed by extensive washing in phosphate buffer at 4°C, embedded in 1.5% (w/v) agarose, and dehydrated in a graded ethanol series (50%, 70%, 90% (v/v), and three times in 100% ethanol) in steps of 20 min each. Samples were then progressively infiltrated with Technovit 7100 resin (Heraeus) supplemented with 1% (w/v) hardener I (benzoyl peroxide) in three separate steps using different mixtures with ethanol at 50%, 33% (v/v), and 0%, respectively, then embedded in molds filled with embedding medium (1 part hardener II mixed with 11 parts infiltration medium), left for 30 min at room temperature and then at 60°C for an additional 30 min until complete polymerization. Serial cross-sections of 4-μm thickness were prepared using a Leica Ultracut UCT microtome (Leica Microsystems) equipped with glass knives. The sections were collected on superfrost glass slides and counterstained for cell walls with 0.05% (w/v) ruthenium red. Roots were observed using a Nikon eclipse 80i microscope, and pictures were taken with a Nikon Digital Camera Sight DS-Fi-1.

For quantification of RNA, total RNA was extracted from plant samples using the NucleoSpin RNA Plant Kit (Macherey-Nagel) following the manufacturer's protocols and treated with RNase-free DNase (Promega) to remove traces of DNA. RNA (2 μg) was reverse-transcribed into cDNA using the Superscript II Reverse Transcriptase and oligo$(dT)_{20}$ primers (Life Technologies) according to the manufacturer's recommendations and stored at −20°C. Quantification analyses were performed with a 7900 HT Fast Real-Time PCR system using the Power SYBR Green PCR Master Mix (Applied Biosystems). Relative mRNA abundance was calculated using the comparative Δ*Ct* method and normalized against the housekeeping gene *UBC21* (At5g25760). The list of primer pairs used is given in Supplemental Table S1.

Phloem Exudate Collection

Six-week-old Arabidopsis plants were used to collect phloem exudates essentially as described by Tetyuk et al. (2013) with minor modifications. Rosette leaf numbers 8 to 15 were used and exudates collected in doubly distilled water spiked with internal standard pyrithiamine or hexamethylenediamine for B_1 vitamer or polyamine determination, respectively, after EDTA treatment. Exudates were collected for 5 h and then lyophilized. Samples were resuspended in doubly distilled water for metabolite determination and normalized to the protein content (Bradford, 1976).

Determination of Vitamin B_1 and Polyamine Content

Determination of B_1 vitamer content was carried out as described by Moulin et al. (2013) with the exception that Arabidopsis tissues (50–100 mg) were used as indicated, extracted in 1% (v/v) trichloroacetic acid, and pyrithiamine was used as an internal standard. Soluble polyamines were extracted and their conjugates hydrolyzed as described by Fontaniella et al. (2001) with minor modifications. Briefly, free polyamines were isolated from plant extracts or phloem exudates by mixing with an equal volume of doubly distilled water and directly derivatized. Conjugated polyamines were first hydrolyzed to free polyamines by treatment with hydrochloric acid for 18 h at room temperature, and the pH was raised to ≅12. Derivatization was performed by mixing samples with two volumes of a saturated sodium carbonate solution, followed by four volumes of 7.5 mg mL^{-1} dansyl chloride in 98% (v/v) acetone and incubation in darkness for 1 h at 60°C, then supplemented with one volume of 100 mg mL^{-1} L-Pro and incubated for a further 30 min at room temperature. Five volumes of ethyl acetate were then added, centrifuged for 5 min, and the supernatant lyophilized. Samples were resuspended in methanol before analysis. Separation was performed on a C_{18} Ultrasphere column (250 mm × 4.6 mm, 2.5 μm; Hichrom) under the following conditions: Solvent A = doubly distilled water; Solvent B = 100% methanol; 60% to 95% B in 23 min, 90% to 100% B in 2 min, 100% B for 5 min, returned to 60% B in 1 min, and reequilibrated for an additional 9 min at a flow rate of 1.0 mL min^{-1} and 27°C. Dansylated polyamines were identified by fluorescence (λ_{ex} = 365 nm; λ_{em} = 515 nm) and quantified by comparison with commercial standards.

Arabidopsis Micrografting

Grafting of young Arabidopsis seedlings was carried out according to the methods described by Bainbridge et al. (2006) and Marsch-Martínez et al. (2013)

with minor modifications. Seeds were surface-sterilized, plated on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962; Duchefa) containing 0.8% (w/v) agar, and stratified for 4 d in the dark at 4°C, then plates were incubated vertically in the dark at 28°C for an additional 2 d to promote hypocotyl etiolation before being moved to standard growth conditions (150 μmol photons m^{-2} s^{-1} for 8 h at 22°C and 16 h dark at 18°C). Grafting was performed under sterile conditions 4 to 5 d after germination using a binocular stereoscope. Hypocotyls were cut using a sharp razor blade, and freshly cut scions and rootstocks were moved to a new plate, aligned, and joined. Sterile silicon tubing with an internal diameter of 0.3 mm (HelixMark) was cut in half longitudinally and transversely into segments of about 2 mm, laid on top of the union site of the grafted seedling, and gently pushed so that it would adhere tightly to both the reconstituted hypocotyl and the gel surface. Plates were then placed vertically, and plants were allowed to heal and grow under standard conditions for an additional 5 to 7 d before grafting efficiency was evaluated. The use of C-shaped sheaths represents an intermediate solution between collar and collar-free grafting procedures in which scions and rootstocks are less damaged during manipulation and more precisely aligned compared with when they are forced into a solid collar, while maintaining the presence of a solid cast in addition to the gel surface, thus limiting movement of the two extremities during the healing process.

Accession Numbers

Sequence data from this article are as follows: At5g05630 (*PUT3/RMV1*), At5g25760 (*UBC21*), At5g54770 (*THI1*), and At1g22940 (*TH1*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Simplified schemes of thiamine and polyamine biosynthesis in plants.

Supplemental Figure S2. Optimum pH and TDP competition assays in yeast.

Supplemental Figure S3. Histochemical localization of *pPUT3-GUS* expression activity.

Supplemental Figure S4. Subcellular localization of PUT3-YFP to the plasma membrane.

Supplemental Figure S5. Proportion of B_1 vitamers in phloem exudates compared with leaves.

Supplemental Figure S6. Quantitative real-time PCR of *PUT3* expression in *rmv1* versus the wild type.

Supplemental Table S1. List of oligonucleotide primers used in this study.

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