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Altered expression of polyamine transporters reveals a role for spermidine in the timing of flowering and other developmental response pathways.

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ABSTRACT

Changes in the levels of polyamines are correlated with the activation or repression of developmental response pathways, but the role of polyamine transporters in the regulation of polyamine homeostasis and thus indirectly gene expression, has not been previously addressed. Here we show that the *A. thaliana* and rice transporters *AtPUT5* and *OsPUT1* were localized to the ER, while the *AtPUT2*, *AtPUT3*, and *OsPUT3* were localized to the chloroplast by transient expression in *N. benthamiana*. *A. thaliana* plants that were transformed with *OsPUT1* under the control the *PUT5* promoter were delayed in flowering by 16 days. In contrast, *put5* mutants flowered four days earlier than WT plants. The delay of flowering was associated with significantly higher levels of spermidine and spermidine conjugates in the leaves prior to flowering. A similar delay in flowering was also noted in transgenic lines with constitutive expression of either *OsPUT1* or *OsPUT3*. All three transgenic lines had larger rosette leaves, thicker flowering stems, and produced more siliques than wild type plants. In contrast, *put5* plants had smaller leaves, thinner flowering stems, and produced fewer siliques. Constitutive expression of PUTs was also associated with an extreme delay in both plant senescence and maturation rate of siliques. These experiments provide the first genetic evidence of polyamine transport in the timing of flowering, and indicate the importance of polyamine transporters in the regulation of flowering and senescence pathways.

Keywords polyamines, *A. thaliana*, rice, flowering time, localization, chloroplast

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; FW, fresh weight; GA, gibberellic acid; GUS, β -glucouronidase; HPLC, high performance liquid chromatography; LD, long days; MTA, methylthioadenosine; PUT, polyamine uptake transporter; SAM, S-adenosylmethionine; WT, wild type;

1. Introduction

The polyamines putrescine, spermidine and spermine were amongst the first metabolites to be isolated from living organisms [1] but how these compounds function at a molecular level is largely uncharted territory. Polyamines have hormone-like properties in that changes in polyamine levels are associated with fundamental physiological

processes such as embryogenesis, flowering, fruit development and ripening, senescence, and tissue responses to biotic and abiotic stresses [2]; but the levels of polyamines are much higher than true hormones such as auxins, gibberellins, or brassinosteroids. In *A. thaliana*, putrescine levels range from 10-50 nM/g FW, while spermidine levels are typically higher (50-80 nM/g FW) and spermine levels fall in the range of 10-50 nmol/gFW [3, 4]. The identification of thermospermine in plant tissues is a more recent development, largely because this compound is not easily resolved from spermine [4]. Levels of thermospermine in different tissues are estimated to be in the range of 0.5 to 2 nM/g FW. Conjugated forms of polyamines that include alkaloids, and caffeic, coumaric, and ferulic acids form the largest pools of polyamines in plants [5-7]. More recent evidence suggests that some phenolamides have specific roles such as pollen development [2] and defense responses to pathogens and insects [6]. The extent of back conversion of such compounds to the polyamines is also unknown [8].

These estimates of pool sizes may be misleading, as polyamines could be concentrated in cellular compartments or bound to macromolecules. The association of polyamines with other macromolecules has been estimated for spermidine and spermine for mammalian cells, and putrescine and spermidine in *E. coli* [9]. While polyamines were found not to have a strong affinity for cytosolic proteins, a significant fraction of polyamines were bound to RNA. In bovine lymphocytes, 57% of the spermidine pool, and 65% of spermine was bound to RNA, while in *E. coli*, 48% of the putrescine pool, and 90% of the spermidine was bound to the RNA. In mammalian cells, the percentage of spermidine bound to DNA, ATP and phospholipids was 13%, 12%, and 3%, and that of spermine was 18%, 9% and 2.5%, respectively. Importantly, the levels of “free” spermidine and spermine in mammalian cells was estimated to be 15% and 5% respectively of the total pools of these polyamines [9]. Putrescine levels were not estimated in mammalian cells. However in *E. coli*, putrescine was found to have a lower affinity for other macromolecules than the larger polyamines. In those cells, the amount of free putrescine was estimated to be 39% of the total pool, while only 4% of the total amount of spermidine in the cell was estimated to be free. In plants, non-aqueous fractionation has been used to fractionate metabolites into a cytosolic compartment, vacuolar, and chloroplast fractions [10]. In this study 61% of the spermidine and 45% of

soluble putrescine were estimated to be localized with the chloroplast fraction, while the cytosolic fractions contained 33% and 16% respectively of the putrescine and spermidine pools.

A role of polyamines as an additional regulator of flowering has been suggested from both feeding experiments and transgenic manipulation of polyamine biosynthesis. In *Sinapsis alba*, the transition to flowering was marked by changes in the levels of polyamines and polyamine conjugates in xylem and phloem exudates [11]. Furthermore, the inhibition of flowering by the application of the ornithine decarboxylase inhibitor DFMO to the leaves and its reversal by application of putrescine to the roots, suggested that putrescine regulated the timing of flowering. In *A. thaliana*, feeding spermidine via the roots under permissive flowering conditions resulted in a delay of flowering [12]. Overexpression of arginine decarboxylase resulted in the accumulation of putrescine in the leaves, and plants that were both dwarfed and delayed in flowering [13]. The dwarfed phenotype suggested that high putrescine levels might be inhibiting either gibberellic acid (GA) synthesis, or the response of leaf tissues to GA. Both the dwarfing phenotype, and the delay in flowering were alleviated by spraying leaves with GA.

In prior work, we identified a clade of Polyamine Uptake Transporters (PUTs) that act as spermidine-preferential transporters [14, 15]. To demonstrate that transport is an essential component of polyamine homeostasis, we first identified PUTs that were altered in their subcellular location in plant leaves. Then, we tested the hypothesis that alterations in the expression of transporters localized to those membranes would result in changes of plant phenotypes. Here, we show that the *A. thaliana* transporter PUT5 (At3G19553) is localized to the ER, while PUT2 (At1G31830) and PUT3 (At5G05630) are localized to the chloroplast. In plants with heterologous expression of *OsPUT1* (localized to the ER) or *OsPUT3* (localized to the chloroplast) we noted an accumulation of spermidine and spermidine conjugates in the leaves prior to the onset of flowering, a delay in the timing of flowering, larger leaves, thicker stems, and more flowers. In contrast, *put5* plants had smaller leaves, flowered earlier than WT plants, had thinner stems, and produced fewer flowers.

2. Materials and Methods

2.1 Plant material and growth conditions

Homozygous mutants of *put5* (Salk_007135) were obtained from the Arabidopsis Biological Resource Center, Columbus, OH. Plants were grown at 22°C under LD conditions (16 h light/8 h dark). Rice clones *OsPUT1* (AK068055) and *OsPUT3* (AK070314) were obtained from the Rice Genome Resource Center (<http://www.rgrc.dna.affrc.go.jp/index.html>).

The full-length coding sequences of *OsPUT1* and *OsPUT3* were amplified by PCR using the primer sequences listed in Table 1 and cloned into the pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, USA) according to the supplier's protocol. The resulting plasmids were used to mobilize target genes into the plant expression vector, pGWB2 [16] to generate pGWB2-*OsPUT1* and pGWB2-*OsPUT3* by LR recombination reaction (Invitrogen, Carlsbad, USA). The plant expression vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101 followed by a transformation into Arabidopsis Col-0 plants using the floral dip method [17]. Transgenic plants were screened by germination of seeds on ½ MS plates containing 100 µg/ml of kanamycin. Seeds from 10 independent T3 lines for each of *OsPUT1* and *OsPUT3* were pooled and used for further analysis. Transgenic plants expressing pPUT5:*OsPUT1* were generated by cloning and transforming, the 5' upstream region (1.3 kb) of At3g19553 (see Table 1) followed by the full-length coding sequence of *OsPUT1* in pEG301 [18] using the same steps as described above. These plants were selected by spraying with BASTA. Seeds from 12 plants after T3 were pooled as all plants were phenotypically similar, and used in subsequent experiments.

2.2. Phenotypic analysis

Seeds were surface sterilized, vernalized at 4°C for 3 days and planted in soil. Plants were grown in a growth chamber at 22°C and a relative humidity of 55%. Stem thickness was measured by using a vernier caliper (Fowler tools and instruments, Boston, USA). Fluorescence measurements were made using a FluorPen FP 100 (Photon System Instruments) [19]. Flowering time was measured as the day when the floral stem was one cm above the rosette. To test the effect of GA on flowering time, the plants were sprayed four times with 100 µM GA, at four day intervals starting at eight days after planting.

2.3. Gene expression analysis

Total RNAs were extracted from leaf tissues using RNase easy plant mini kit (Qiagen, CA). The purity and concentration was measured using the Nanodrop 2000 (Thermo Fisher, DE). Gel electrophoresis was used to ascertain the integrity of the RNA. cDNA synthesis was performed using SuperScript® III First-Strand Synthesis SuperMix and oligo(dT) primers (Invitrogen, CA) following the manufacturer's protocol. Gene expression analyses were performed via RT-PCR using Eppendorf™ Mastercycler™ pro PCR System. All primers used are listed in Table 1. PCR was performed using Taq 2X Master Mix (NEB, MA). PCR conditions were 20 cycles of 95°C for 20 s, 55°C for 30 s, 72°C for 1 min, with final extension of 72°C for 5 min. The PCR amplicons were assessed on 1% agarose gel electrophoresis.

2.4 GUS analysis

The 5' upstream region of *AtPUT5*, 1320 bp in length, was amplified by PCR. The amplified product was cloned into the Gateway promoter analysis vector pBGWFS7 by Gateway technology [20]. The resulting construct was then transformed in *A. thaliana* (Col-0) by floral dip method [17]. BASTA resistant seedlings were selected, transferred to soil and grown at 22^o C in a growth chamber. Histochemical staining of GUS expression from eight resistant lines was performed by infiltration with X-Gluc buffer (2 mM X-Gluc, 50 mM NaPO₄ pH 7.0, Triton-X (0.5%), 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide) [21].

2.5. Subcellular Localization of PUTs

Full length sequences of *AtPUT5*, *AtPUT2* (At1G31820); *AtPUT3* (At5G05630); *OsPUT1* and *OsPUT3.1* were cloned into the GFP expression vectors pGWB6 or pGWB5 using the GATEWAY® recombination system [16]. Inserts were verified by sequencing, and vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. [22]. The mCherry-ER Marker (ER-rbCD3960) [23] was obtained from ABRC and used as an organellar marker. Images of one micron sections through the leaf were acquired using a Leica TCS SP5 multi-photon laser scanning confocal microscope at 12-72 hours after infiltration. Samples were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) and the Leica Application Suite Advanced Fluorescence (LASAF) program. Images were acquired in the XYZ

plane in 1 μm steps with a 63X oil objective (NA 1.40) using the sequential scan mode to eliminate any spectral overlap in the individual fluorophores. Specifically, GFP was excited at 488 nm and detected at 510 nm. The mCherry was excited at 561nm and detected at 610 nm. Plastids were excited at 633nm and detected at 670 nm. GFP signals were false-colored green and mCherry signals were false-colored red. Background fluorescence from untransformed leaves of plants at similar laser excitation settings were acquired and subtracted from images to identify fluorescence generated by tagged proteins. Images were merged using ImageJ [24]. Images were acquired from multiple leaf sections at different time points following infiltration on at least two separate occasions for each construct.

2.6. Polyamine analysis

Leaves of Arabidopsis plants were extracted in 5% cold perchloric acid (300 mg fresh weight/ml) in an ice bath. Plant extracts were centrifuged at 10,000 g for 10 minutes. The supernatant, containing the soluble polyamines and hydrolysable conjugates, was stored at -20°C. The pellet containing insoluble conjugates was re-suspended in 1 M NaOH (300 mg fresh weight/ml) and stored at -20°C until dansylation. Soluble conjugated polyamines and insoluble conjugated polyamines were released from the supernatant and pellet fractions respectively, by hydrolysis with equal volumes of 12 M HCl at 100 °C for 20 h. Dansyl derivatization was performed as described (Marce et al., 1995). 200 μl of supernatant was mixed with an internal standard (40 μl of 0.05 mM 1,7-heptanediamine, Sigma-Aldrich), 200 μl of saturated sodium carbonate (13%, w/v) and 400 μl of dansyl chloride in acetone (10 mg/ml). After vortexing, the mixture was incubated overnight at room temperature in the dark. Excess dansyl chloride was removed by adding 100 μl of L-proline (100 mg/ml) followed by an incubation for 30 min at room temperature in the dark. The dansylated polyamines were further extracted with 500 μl of toluene. The extraction was repeated two times. The organic phase containing polyamines was vacuum evaporated, and the residue was dissolved in 500 μl of methanol. The dansylated polyamines were separated and quantified by HPLC with an Agilent Technologies 1120 series HPLC and Applied Biosciences 980 programmable fluorescence detector (excitation at 340 nm, emission at 500 nm). A Gemini® 5 μm C18 110 Å, 250 $\times$ 4.6 mm LC column (Phenomenex, Torrance, CA) was used for the separation of polyamines.

Dansylated polyamines were separated with the HPLC method of Smith et al., (1985) with modifications. Samples were eluted from the column with a programmed water: methanol solvent gradient over 40 minutes. The initial conditions were 10% methanol and 90% water pumping at a flow rate of 0.75 ml/min. The methanol concentration was increased to 60% over 4 minutes and then up to 80% over 11 minutes. Then the concentration of methanol was increased to 95% over 17 minutes. A standard curve was generated by measuring known amounts of polyamines and used for the estimation of polyamine content.

2.7. Statistical analysis

All data analyses were performed using the R Studio software program, V. 0.98.1062. A one-way T test was applied, and the difference between means were considered to be significant for $P < 0.05$.

3. Results

3.1. Promoter analysis of *AtPUT5*

Microarray analysis of *PUT5* indicates that this gene shows highest expression in mature leaves, the cauline leaf, and the stem [25]. To determine the cellular distribution of this transporter in plant tissues, we fused the upstream sequence of *PUT5* to GUS, and transformed these constructs into *A. thaliana*. At two and four weeks, the highest level of staining was observed in the major veins of the leaves (Fig. 1). At six weeks, staining was observed throughout the leaf, at the tops of flower petals, and the root shoot junction. However in our constructs we did not note any expression in the stems.

3.2 Subcellular localization of PUTs by transient expression in *N. benthamiana*

Confocal analysis of *GFP-AtPUT5* and *GFP-OsPUT1* fusion constructs revealed a compartmental localization within the cytoplasm. Co-expression with an mCherry ER-marker indicated that both *AtPUT5* and *OsPUT1* were localized to the ER (Fig. 2). In contrast, confocal analysis of GFP fusions of *AtPUT2*, *AtPUT3*, and *OsPUT3* showed that these PUTs were localized to the chloroplast in the leaves of *N. benthamiana* (Fig. 3).

3.3. Phenotypic changes resulting from altered expression of PUTs

The rice gene OsPUT1 was the first characterized member of the PUT family [14] and shares 56% identity and 71% homology with PUT5 (Fig.S1). Since both *AtPUT5* and *OsPUT1* are localized to the ER, *put5* plants and *A. thaliana* plants transformed with *pPUT5:OsPUT1* and *p35S:OsPUT1* were used to assess how variation in the expression of an ER-localized polyamine transporter might affect plant phenotype. Plants with *PUT5:OsPUT1* or *p35S:OsPUT1* constructs enabled us to examine the consequences of tissue-specific vs constitutive changes in gene expression on plant phenotypes. The top BLAST hits for the rice gene OsPUT3 rice gene in the *A. thaliana* genome are to PUT2 and PUT3. OsPUT3 shares 64% identity and 76% similarity to PUT2 and 62% identity and 77% similarity to PUT3 (Fig. S2). Thus transformation of plants with the construct *p35S:OsPUT3* was considered the best choice to evaluate the effect of overexpression of a plastid-localized polyamine transporter on plant development. To confirm expression of transgenes in transformant lines, and the absence of expression of PUT5 in *put5* plants, we did RT-PCR of mRNA from rosette leaves (Fig. S3).

The phenotypes of the three overexpression lines were compared with wild type (WT) and *put5* plants. Under LDs at 22°C, *put5* flowered 4 days earlier than WT, which flowered at 27 ± 3 days (Table 2). All of the overexpression lines were significantly delayed in flowering. Plant expressing *pPUT5:OsPUT1* flowered at 43 ± 3 days, while plants expressing *p35S:OsPUT1* and *p35S:OsPUT3* flowered at 38 ± 3 and 44 ± 4 days respectively. The number of rosette leaves was positively correlated with time of flowering. At five weeks, the rosette leaves of the *put5* were smaller than WT, while the leaves of three overexpression lines were two-fold larger than WT leaves (Fig. 4 and Fig. 5). We also noted that overexpression lines had thicker and more rigid floral stems (Table 2, Fig. 4). The stems of the *put5* plants near the base of the rosette were slightly thinner (0.74 mm) than WT plants (1.16 mm) but the floral stems in the overexpression lines ranged from 1.7 to 1.9 mm.

3.4. Constitutive expression of PUTs delays senescence

Constitutive expression of PUTs was also associated with an extreme delay in senescence. At 97 days, rosette leaves of *p35S:OsPUT1* and *p35S:OsPUT3* were still green, and had some photosynthetic electron transport capacity as assessed by F_v/F_m (Fig 6) compared to WT plants. The siliques of these transformants also remained green and

desiccated much more slowly. However, no delay of senescence relative to WT plants was noted for *pPUT5:OsPUT1* plants.

3.5. Effect of PUT expression on polyamine levels in rosette leaves

Polyamine levels in leaves fluctuated during different developmental stages of the plant. Polyamine levels were assessed at two weeks, four weeks, and six weeks to correspond with three periods in the life cycle of WT plants. At two weeks, the WT plants were at the early stages of development with no floral stems. At four weeks, WT plants were at the flowering stage, and at six weeks, WT plants had a large number of dried siliques. At week two in WT plants, pools of putrescine and spermine conjugates were less than 10 nmoles/g FW and did not change significantly over the three time points. Levels of spermidine conjugates were 34 ± 2 nmoles/g FW at two weeks and then declined to 15 ± 3 and 9 ± 2 nmoles/g FW at four and six weeks respectively (Fig. 7). While the phenotypes of *put5* plants were distinguishable from WT plants, there were no significant differences from WT plants in any of the polyamine pools (Fig 7). In contrast, at two weeks, each of the overexpression lines had significantly higher levels of spermine, spermidine and spermidine conjugates than WT plants. *pPUT5:OsPUT1* plants had significantly higher putrescine levels than WT plants. At four weeks, when WT were already flowering (Fig. 7) spermidine and spermidine conjugate levels, were not significantly different in *pPUT5:OsPUT1* plants from WT plants, but they remained significantly higher in both of the *CaMv* overexpression lines. At six weeks, the two lines with constitutively expressed *PUTs* had significantly higher levels of putrescine, spermidine and spermidine conjugates. The elevated levels of substrates in these lines were correlated with a delay in senescence that was not seen in *pPUT5:OsPUT1* plants.

3.6. Gibberellins suppress the delay of flowering in pPUT5:OsPUT1 plants

Previous studies have shown that overexpression of arginine decarboxylase (the first step in the putrescine synthesis pathway) resulted in plants that accumulated putrescine in the leaves, and exhibited a delay of flowering, and a dwarf phenotype [13]. In contrast, plants with the *pPUT5:OsPUT1* construct had significantly higher levels of putrescine than the WT at two weeks. To test whether the delay of flowering in *pPUT5:OsPUT1* constructs could be alleviated by GA, leaves of these plants were sprayed four times with GA. At the time, when most of the *put5* plants were starting to flower, all of the floral stems of

GA-treated plants were more than 10 cm high. Thus spraying the leaves of these plants enabled them to flower earlier than both *put5* and WT plants, and more than two weeks earlier than expected (Fig. 8 and Table 1).

4. Discussion

This study provides the first experimental evidence that the regulation of polyamine transport plays a key role in both polyamine homeostasis and the downstream consequences of changes in cellular levels of polyamines and polyamine conjugates. The polyamine transporter mutant *put5* flowered slightly earlier than WT plants, while flowering was delayed by 16 days in *pPUT5:OsPUT1* plants. The delay of flowering was also noted in the constitutively expressed transgenic lines. The profiles of polyamine levels were unique for each of the three transgenic lines. However, a common feature that correlated with the phenotype of delayed flowering, was significantly higher leaf levels of both spermidine and spermidine conjugates at two weeks, and higher levels of spermidine conjugates in the rosette leaves at four weeks, in the two constitutively overexpressed lines.

PUT transporters have been characterized as unidirectional, preferential transporters of spermidine [14, 15]. Here, we noted that OsPUT1 and AtPUT5 were localized to the ER, when transiently expressed in the leaves of *N. benthamiana*. Localization of AtPUT5/LAT3 to the ER by transfection of *A. thaliana* protoplasts was also reported [26]. While we used transient expression analysis in *N. benthamiana* of full length cDNAs of *AtPUT2*, *AtPUT3*, and *OsPUT3*, to localize these genes to the chloroplast, other groups have examined the localization of these genes in other plant tissues [26-29]. Confocal microscopy of AtPUT3/RMV1-GFP fusions in transgenic *A. thaliana* showed this protein to be localized to the plasma membrane in root cells [27, 29]. Callus cultures of *lhr1/put3* plants were also reduced in their ability to take up 2 μ M spermidine relative to WT cells, consistent with the localization of this transporter to the plasma membrane in those tissues [29]. In stably transformed *A. thaliana*, GFP ATPUT2/PAR1-GFP fusions were localized to a subcellular compartment of roots cells, that in protoplasts prepared from leaf tissues was found to be co-localized with an ER marker [26]. The same research group reported that OsPAR1-GFP fusions were localized

to the Golgi in both rice and *A. thaliana* protoplasts. There are many potential explanations that may account for the dual localization of proteins. For example, there are many proteins that are targeted to the chloroplasts or mitochondria in different tissues despite the striking differences in the import apparatus of these two organelles [30]. The plant glutamate receptor AtGRL3.4 was localized to both the plasmamembrane and chloroplast using both a specific antibody and YFP-tagged fusion constructs [31]. The dual targeting of this protein was observed in WT plants, transiently expressed protoplasts of *A. thaliana* and agroinfiltrated tobacco leaves. Some proteins such as the rice alpha amylase protein, and nucleotide pyrophosphatase/phosphodiesterases [32] are targeted to the plastid after first passing through the Golgi [33]. Protoplasts have been a valuable resource for localization studies, but protoplasts prepared from leaf tissues are not identical cell types, and the localization of some proteins is both tissue and cell-type dependent [34]. Taken together, the localization experiments suggest that these transporters may, like other proteins be localized to more than one membrane under different conditions. This conclusion needs to be verified independently by immunolocalization experiments.

However the *in vivo* experiments using detached leaves or isolated chloroplasts, directly supports the chloroplast localization of PUT2 and PUT3 in these tissues. The leaves of overexpression constructs of *PUT3/RMV1* were found to be hypersensitive to paraquat ([27], Fig 3) a phenotype that would also be expected for a chloroplast-localized paraquat transporter. Similarly, Li et al (2013) reported that paraquat accumulation in the chloroplast was markedly increased in overexpressing *PAR1/PUT2* plants ([26]Fig. 6B). Both groups also noted that null mutations did not prevent the paraquat sensitivity of chloroplasts, a finding that is also consistent with our observations that both AtPUT2 and AtPUT3 are localized to the chloroplast.

It is interesting to note that the overexpression lines all had delayed flowering times, larger leaves and thicker stems, although the subcellular distribution of AtPUT5 and OsPUT1 is different from that of OsPUT2, AtPUT3, and OsPUT3. The direction of substrate transport for these proteins has not been clearly established, but the increased sensitivity of detached leaves to paraquat in transgenic lines overexpressing *PUT2/RMV1* [27] or *PUT3/PAR1* [26] is consistent with their localization to the chloroplast, and that

they function as importers. We have not yet demonstrated that PUT5 and OsPUT1 are functionally active on the ER, but the shared phenotypes of these transporters with those of plants overexpressing *OsPUT3*, could mean that ER and chloroplast-localized transporters act to sequester spermidine from the cytoplasm. Prior studies of long distance transport of polyamines, suggests a rapid turnover of polyamine pools due to export from the leaves [35-37]. Since our experiments involved the genetic manipulation of transporters located in either the ER or the chloroplast membrane, slight changes in the compartmentation of polyamines could easily affect export rates from mesophyll cells. This hypothesis will be evaluated in future metabolic flux experiments.

Polyamines are not often referenced as significant regulators of flowering in plants [38, 39]. However a premature flowering phenotype which was also correlated with a significant increase in putrescine levels was recently reported [40]. The increase in putrescine levels was observed in plants that were deficient or silenced in N2-acetylornithine deacetylase which converts acetylornithine to ornithine, but the mutant plants also had lower seed production. In contrast, we observed increased levels of putrescine in *pPUT5:OsPUT1* plants at two weeks, but these plants exhibited a delay in flowering (Fig. 6). A delay of flowering has also been reported in *A. thaliana* that were supplemented with spermidine via the roots [12]. Flowering was also delayed in transgenic lines overexpressing *AtADC2* [13]. The dwarf phenotype of those plants suggested that the phenotype might be caused by an inhibition of gibberellin biosynthesis. Down regulation of genes in the gibberellin biosynthetic pathway was noted in the dwarf plants, and gibberellin treatment of the leaves reversed the inhibition of flowering [13]. By four weeks, the rosette leaves of all three transgenic lines were larger than WT plants, which would not be expected if the accumulation of spermidine or spermidine conjugates resulted in an inhibition of gibberellin synthesis. We also noted that the application of gibberellins clearly promoted the onset of flowering, and these plants flowered even earlier than *put5* plants. We therefore hypothesize that the inhibition of flowering due to the higher levels of spermidine and spermidine conjugates in young rosette leaves, is upstream of the activation of flowering due to the gibberellin response pathway. Sustained levels of spermidine or spermidine conjugates in the rosette leaves at four weeks appear not to be needed to cause the delay in flowering, as polyamine levels

in *pPUT5:OsPUT1* plants were not significantly different from WT plants. Yet these plants flowered later than *p35S:OsPUT1* plants and at the same time as *p35S:OsPUT3* plants.

The three overexpressor lines described here displayed a common set of other phenotypes, notably, larger leaves, and thicker stems. Taken as a whole, these developmental changes suggest the involvement of several regulatory pathways. One possibility is that spermidine and or spermidine conjugates are acting as signaling molecules. Changes in the levels of these signaling molecules are sensed by the cell and serve as a signal to activate hormone responsive pathways [41]. Plants overexpressing *ADC2* were found to have increased tolerance to both freezing and drought stress [3, 42]. In these plants, microarray analysis pointed to altered expression of genes involved in auxin synthesis, auxin transport, auxin-related genes, along with genes associated with abiotic stress in plants [41]. Gene expression in hormone-responsive pathways were also affected. These included ABA, jasmonic acid, and ethylene pathways.

The thinner stems that we noted in *put5* plants, and the thicker, more robust stems that are a feature of all of the overexpression lines of PUTs, may be related to alterations in thermospermine levels. Thermospermine is synthesized by *ACL5* in vascular tissues [43] and vascular development occurs as a consequence of a feedback loop between auxin levels, *HD-ZIPIII* genes and thermospermine [44]. Auxin promotes the synthesis of both *ACL5* and *BUD2* [45], genes that are needed for thermospermine synthesis. Thermospermine in turn, promotes the expression of *SAC51* and other *bHLH* transcription factors, that together act as negative regulators of auxin signaling and perivascular divisions [46]. Increased expression of a cotton *ACL5* in *A. thaliana* resulted in taller plants [47] while silencing of the gene resulted in a severe dwarf phenotype [43]. Spermidine is a precursor to thermospermine, and increased levels of spermidine in stem tissues may result in increased synthesis of thermospermine, and thus taller plants. Changes in the expression of PUT transporters may also affect the redistribution of thermospermine. The phenotypes produced by enhanced PUT transport are consistent with an overall increase in the expression of genes in the auxin, *HD-ZIPIII*, and thermospermine regulatory loop described above. The role of spermidine and spermidine transport in promoting vascular development is presently under investigation.

One of the great challenges for plant scientists is to be able to explain the phenotypic diversity within a single plant species. Here, we have shown that mutations that change the expression of a single spermidine transporter have pleiotropic effects. Thus transporters that regulate the movement of hormone and other signaling compounds could provide a mechanism for generating phenotypic variation across species.

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Table 1: Primer sequences used in this study.

Gene	Forward Primer	Reverse Primer	Purpose
OsPUT1	CACCATGGCGGACACCGGCGGACG	CTACGGAACCAACGGCTCGTCCG	Overexpression in Arabidopsis
OsPUT3	CACCATGACGAACGCATGGATCTC	TCAGCACACAAGTGGGATTGTGC	Overexpression in Arabidopsis
AtPUT5 promoter	CACCATCCTTCCATCGTTTGCAAG	ACAAATATCACCGACAGAGAGACAG	GUS Expression
At3g18780	ATCAATTCGATCACTCAGAGC	CTATGATGCACTTGTGTGTGA	RT-PCR
OsPUT1	ATCTTCCTCATCTTCTACGAGGTGT	CGGACTTGAGGTAGTCCAAGAAG	RT-PCR
OsPUT3	TTACATGAACTACAGAGGGTTGACA	GGTAAGAATCACTGCTCATTTCAGT	RT-PCR
AtPUT5	TGGAGTTTCAATGCTGTGCCT	ACCCTCCTGCTTCTACACTCT	RT-PCR

Table 2. Phenotypic variation of *A. thaliana* genotypes.

Accession	Number of Rosette Leaves	Time to Flowering (days)	Stem Thickness (mm)
<i>put5</i>	6.9 ± .87	23 ± 2.5	0.74 ± 0.13
Wild Type	8.8 ± 1.01	27 ± 3	1.16 ± 0.18
<i>pPUT5:OsPUT1</i>	11.8 ± 1.4	43 ± 3	1.74 ± 0.14
<i>p35S:OsPUT1</i>	10.9 ± 1.2	38 ± 3	1.70 ± 0.16
<i>p35S:OsPUT3</i>	13.1 ± 1.13	44 ± 4	1.92 ± 0.23

Values are based on 10 plants for *put5* and *pPUT5:OsPUT1* and 24 plants for other genotypes

Figure Legends

Fig. 1 GUS-expression driven by the *AtPUT5* promoter in *A. thaliana*. (A) In two week rosette leaves, staining is observed in the veins; (B) At four weeks, staining restricted to the major vein of rosette leaves; (C) At six weeks, staining is visible throughout the leaf blade; (D) Staining is observed at the tip of the flower petals. (E) Staining is visible at the base of leaf petiole.

Fig. 2. Subcellular localization of AtPUT5 and OsPUT1 to the ER by transient expression in *N. benthamiana* leaves. (A) Expression of GFP-AtPUT5 (B) Localization of mCherryER marker (C) Merged images showing co-localization of GFP and mCherry markers (yellow). (D) Expression of GFP-OsPUT1 (E) Localization of mCherry-ER marker (F) Merged images showing co-localization of GFP and mCherry markers. Z-stack images were generated with Image J [24].

Fig. 3. Transient expression of AtPUT2, AtPUT3, and OsPUT3, tagged with GFP, in *N. benthamiana* leaves indicate that these transporters are localized to the chloroplast. (A) Expression of GFP-AtPUT2 (B) Chlorophyll autofluorescence (excitation 633 nm, emission 670 nm) (C) Merged images showing GFP localization and chlorophyll autofluorescence (yellow). (D). Expression of GFP-AtPUT3; (E) chlorophyll autofluorescence; (F) Merged images showing GFP localization and chlorophyll autofluorescence (yellow); (G) Expression of OsPUT3 (H) chlorophyll autofluorescence; (I) Merged images showing GFP localization and chlorophyll autofluorescence (yellow). Z-stack images were generated with Image J [24].

Fig. 4. Increased expression of PUTs results in larger leaves. Rosette leaves of plants at 27 days. Note the reduction in petiole length in the overexpression lines.

Fig. 5. Increased expression of PUTs results in a delay of flowering. Photograph of *A. thaliana* plants at six weeks. Note the thicker stems in the overexpressed lines.

Fig. 6. *A. thaliana* plants with constitutive expression of *OsPUT1* or *OsPUT3* are delayed in senescence. Fluorescent measurements of the rosette leaves of *p35S:OsPUT1* and *p35S:OsPUT3* indicated that they were still photosynthetically active at 97 days.

Fig. 7. Polyamine levels in rosette leaves of *A. thaliana* plants. Soluble (S), soluble conjugated (SC) and insoluble conjugated (IC) polyamines at two weeks (A) four weeks (B) and 6 weeks after planting(C). * indicates a significant difference from the WT pool at that time period.

Fig. 8. Gibberellins promote early flowering in *pPUT5:OsPUT1* plants. Plants expressing *pPUT5:OsPUT1* that were sprayed with gibberellins flowered slightly ahead of *put5* plants.

GFP

ER-mCherry

Merged image

A

B

C

AtPUT5

0 μ m 25

0 μ m 25

0 μ m 25

D

E

F

OsPUT1

0 μ m 75

0 μ m 75

0 μ m 25

put5

Wild type

p35S:OsPUT1

pPUT5:OsPUT3

p35S:OsPUT3

A) 2 weeks

B) 4 weeks

C) 6 weeks

put5

Wild type

pPUT5:OsPUT1

pPUT5:OsPUT1 - GA