THE ROLE OF POLYAMINE UPTAKE TRANSPORTERS ON GROWTH AND DEVELOPMENT OF ARABIDOPSIS THALIANA

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ABSTRACT

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Transgenic manipulation of polyamine levels has provided compelling evidence that polyamines enable plants to respond to environmental cues by activation of stress and developmental pathways. Here we show that the chloroplasts of *A. thaliana* and soybeans contain both an arginine decarboxylase, and an arginase/agmatinase. These two enzymes combine to synthesize putrescine from arginine. Since the sequences of plant arginases show conservation of key residues and the predicted 3D structures of plant agmatinases overlap the crystal structure of the enzyme from *Deinococcus radiodurans*, we suggest that these enzymes can synthesize putrescine, whenever they have access to the substrate agmatine. Finally, we show that synthesis of putrescine by ornithine decarboxylase takes place in the ER. Thus *A. thaliana* has two, and soybeans have three separate pathways for the synthesis of putrescine. This study also describes key changes in plant phenotypes in response to altered transport of polyamines.
Dedicated to my father, Jayantilal Haribhai Patel
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# TABLE OF CONTENTS

**GENERAL INTRODUCTION** ....................................................................................................... 1

References .................................................................................................................................... 9

**CHAPTER 1: HIDING IN PLAIN SIGHT: A THIRD ROUTE FOR PUTRESCINE BIOSYNTHE**
**SIS IN PLANTS** .......................................................................................................................... 25

Abstract ...................................................................................................................................... 25

Introduction, Results and Discussion .................................................................................... 26

References .................................................................................................................................... 35

Supplementary Material for Hiding in Plain Sight: A Third Route for Putrescine

Biosynthesis in Plants .......................................................................................................................... 39

Supplementary materials and methods .................................................................................. 39

Supplementary figures and tables ......................................................................................... 42

Supplementary references ............................................................................................................... 49

**CHAPTER 2: CONTROL OF FLOWERING IN *A. THALIANA* BY ALTERED EXPRESSION OF POLYAMINE TRANSPORTERS.** .................................................................................................................. 51

Introduction .................................................................................................................................. 51

Materials and Methods .................................................................................................................. 55

Plant material and growth conditions ....................................................................................... 55
CHAPTER 3: OVEREXPRESSION OF OSPUT3 CONFERS DROUGHT TOLERANCE IN ARABIDOPSIS THALIANA

Introduction ....................................................................................................................... 84

Materials and Methods ................................................................................................. 87

Plant material and growth conditions ......................................................................... 87

Subcellular localization analysis ................................................................................. 87

Generation of transgenic *Arabidopsis* plants ......................................................... 88

Phenotypic analysis ........................................................................................................ 88

Drought stress tolerance assay .................................................................................... 88

Results .............................................................................................................................. 89
Subcellular localization of OSPUT3

Overexpression of OsPUT3 in Arabidopsis increases leaf and stem size

Overexpression of OsPUT3 in Arabidopsis enhances drought stress tolerance along with an increase in yield and biomass.

Discussion

References

SUMMARY
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polyamine biosynthetic pathways in plants.</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Localization analysis of AtADC2 and AtARGAH2 by transient expression in N.</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>Characterization of Arabidopsis agmatinase by complementation in yeast</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Predicted 3D structure of AtARGAH2 is highly similar to the crystal structure of Deinococcus radiodurans agmatinase</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Subcellular localization of OsODC</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>Localization of full-length OsPUT1</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td>Localization of half OsPUT1-GFP fusion protein</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>AtPUT3 and AtPUT2 are localized to the chloroplast</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>Transgenic plants overexpressing OsPUT1 and OsPUT3 are delayed in flowering.</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>Transgenic plants with increased expression of OsPUT1 and OsPUT3 had larger leaves</td>
<td>65</td>
</tr>
<tr>
<td>11</td>
<td>Transgenic plants with increased expression of OsPUT3 had thicker stems</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>Transgenic plants with increased expression of OsPUT3 had increased yield</td>
<td>66</td>
</tr>
<tr>
<td>13</td>
<td>Transgenic plants with increased expression of OsPUT1 and OsPUT3 were delayed in senescence</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>Gus expression of promoter region of AtPUT5 in A. thaliana</td>
<td>68</td>
</tr>
<tr>
<td>15</td>
<td>Plants expressing AtPUT5::OsPUT1 were delayed in flowering</td>
<td>69</td>
</tr>
<tr>
<td>16</td>
<td>Plants expressing AtPUT5::OsPUT1 were taller compared to WT and CS869507</td>
<td>70</td>
</tr>
</tbody>
</table>
17 Plants expressing AtPUT5::OsPUT1 had larger leaves compared to WT and CS859607.

18 Expression of AtPUT5::OsPUT1 in A. thaliana resulted in increased silique production.

19 Plants expressing AtPUT5::OsPUT1 had thicker stems as compared to WT and CS859607.

20 Levels of polyamines in WT, OsPUT1, and OsPUT3 plants before, during, and after flowering under continuous light.

21 Polyamine levels in CS859607, WT, and OX 19553 were assessed at two, four and six weeks.

22 Overexpression of OsPUT3 in Arabidopsis enhances drought stress tolerance.

23 Plants overexpressing OsPUT3 are taller as compared to WT and have increased silique length.

24 Overexpression of OsPUT3 in Arabidopsis leads to 5 fold increase in yield as compared to wild type.

25 OsPUT3 is localized to the chloroplast.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Phylogenetic analysis of plant arginine decarboxylases</td>
<td>45</td>
</tr>
<tr>
<td>S2</td>
<td>Phylogenetic analyses of Plant arginase/agmatinases</td>
<td>46</td>
</tr>
<tr>
<td>S3</td>
<td>Characterization of Soybean agmatinase by complementation in yeast</td>
<td>47</td>
</tr>
<tr>
<td>S4</td>
<td>Comparison of active site residues of Dienococcus radiodurans agmatinase and arginases from Arabidopsis, Glycine max, and Populus trichocarpa</td>
<td>48</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>List of polyamine biosynthetic genes in Arabidopsis and the factors that enhance the expression of those genes</td>
</tr>
<tr>
<td>S1</td>
<td>List of primers used in this study</td>
</tr>
<tr>
<td>S2</td>
<td>Predicted localization of Arginine decarboxylase genes from sequenced plant genomes</td>
</tr>
<tr>
<td>S3</td>
<td>Predicted localization of Arginase/agmatinase genes from sequenced plant genomes</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Plants are sessile organisms that have acquired the ability to adapt to a continuously changing environment. Plants can survive, grow, and propagate even in harsh environmental conditions by activating specific signaling pathways. Polyamines (PAs) are emerging as essential plant growth and development regulators (Mattoo, Minocha, Minocha, & Handa, 2010). PAs play significant roles in physiological and developmental processes (Nambeesan, Mattoo, & Handa, 2008). Increase in PA concentration in plants during growth development, and stress responses have been well documented (Shi & Chan, 2014a). The rise in PA levels, in response to development and stress, is usually attributed to increased activity of PA biosynthetic or catabolic enzymes (Kusano & Suzuki, 2015). While the role of PA biosynthesis and PA catabolism in maintaining cellular PA levels is broadly understood, PA transport in plants has not been thoroughly dissected yet.

PAs are low molecular weight, polycationic molecules bearing amino groups. Diamine putrescine, triamine spermidine (Spd), and tetraamine spermine (Spm) are the most common PAs in higher organisms, and exist in almost all organisms including, bacteria, animals, and plants (Hussain, Ali, Ahmad, & Siddique, 2011). Additionally, PAs such as cadaverine, norspermidine, and thermospermine are present in many organisms. In plants, PAs are widely implicated in chromatin organization, DNA synthesis, gene transcription, protein translation, cell division and differentiation, programmed cell death, root elongation, floral development, and leaf senescence (Alcázar, Altabella et al., 2010; Alet et al., 2012; Shi & Chan, 2014a; Tavladoraki et al., 2012; Wimalasekera, Villar, Begum, & Scherer, 2011; Z. Zhang et al., 2011). In addition, PAs play a significant role in many environmental stresses, including salt, drought, temperature, ozone, wounding, and metal oxidative stresses (Cuevas et al., 2008; J. Liu, Kitashiba, Wang, Ban, &
Moriguchi, 2007; Rider et al., 2007; Tavladoraki et al., 2012; Urano et al., 2004; Wimalasekera et al., 2011; Yamaguchi et al., 2007a). Recent molecular genetic studies, involving transgenic plants with altered PA biosynthesis and mutants deficient in PA biosynthesis, have provided convincing evidence that PAs act as critical modulators of various physiological processes during plant growth and development (Alcázar, Planas et al., 2010; Kusano, Berberich, Tateda, & Takahashi, 2008b; Takahashi & Kakehi, 2010b). In plants, homeostasis of PAs is crucial for optimal growth and development, as well as stress responses (Tiburcio, Altabella, Bitrián, & Alcázar, 2014). Intracellular homeostasis of PAs is regulated by biosynthesis, catabolism, conjugation and transport (Wallace, Fraser, & Hughes, 2003). The current study is focused on understanding the role of PA transport on plant growth, development and stress responses.

It is essential to understand how PA homeostasis is achieved by regulation of genes and enzymes involved in the biosynthesis, catabolism, conjugation and transport of PAs, before considering the involvement of PAs in development and stress. In plants, putrescine is synthesized from ornithine or arginine. Conversion of ornithine to putrescine is mediated by ornithine decarboxylase (ODC), also known as ODC pathway, and this pathway is also used by almost all eukaryotes to synthesize putrescine (Fig. 1). Plants also have an alternative pathway for putrescine synthesis, in which putrescine is synthesized from arginine by a three-step enzymatic reaction, the ADC pathway. Arginine is converted to agmatine by arginine decarboxylase (ADC) followed by two successive steps catalyzed by agmatine iminohydrolase (AIH – agmatine to $N$-carbamoyl-putrescine) and $N$-carbamoyl-putrescine amidohydrolase (CPA – $N$-carbamoyl-putrescine to putrescine) (Kusano & Suzuki, 2015) (Fig. 1). Spd is synthesized from putrescine by the transfer of an aminopropyl group from decarboxylated $S$-adenosylmethionine (deSAM), in a reaction catalyzed by spermidine synthase (SPDS; Fig. 1).
Spm is synthesized from Spd by spermine synthase (SPMS; Fig. 1). Thermospermine is formed by the action of thermospermine synthase (TSPMS) (Fig. 1). Cadaverine is generated from lysine through the action of lysine decarboxylase (LDC; Fig. 1).

Figure 1. Polyamine biosynthetic pathways in plants. Aminopropyl and aminobutyl groups in polyamines are shown as NC₄ and NC₃, respectively. (Adopted from Kusano and Suzuki, 2015).

ADC, AIH and CPA, genes involved in the ADC pathway in plants are postulated to be derived from the cyanobacterial ancestor of the chloroplast by endosymbiotic gene transfer to the nuclear genome of the plant cell (Illingworth et al., 2003). Plant ADC differs from bacterial ADC because it has a chloroplast transit peptide, and ADC proteins have been detected in chloroplasts of oat and tobacco (Borrell et al., 1995; Bortolotti et al., 2004). ODC genes have been identified in many plant species (Fuell, Elliott, Hanfrey, Franceschetti, & Michael, 2010), but ODC is absent in many species of Brassicaceae (Hanfrey, Sommer, Mayer, Burtin, & Michael, 2001). In bacteria, a third pathway for putrescine biosynthesis exists, in which putrescine is generated from arginine by a two-step reaction. Arginine is converted to agmatine by the action of ADC.
followed by direct conversion of agmatine to putrescine by agmatinase (AUH) (Ahn et al., 2004). There is no evidence, yet, that plants have agmatinases, but bioinformatics analysis has revealed that plant arginases are very similar to bacterial agmatinases (Chen, McCaig, Melotto, He, & Howe, 2004).

The differential contribution of ADC and ODC pathways to stress, development and tissue specificity may be a reason for the co-existence of ADC and ODC pathways in some plants. ODC pathway is required for cell proliferation and putrescine biosynthesis in roots whereas ADC pathway is activated under stress (Carbonell & Blázquez, 2009). In plants missing \(O\text{DC}\), there is a duplication of \(A\text{DC}\), and it has been suggested that it may be a compensatory mechanism (Galloway, Malmberg, & Price, 1998; Hanfrey et al., 2001). Plant ODC is insensitive to exogenous application of Spd, or Spm and the degradation of ODC is not feedback regulated by PAs (Fuell et al., 2010; Hiatt, McIndoo, & Malmberg, 1986; Illingworth et al., 2003). The expression of \(A\text{DC}\), is induced in many biotic and abiotic stress responses, however \(A\text{IH}\) and \(C\text{PA}\) transcripts are usually not increased under stress (Alcázar, García-Martínez, Cuevas, Tiburcio, & Altabella, 2005; Alcázar et al., 2010). A list of genes involved in polyamine biosynthesis and factors that enhance their expression is shown in table 1 (Kusano & Suzuki, 2015). Many studies have shown that change in biosynthesis of PAs is co-related with plant development and stress responses but the underlying mechanism is not completely understood (Tiburcio et al., 2014).
Table 1. List of polyamine biosynthetic genes in *Arabidopsis* and the factors that enhance the expression of those genes. (Adopted from Kusano and Suzuki, 2015).

Apart from biosynthesis, the homeostasis of PAs in plants can also be maintained by conjugation and catabolism. PAs in plants are present as perchloric acid (PCA)-soluble of PCA-insoluble conjugates with cinnamic acids such as *p*-coumaric, caffeic, and ferulic acids. PA conjugates such as hydroxycinnamic acid amides (HCAA) and phenolamides or phenylamides, are known to play a significant role in promoting flowering, protection against pathogens, detoxifying phenolic compounds, acting as a bridge between different cell wall polymers, and serving as a reserve of polyamines for proliferating tissues (Bagni & Tassoni, 2001; Bassard, Ullmann, Bernier, & Werck-Reichhart, 2010; Martin-Tanguy, 1985). Transglutaminase (TGase) activity also plays a significant role in conjugation of PAs. PAs can be covalently bound to glutamine residues of proteins by TGase activity. TGase activity is widespread in all plant tissues, which suggests the importance of inter- or intramolecular cross-link formation of the proteins by PAs (Serafini-Fracassini & Del Duca, 2008). The photosynthetic protein complexes, in the chloroplast, may be stabilized by TGases (Serafini-Fracassini, Di Sandro, & Del Duca, 2010).

<table>
<thead>
<tr>
<th>Catalytic function</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Factors that enhance expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine decarboxylase</td>
<td>ADC1</td>
<td>At2g16500</td>
<td>Cold</td>
</tr>
<tr>
<td></td>
<td>ADC2</td>
<td>At4g34710</td>
<td>Drought, osmosis, salt, wounding, cold, ABA, MeJA, K⁺ deficiency, bacterial pathogen</td>
</tr>
<tr>
<td>Agmatine iminohydrolase</td>
<td>AII</td>
<td>At5g08170</td>
<td></td>
</tr>
<tr>
<td>N-Carbamoylputrescine amidohydrolase</td>
<td>CPA</td>
<td>At2g27450</td>
<td></td>
</tr>
<tr>
<td>Arginase</td>
<td>ARGH1</td>
<td>At4g08900</td>
<td>MeJA, pathogen</td>
</tr>
<tr>
<td></td>
<td>ARGH2</td>
<td>At4g08870</td>
<td></td>
</tr>
<tr>
<td>S-Adenosylmethionine decarboxylase</td>
<td>SAMDC1</td>
<td>At3g02470</td>
<td>Cold, heat shock</td>
</tr>
<tr>
<td></td>
<td>SAMDC2</td>
<td>At5g15950</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAMDC3</td>
<td>At3g25570</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAMDC4/BUD2</td>
<td>At5g18930</td>
<td>Thermospermine depletion, auxin</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>SPDS1</td>
<td>At1g23820</td>
<td>Drought, ABA</td>
</tr>
<tr>
<td></td>
<td>SPDS2</td>
<td>At1g70310</td>
<td>Cytokinin</td>
</tr>
<tr>
<td>Spermine synthase</td>
<td>SPMS</td>
<td>At5g53120</td>
<td>Drought, ABA, heat shock</td>
</tr>
<tr>
<td>Thermospermine synthase</td>
<td>ACL5</td>
<td>At5g19530</td>
<td>Thermospermine depletion, auxin</td>
</tr>
<tr>
<td>Deoxyhypusine synthase</td>
<td>DHS</td>
<td>At5g05920</td>
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</tr>
</tbody>
</table>
Catabolism (degradation) of PAs is catalyzed by two classes of amine oxidases: the copper-containing amine oxidases (CuAOs) and the polyamines oxidases (PAOs) (Kusano & Suzuki, 2015). CuAOs are involved in terminal catabolism, which converts putrescine or Spd to 4-aminobutanal, ammonia and H₂O₂. CuAOs in *Arabidopsis* are localized to the peroxisomes and apoplast (Møller & McPherson, 1998; Planas-Portell, Gallart, Tiburcio, & Altabella, 2013). PAOs are involved in terminal catabolism as well as back conversion of PAs. Back conversion of PAs is an oxidation reaction in which higher polyamines (such as Spd and Spm) are converted to putrescine along with the production of 3-aminopropanal and H₂O₂. PAOs have been localized to cytoplasm, peroxisomes, and apoplast in *Arabidopsis* and *Oryza sativa* (rice), so far (Kusano & Suzuki, 2015). The H₂O₂ production through PA degradation has been correlated with cell death, lignification, xylem differentiation, and hypersensitive response (Angelini et al., 2008; Kusano & Suzuki, 2015; Moller & McPherson, 1998; Walters, 2003). It has been suggested that the H₂O₂ produced through PA catabolism is used as a signaling molecule for stress signal transduction (Mitsuya et al., 2009; Yoda, Hiroi, & Sano, 2006). The back conversion of Spm to Spd, and Spd to putrescine results in elevated putrescine levels in plants under stress (Cona, Rea, Angelini, Federico, & Tavladoraki, 2006; Moschou, Paschalidis, & Roubelakis-Angelakis, 2008). Most reports of physiological roles of *CuAO* and *PAO* genes are based on data using CuAO or PAO inhibitors. Transgenic lines with altered expression of genes involved in PA oxidation may provide more insights on the role of PA catabolism in plant development and stress responses.

Intracellular levels of PAs in plants are also regulated by PA uptake and transport (Igarashi & Kashiwagi, 2010). In plant cells, PAs are localized in cell walls, vacuoles, nuclei, mitochondria and chloroplasts (Slocum, 1991). PAs are now considered as signaling molecules, and an important criterion for signaling molecules is translocation from the site of synthesis to
the site of function. Therefore, understanding the transport of PA is critical. There is a significant amount of information about PA transport systems in bacteria, yeast, and mammals, but PA transport system in plants has not been well characterized. The detection of PAs in phloem and xylem sap in different plant species indicated that a PA transport system must exist in plants (Antognoni, Fornalè, Grimmer, Komor, & Bagni, 1998; Pistocchi, Bagni, & Creus, 1987).

Our lab was the first to identify PA transporters in plants (Mulangi, Phuntumart, Aouida, Ramotar, & Morris, 2012a). Orthologous sequences of known systems in other organisms were used to identify PA transporters in plants. OsPUT1, a Polyamine Uptake Transporter (PUT) in rice, was characterized using heterologous expression and yeast complementation assays (Mulangi et al., 2012a). PAs (such as Putrescine and Cad) as well as the PA precursors ornithine and agmatine did not compete for Spd uptake mediated by OsPUT1 (Mulangi et al., 2012a). However, OsPUT1 could not be localized in transiently transformed onion or rice protoplasts (Mulangi et al., 2012a). Research in our lab, has also identified and characterized five additional PA transporters from Arabidopsis and rice, OsPUT2, OsPUT3, AtPUT1, AtPUT2, and AtPUT3, as PUTs, these transporters cluster in the same clade as OsPUT1 (Mulangi, Chibucos, Phuntumart, & Morris, 2012). Based on conservation of key residues within the members of this clade and experimental evidence it has been postulated that all plant proteins in this clade are PA transporters (Mulangi et al., 2012a). Meanwhile, Fujita et al. (2012) have identified an Arabidopsis L-type amino acid transporter (LAT) family transporter, named RMVI as a transporter of PAs and its structural analog paraquat (Fujita et al., 2012). A mutation in AtPAR1 (At1G31830), identical to AtPUT2, results in lower levels of paraquat accumulation in the chloroplasts, suggesting that AtPUT2/AtPAR1 may be involved in intracellular transport of PQ into chloroplast (J. Li et al., 2013). The same group has reported that OsPAR1, identical to
OsPUT3, regulates rice sensitivity to paraquat just like AtPUT2/AtPAR1 (J. Li et al., 2013). Paraquat is translocated in plants via PA transporters (Fujita & Shinozaki, 2014). However, the translocation of paraquat is inhibited by exogenous application of PAs (Hart, Ditomaso, Linscott, & Kochian, 1992; Kurepa, Smalle, Van Montagu, & Inzé, 1998). The understanding of PA transport system in plants is still in its early phase and requires more research. There is a lack of studies involving analysis of growth, development, and stress responses in plants with increased or decreased expression or PA transporters. Here we show that:

1) Selected plants have a third pathway for putrescine biosynthesis.

2) Change in expression of Polyamine Uptake Transporters alters the growth and development of Arabidopsis thaliana.

3) Increased expression of OsPUT3 enhances drought tolerance in Arabidopsis thaliana.
References


expressing the homologous arginine decarboxylase 2 gene. *Plant Physiology and Biochemistry, 48*(7), 547-552.


CHAPTER 1: HIDING IN PLAIN SIGHT: A THIRD ROUTE FOR PUTRESCINE BIOSYNTHESIS IN PLANTS

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Abstract

Transgenic manipulation of polyamine levels has provided compelling evidence that polyamines enable plants to respond to environmental cues by activation of stress and developmental pathways. While it has been thought that A. thaliana has only a single cytosolic pathway for putrescine synthesis, here we show that both A. thaliana and soybeans have a plastid-localized putrescine pathway consisting of an arginine decarboxylase and an enzyme with arginase/agmatinase activity. Since the sequences of plant arginases show conservation of key residues and the predicted 3D structure of plant agmatinases overlaps the crystal structure of the enzyme from Deinococcus radiodurans, we suggest that these enzymes can synthesize putrescine, whenever they have access to the substrate agmatine. Finally, we demonstrate that the synthesis of putrescine by ornithine decarboxylase is localized to the endoplasmic reticulum. Our observations point to a complex compartmentalization of putrescine synthesis in plants.
Introduction, Results and Discussion

A distinguishing feature of eukaryotic cells is the presence of membrane-bound organelles and vesicles. In plants, these cellular rooms have multiple functions that include the specialized compartmentation of metabolic processes such as carbon fixation, and respiration, thylakoid membranes to capture light energy, and the sequestering of waste products or anti-feedants that are normally released only upon cell lysis (Lunn 2007).

In addition to its role in photosynthesis, the plastid plays a major role in the biosynthesis of amino acids, nucleotides, fatty acids, and vitamins. This compartmentation of metabolism requires the organized import and export of metabolites, but also prevents futile cycling of metabolites (Linka and Weber 2010). An additional feature of plant metabolism is the redundancy of certain metabolic pathways (Plaxton 1996, Krueger, Niehl et al. 2009, Ros, Munoz-Bertomeu et al. 2014). This is also the case for polyamine biosynthesis. Ornithine decarboxylase (ODC) converts ornithine directly to putrescine (Fuell, Elliott et al. 2010) and then putrescine serves as one of the building blocks for spermidine, thermospermine, and spermine. A second route for putrescine biosynthesis, utilizes arginine decarboxylase (ADC) to convert arginine to agmatine, and two additional enzymes, agmatine deiminase (AtAI) and N-carbamoyl putrescine aminohydrolase (At-NLP1) are needed to complete the pathway.

Polyamines are essential for cell viability (Imai, Matsuyama et al. 2004), and levels of putrescine along with the other polyamines spermidine, and spermine are strongly correlated with cellular responses to development and various environmental stresses (Tiburcio, Altabella et al. 2014). Two independent pathways for polyamines enable plants to differentially activate the pathways in response to an environmental stimulus. In the course of evolution, A. thaliana has lost ODC, and thus the second known pathway, but now has two ADC’s (Hanfrey, Sommer et al. 2001).
The two ADCs also show a strong divergence in expression, and up-regulation of ADC2 has been implicated in multiple stress responses (Winter, Vinegar et al. 2007). Presently there is no experimental evidence to indicate where these proteins are localized in the plant cell (Sun, Zybaiov et al. 2009). However in oats and tobacco, an ADC was localized to the chloroplast (Borrell, Culiañez-Macià et al. 1995, Bortolotti, Cordeiro et al. 2004). Spatial separation of the two ADCs between the chloroplast and the cytosol in A. thaliana could indicate that agmatine produced by the two enzymes is diverted to different metabolic pathways.

In E coli and humans, putrescine can be synthesized in a two-step process from arginine by arginine decarboxylase, which makes agmatine; and agmatinase, which converts agmatine to putrescine with the release of urea (Heller, Rostomily et al. 1983, Satishchandran and Boyle 1986, Mistry, Burwell et al. 2002). Phylogenetic analyses suggest that plant arginases may be more similar to bacterial agmatinases than bacterial or mammalian arginases (Chen, McCaig et al. 2004). Enzymes with agmatinase activity are members of the ureohydrolase superfamily that also include enzymes with arginase, forminoglutamase, and proclavaminate amidoinohydrolase activities (Ahn, Kim et al. 2004). However plant enzymes with arginase activity appear to have little or no agmatinase activity (Hwang, Kim et al. 2001, Chen, McCaig et al. 2004).

A. thaliana contains two predicted genes with Arginase/Agmatinase activity (At4g08870 and At4G0890) that have not been fully characterized. Both proteins have been localized to the plastid in independent proteome experiments (Sun, Zybaiov et al. 2009). Non-aqueous fraction, followed by MS analysis has emerged as a means of predicting the localization of metabolites to specific subcellular compartments (Krueger, Giavalisco et al. 2011). Using this approach, arginine, and the polyamines, putrescine and spermidine, were found to be enriched in the chloroplast fraction of A. thaliana. To summarize, localization of the two ADCs, has not been
established, but both the substrate and product for ADC are enriched in the plastid fraction (Krueger, Niehl et al. 2009). MS analysis provides support for one enzyme with potential agmatinase activity being associated with the chloroplast (At4g08870), but this enzyme has also been associated with mitochondrial fractions, and its activity as an agmatinase has not been demonstrated.

To address gaps in the localization of polyamine biosynthesis, we first sought to confirm the cytosolic location of ADC1 by transient expression of N-terminal and C-terminal GFP fusions in tobacco leaves. Both constructs were found to localize to the cytosol. Since ATAIH and ATNLP have been previously localized to the cytosol in a proteomic study (Ito, Batth et al. 2011) all of the enzymes of this putrescine pathway are localized in the cytosol. The Arabidopsis genome contains two ADCs, one of which has a predicted chloroplast translocation signal (Table S2) and two genes with predicted arginase/agmatinase activity. One of these, (ARGAH2), was found to be associated with the chloroplast proteome (Kleffmann, Russenberger et al. 2004). Transient expression of ADC2-GFP and ARGAH2-GFP in N. benthamiana leaves showed that both these proteins are localized to the chloroplast (Fig. 2). To investigate whether these two enzymes could function together in a cell to synthesize putrescine, the two genes were tested together using a yeast complementation assay. Yeast mutants lacking ornithine decarboxylase (Spe1) are deficient in the synthesis of putrescine, and cease growth in the absence of exogenous polyamines (Schwartz, Hittelman et al. 1995). Co-transformation of an oat ADC and either a human or an E. coli agmatinase gene has previously been used to reconstitute a putrescine biosynthetic pathway in yeast (Mistry, Burwell et al. 2002). Using a similar strategy we transformed AtADC2 and AtARGAH2 in the yeast mutant Spe1. Transformants expressing only AtADC2 or AtARGAH2 did not grow in the absence of exogenous putrescine (Fig. 3).
Expression of both genes fully complemented the growth defect of *Spe1*. Thus the two chloroplast-localized genes are capable of working in concert to synthesize putrescine.

**Figure 2.** Localization analysis of AtADC2 and AtARGAH2 by transient expression in *N. benthamiana* leaves. *N. benthamiana* leaves were infiltrated by *Agrobacterium tumefaciens* (GV3101) containing expression plasmids harboring the full-length AtADC2 or AtARGAH2 fused to *GFP* at its C terminus under the control of the CaMV 35S promoter. GFP fluorescence and chlorophyll autofluorescence of transformed leaves were observed by confocal microscopy.

**Figure 3.** Characterization of *Arabidopsis* agmatinase by complementation in yeast. WT, *spe1, spe1+ AtADC2, spe1+AtARGAH2* and *spe1 + AtADC2 +AtARGAH2* were plated on SC minimal media in the presence and absence of exogenous putrescine.

To investigate whether this pathway might exist in other plants, we examined the genomes of several species for evidence of multiple ADCs and multiple genes with predicted agmatinase activity. We searched 43 sequenced and annotated green plant genomes from Phytozome for a plastid-localized putrescine pathway. We found that, 23 of 43 sequenced and annotated plant genomes had more than one *ADC*, and 12 of these genomes had at least one *ADC* with predicted localization to the plastid (Table S2). Within in this subset of plant genomes, 13
genomes included more than one gene with predicted ARGAH activity. Phylogenetic analysis showed that all of the ADCs from seven Brassica genomes grouped together in two clades of nine and five sequences (Fig. S1). *AtADC2* was in a clade separate for AtADC1 and all the members of the clade with *AtADC2* which included at least one gene from each species, were predicted to be localized to the plastid. Other dicot species with two ADC genes included cacao, strawberry, tomato, potato, soybeans and *Mimulus guttatus*. With the exception of soybeans, at least one of the ADCs in each of these species had a predicted plastid localization. The monocots; corn, rice and *Panicum virgatum* also contained two ADC genes, but none of these genes were predicted to be targeted to the chloroplast. We also noted that moss has three predicted ADC genes, and one of them is predicted to be localized in the chloroplast. A smaller number of species were found to have two genes with predicted ARGAH activity (Fig. S2). Species that contained two members of both ADC and ARGAH included four members of the Brassicaceae, along with tomatoes, and soybeans. Dicots that had duplications in only ARGAH, but not ADC, included cassava, flax, and poplar. No monocot species were identified with more than one ARGAH.

To determine whether a complete biosynthetic pathway for putrescine in the chloroplast might exist outside of the Brassicaceae, we used transient expression assays of both Glyma.03G028000, (ADC2); and Glyma03g03270, (ARGAH) in *N. benthamiana* leaves to show that these two genes are localized to the chloroplast (Fig. S4.). In the yeast complementation assay, the expression of *ATADC2* and Glyma03g03270 in the yeast mutant strain Spe1 enabled the yeast strain to grow in the absence of exogenous putrescine. Thus soybeans, along with *A. thaliana* have a complete chloroplast-localized putrescine biosynthetic pathway. Sequence alignment of plant agmatinases retrieved from phytozome showed that these proteins have a high
level of sequence conservation. Protein homology modeling of ARGAHs from *A. thaliana*, *Glycine max*, and *Populus trichocarpa* using PHYRE2 (Kelley and Sternberg 2009) showed that these proteins are structurally similar to agmatinase from *Deinococcus radiodurans* (DR) (Fig 4). These plant genes along with the human mitochondrial agamatinase (Ahn, Kim et al. 2004) have conservation of the critical Mn binding sites and the predicted catalytic residues. PHYRE2 analysis also revealed that the Mn binding sites and catalytic sites are highly conserved amongst the ARGAHs investigated and DR agmatinase (Fig S4). Thus we predict that the ARGAHs from *A. thaliana*, *Glycine max*, and *Populus trichocarpa* must have agmatinase activity.

**Figure 4.** Predicted 3D structure of *AtARGAH2* is highly similar to the crystal structure of *Deinococcus radiodurans* agmatinase. (A) DR agmatinase, (B) AT agmatinase (C) superpose of A and B. Structure of AT agmatinase was predicted using PHYRE2 and superposition of DR agmatinase and AT agmatinase was done using Chimera.

As an additional test of this hypothesis, we also tested the other *AtARGAH* gene in the yeast complementation assay. As expected, this gene was also able to complement the yeast mutant (Data not shown). Plant arginases exhibit a clear substrate preference for arginine over agmatine at mM levels (Chen, McCaig et al. 2004). The dual activities of these enzymes enables them to function both as arginases in the urea cycle during tissue senescence, and also serve as an agmatinase to synthesize putrescine at other times. Plant agmatine transporters have
not yet been characterized, but the transport of agmatine into the mitochondria could enable agmatinases located there, to synthesize putrescine.

The localization of ornithine decarboxylase has not yet been clearly established. However, since we noted spatial separation of the two putrescine biosynthesis pathways starting with ADC, we postulated that synthesis of ornithine decarboxylase might be spatially separated from the cytosolic ADC pathway. The subcellular localization of OsODC was determined by transient expression of GFP fusions in *Nicotiana benthamiana* leaves mediated by *Agrobacterium*. The GFP signals overlapped with the mCherry signals of the endoplasmic reticulum (ER) marker (Fig 5. A&B). Bombardment assay of onion epidermal cells confirmed the localization of ODC to the ER. Localization of ornithine decarboxylase to the ER may enable simultaneous operation of the urea cycle, and polyamine synthesis, which share a common substrate. It may also enable putrescine that is synthesized in the ER to be directly exported from the cell by secretion to the apoplast.
Figure 5. Subcellular localization of OsODC. A. Confocal images of *Nicotiana benthamiana* leaf cells expressing OsODC-GFP under control of a cauliflower mosaic virus (CaMV) 35S constitutive promoter and fused with a C-terminus GFP tag. Scale bars, 25μm. Pictures were taken 3 days after the infiltration of Agrobacterium into tobacco leaves. B. Confocal images of onion epidermis cells expressing OsODC-GFP under control of a cauliflower mosaic virus (CaMV) 35S constitutive promoter and fused with N-terminus GFP tag. Scale bars, 50μm. Pictures were taken 16 hours after the particle bombardment.

In this study we have confirmed the localization of two enzymes that enable the synthesis of putrescine in the chloroplast of *A. thaliana* and *G. max*. Our study also identified many plant species with two ADCs, some of which have been predicted to be localized to the plastid. However, plant genomes with two ADCs and two agmatinases are not common, and notably, none of the sequenced monocot genomes contain two agmatinases. Thus the retention of a complete plastid pathway is relatively uncommon in plant genomes. Of the seven sequenced *Brassica* genomes with two ADC’s, only four were found to have retained two ARGAH genes. In this scenario, agmatine would then have to be exported from the plastid to be metabolized in the cytoplasm by the cytosolic pathway, or imported into the mitochondria to be converted to
putrescine by agmatinases located there. Given the importance of interspecies hybridization and whole genome duplication events in the evolutionary history of plants, the absence of a separate plastid pathway hints that for most plants, this pathway would be disadvantageous. Perhaps this is a consequence of that putrescine may also act as a permeant buffer in the thylakoid spaces, and under certain conditions impair the normal activity of the ETC (Ioannidis, Cruz et al. 2012, Ioannidis and Kotzabasis 2014). Some plant genomes such as flax and cassava have two agmatinases. In these plants, synthesis of putrescine may also occur, if there is a plastid-localized agmatine transporter or exchanger.

The persistence of two or more biosynthetic pathways for polyamines is a likely consequence of the multiplicity of roles that these compounds have been shown to have in developmental and stress responses of plants. The spatial organization of these pathways also highlight the importance of PA transporters in providing an additional means of regulating localized changes of these signaling compounds. We hope that the elucidation of this pathway may renew interest in manipulating PA levels to optimize crop responses to climate change.
References


Supplementary Material for Hiding in Plain Sight: A Third Route for Putrescine Biosynthesis in Plants

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Supplementary materials and methods

Genes and constructs
cDNA clones of Arabidopsis thaliana ADC2 (At4G34710) ARGAH 1 (At4G08900) ARGAH2 (At4G08870) were obtained from ABRC. Genes were amplified via PCR using gene-specific primer pairs listed in Table S1. AtADC1 was amplified from genomic DNA of A. thaliana Col-0. Rice genomic DNA was isolated from the rice cultivar Nipponbare using the CTAB method (Clarke 2009). The full length OsODC gene was amplified from the genomic DNA. GmODC was amplified by PCR using genomic DNA from the cultivar Williams. The sequence of Glyma.03g028000 (GmARGAH) was codon-optimized for expression in yeast and synthesized by GenScript, Piscataway, NJ. GmADC (Glyma.06G007500)...

Subcellular localization analysis
AtADC2, AtARGAH2, AtADC1, OsODC, GmADC, GmARGAH, and GmODC were cloned into plant expression vectors pGWB5/pGWB6 to generate constructs with C or N terminal GFP, using GATEWAY recombination system (Nakagawa et al. 2007). Inserts were verified by PCR.
and/or sequencing. The resulting constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into tobacco (*Nicotiana benthamiana*) leaves (Sparkes et al. 2006). Infiltrated leaves were subjected to confocal laser-scanning analysis after 12-48 hours of infiltration with a TCS SP5 multi-photon laser scanning confocal microscope. Fluorescence of chlorophyll or mCherry tagged ER Marker (ER-rbCD3960) (Nelson et al. 2007) was used as organelle markers. Excitation wavelengths of 488 nm for GFP and 590 nm for mCherry were used. Fluorescence was detected at 510 nm for GFP and at 590 nm for mCherry. Images were merged using ImageJ (Rasband, 2008).

**Yeast complementation assay**

The *SPE1* yeast knockout strain that lacks ornithine decarboxylase (YSC6273-201936543) was obtained from GE Dharmacon, Lafayette, CO. BY4741 served as a wild-type control. Yeast strains were maintained on enriched medium (YEPD) or SC minimal medium supplemented with 1 mM putrescine. The two plasmid constructs pAG303-ADC2 and pYES-DEST52-ARGAH2 were introduced to yeast SPE1 mutant strain separately. Competent yeast spe1 mutant cells were grown on liquid YEPD medium and incubated overnight at 30°C. The pAG303-ADC2 plasmid construct was introduced to yeast spe1 mutant cells by electroporation. The resulting transformants were selected on SC minimal medium lacking histidine and containing putrescine (1 mM). Selected colonies were transferred to liquid YEPD medium to obtain competent cells. The pYES-DEST52-ARGAH2 plasmid construct was then introduced to the above competent cells by electroporation. The resulting transformants were again selected on SC minimal medium plates lacking uracil and containing putrescine (1mM). Finally, selected transformants were tested for growth in SC minimal media with 1% raffinose and 2% galactose lacking exogenous polyamines.
Phylogenetic analysis

Protein sequences of ADC and agmatinase/arginase were obtained from assembled plant genomes (Goodstein et al. 2012). The amino acid sequence alignment was created using MUSCLE (Edgar 2004). Phylogenetic trees were constructed by MEGA 6.06 (Tamura et al. 2013) using the maximum likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model. The reliability of the trees was tested using a bootstrapping test with 1000 duplicates.

Structural modelling

Three-dimensional structures of arginases/agmatinases from Arabidopsis, soybeans, and poplar were predicted using Phyre2 (Kelley & Sternberg, 2009). The predicted structures were then compared to three-dimensional structure of Dienococcus radiodurans agmatinase (PDB entry 1WOHA) using Chimera (http://www.cgl.ucsf.edu/chimera). The 3D structures were superimposed using matchmaker tool of Chimera. Active site regions of DR. agmatinase and plant arginases were compared using match-align tool of Chimera.
Supplementary figures and tables

**Table S1.** List of primers used in this study.

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Table S2. Predicted localization of Arginine decarboxylase genes from sequenced plant genomes

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Figure S1. Phylogenetic analysis of plant arginine decarboxylases.
Figure S2. Phylogenetic analyses of Plant arginase/agmatinases.
**Figure S3.** Characterization of Soybean *agmatinase* by complementation in yeast. WT, *spe1*, *spe1* + *AtADC2*, *spe1* + *GmARGAH*, and *spe1* + *AtADC2* + *GmARGAH* were plated on SC minimal media in the presence and absence of exogenous putrescine.
Figure S4. Comparison of active site residues of *Dienococcus radiodurans* agmatinase and arginases from *Arabidopsis, Glycine max,* and *Populus trichocarpa*. Red boxes show the active site residues.
Supplementary references


CHAPTER 2: CONTROL OF FLOWERING IN *A. THALIANA* BY ALTERED EXPRESSION OF POLYAMINE TRANSPORTERS.


* These individuals contributed equally to this manuscript.

Introduction

The polyamines (PAs) putrescine, spermidine and spermine were amongst the first metabolites to be isolated from living organisms (Wallace, Fraser et al. 2003) but how these compounds function at a molecular level is largely uncharted territory. Polyamines have hormone-like properties in that changes in PA levels are associated with fundamental physiological processes such as embryogenesis, flowering, fruit development and ripening, senescence, and tissue responses to biotic and abiotic stresses (Tiburcio, Altabella et al. 2014); however, their concentrations are much higher than true hormones such as auxins, gibberellins, or brassinosteroids. In *A. thaliana*, putrescine levels range from 10-50nM/g FW while spermidine levels are typically higher (50-80nmol/g FW) and spermine levels fall in the range of 10-50 nmol/gFW (Naka, Watanabe et al. 2010, Alet, Sanchez et al. 2011). The identification of thermospermine in plant tissues is a more recent development, largely because this compound is not easily resolved from spermine (Naka, Watanabe et al. 2010). Levels of thermospermine in different tissues are estimated to be in the range of 0.5 to 2 nmol/g FW. Conjugated forms of polyamines that include alkaloids, and caffeic, coumaric, and ferulic acids form the largest pools of polyamines in plants (Paschalidis and Roubelakis-Angelakis 2005, Kusano, Berberich et al.
2008, Bassard, Ullmann et al. 2010). More recent evidence suggests that some phenolamides have specific roles that include pollen development (Tiburcio, Altabella et al. 2014) and defense responses to pathogens and insects (Bassard, Ullmann et al. 2010). The extent of back conversion of such compounds to the polyamines is also unknown.

These estimates of pool sizes may be misleading, as polyamines are positively charged at cellular pH and can interact with DNA, RNA, nucleotides, phospholipids and some proteins (Igarashi and Kashiwagi 2010). Thus, the pool of free polyamines in the cytosol is likely much smaller. The association of polyamines with other macromolecules has been estimated for spermidine and spermine for mammalian cells, and putrescine and spermidine in E coli. While polyamines were found to not 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 PAs. Putrescine levels were not estimated in mammalian cells. However in E coli, this polyamine was found to have a lower affinity for other macromolecules that 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 (Krueger, Giavalisco et al. 2011). In this study 61% of the spermidine and 45% of soluble putrescine were estimated to be localized with the chloroplast fraction, while the
cytosolic fraction contained 33% of the putrescine and 16% of the spermidine was associated with the cytosolic fraction.

In *A. thaliana*, polyamine synthesis is initiated by one of two arginine decarboxylases (ADCs). The product of this enzyme agmatine is then converted to putrescine by an agmatine deiminase (At-AI) and N-carbamoyl putrescine aminohydrolase (At-NLP1). In most plants, but not *A. thaliana* and other members of the Brassica family, an ornithine decarboxylase converts ornithine directly to putrescine. Knockouts of both ADC genes results in an embryo lethal phenotype. The lethality of this phenotype may be due to the fact that spermidine is a precursor of deoxyhypusine which is covalently linked to the conserved eukaryotic translation initiation factor eIF5A (Park, Nishimura et al. 2010). Loss of thermospermine synthesis in double knockouts of *ACL5/ACAULIS5* results in a stunting phenotype (Ortiz-Lopez, Chang et al. 2000). The expression of *ACL5* is restricted to vessel elements, so thermospermine acts in the same cells where it is synthesized (Muñiz, Minguet et al. 2008). The synthesis of *ACL5* is induced by auxin, and the accumulation of thermospermine acts to enhance the translation of the transcription factor SAC51. The leader sequence of SAC51 contains 5uORFs that acts to negatively inhibit the translation of the main ORF (Imai, Hanzawa et al. 2006). A mutation in one of these uORFs leading to a premature stop codon was found to suppress the thermospermine deficient phenotype. Since polyamines are known to bind to RNA (Igarashi and Kashiwagi 2010) it has been suggested that modification of the secondary structure of mRNA by thermospermine could enable the ribosome to translate the main ORF more efficiently (Takano, Kakehi et al. 2012). The expression of this gene negatively regulates the synthesis of auxin-responsive transcription factors (Yoshimoto, Noutoshi et al. 2012, Milhinhos, Prestele et
Taken together, these data suggest that control of thermospermine levels are essential for normal patterns of xylem cell differentiation.

The importance of polyamines in mediating the plant tissue responses to both abiotic and biotic stresses is now well established (Gill and Tuteja 2010) (Sagor, Berberich et al. 2013). Up-regulation of arginine decarboxylase in rice was found to confer enhance salt and drought tolerance in rice (Roy and Wu 2001, Capell, Bassie et al. 2004). Mutants deficient in arginine decarboxylase showed increased sensitivity to freezing, while transgenics overexpressing ADC showed increased tolerance to freezing (Cuevas, Lopez-Cobollo et al. 2008). In these experiments, feeding polyamines via the roots was also found to increase the freezing tolerance of the shoots in mutant plants. Thus long distance transport of polyamines synthesized in the roots may play a role in the cold acclimation response. In a follow-up experiment, the introduction of the oat arginine decarboxylase gene under the control of a stress inducible promoter has recently been shown to promote freezing tolerance without causing adverse plant growth effects (Alet, Sanchez et al. 2011). Spermidine levels in plants can be increased by increasing the activity of S-adenosylmethionine decarboxylase or spermidine synthase and these strategies have been employed to produce transgenic plants with increased tolerance to both biotic and abiotic stresses (Roy and Wu 2002, Cheng, Zou et al. 2009, Hazarika and Rajam 2011, Sagor, Berberich et al. 2013). Increased polyamine levels don’t simply increase tolerance to abiotic stress through their interactions with other macromolecules, they also impact genetic expression by modulation of signaling pathways (Marco, Alcazar et al. 2011, Sagor, Berberich et al. 2013, Tiburcio, Altabella et al. 2014).

In prior work, we identified a clade of transporters that act as highly specific polyamine uptake transporters (PUTs) (Mulangi, Chibucos et al. 2012, Mulangi, Phuntumart et al. 2012).
We hypothesized that since PUTs function as highly specific polyamine transporters that are present in all plants, natural variation in the expression of these transporters, or their substrate specificity would likely exist across plant accessions. If different rates of PA transport between tissues was not compensated for by changes in biosynthesis or metabolism, then these changes in PA levels would trigger phenotypic changes in the plants. Indeed, genetic variation in one PUT transporter across different accessions of *A. thaliana* was found to confer increased tolerance to the herbicide paraquat (Fujita, Fujita et al. 2012, Fujita and Shinozaki 2014).

The role of polyamines as an additional regulator of flowering has established using both feeding experiments and transgenic manipulation of polyamine biosynthesis. In *A. thaliana*, overexpression of ADC2 produced a dwarfing phenotype and a delay in flowering, both of which were alleviated by the addition of gibberellic acid (GA) (Alcazar, Garcia-Martinez et al. 2005). In *Sinapsis alba*, the transition to flowering was marked by changes in the levels of polyamines and polyamine conjugates in xylem and phloem exudates. Furthermore the inhibition of flowering by the application of the putrescine inhibitor DFMO to the leaves and its reversal by application of putrescine to the roots suggest that putrescine production and export from the leaves regulated the timing of flowering (Havelange, Lejeune et al. 1996). In this study we demonstrate that flowering can be controlled by differential expression of PUTs.

Materials and Methods

**Plant material and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used for gene transformations and phenotypic analysis. Seeds were surface sterilized with 70% alcohol for 2 minutes and 10% bleach solution for 10 minutes, rinsed five times with sterile distilled water and sown onto ½
strength MS plates for germination or planted on soil directly. Plants were grown in a growth chamber at 22°C with relative humidity of 55% under long-day conditions (16 h light/8 h dark).

**Subcellular localization analysis**

Rice clone OsPUT1 was obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/index.html). Plasmids expressing AtPUT2 and AtPUT3 were obtained from Arabidopsis Biological Resource Center (ABRC, Ohio, USA) (Alonso et al., 2003). Full-length cDNA of these transporters was amplified (see Appendix for primer sequences), for half transporters 750bp of OsPUT1 were amplified from C or N terminal. The resulting PCR products were cloned into pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, USA) according to the supplier’s protocol. Subsequently, the target gene was transferred to a destination/expression vector, pGWB5/pGWB6 by LR recombination reaction (Invitrogen, Carlsbad, USA) to generate a construct containing the cDNA flanked with C or N-terminal GFP under the control of CaMV 35S promoter. The resulting constructs were then transformed into Agrobacterium strain GV3101 and infiltrated into tobacco (Nicotiana benthamiana) leaves (Sparkes, Runions, Kearns, & Hawes, 2006). The GFP fluorescence was visualized and photographed with a laser scanning confocal microscope (TCS SP5 multi-photon laser scanning confocal microscope - Leica Microsystems). Overlay of GFP and chlorophyll autofluorescence was created using ImageJ (Rasband, 2008).

For transient expression in onion epidermal cells, onions were cut in to ~1cm² pieces and placed on a wet filter paper in a petri dish, with the inner side oriented upward. Purified DNA was then coated on gold particles and the resulting gold particles were placed on the delivery disk. Onion cells were bombarded at 1300 psi using the PDS-1000 He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA, USA). Following bombardment, the cells were incubated in the
same Petri dishes for 16-24 h at room temperature in dark, before observation. GFP fluorescence was observed using a Zeiss Axiophot microscope equipped with a Micromax cooled CCD digital camera, and electronic shutter control. Images were recorded with Metamorph software (http://www.spectraservices.com/METAMRPH.html).

Generation of transgenic *Arabidopsis* plants

The full-length coding sequences of *OsPUT1* and *OsPUT3* were cloned into pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, USA) according to the supplier’s protocol. The resulting plasmids were used to mobilize target genes into plant expression vector, pGWB2, to generate pGWB2-*OsPUT3* and pGWB2-*OsPUT1* by LR recombination reaction (Invitrogen, Carlsbad, USA). The plant expression vectors were then transformed into *Agrobacterium* strain *GV3101* followed by a transformation into *Arabidopsis* Col-0 plants using the floral dip method (Zhang, Henriques, Lin, Niu, & Chua, 2006). Transgenic plants were screened by germination of seeds on ½ MS plates containing 100ug/ml of kanamycin. T3 homozygous lines were used for further analysis.

Transgenic plants expressing *AtPUT5::OsPUT1* were generated by cloning and transforming, the 5’ upstream region (1 kb) of At3g19553 followed by the full-length coding sequence of *OsPUT1* in pEG301 (Earley et al., 2006) using the same steps as described above. These plants were selected by spraying with BASTA.

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). Leaf chlorophyll content was measured by a Fluorpen FP 100 (Photon System Instruments) (Woo,
Badger, & Pogson, 2008). Leaf area was measured by taking pictures of leaves from 4 week old plants and using ImageJ (Rasband, 2008).

GUS Analysis

The 5’ upstream region of At3g19553, 1 kb in length, was amplified by PCR. The amplified product cloned into the Gateway promoter analysis vector pBGWFS7 (Karimi, Inze, & Depicker, 2002) by Gateway technology. The resulting construct was then transformed in A. thaliana (Col-0) by floral dip method (Zhang et al., 2006). Transformants were selected by using BASTA (Sigma-Aldrich, St. Louis, MO). BASTA resistant seedlings were selected, transferred to soil and grown at 22°C in a growth chamber.

Histochemical staining of GUS expression was performed by staining 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) (Jefferson, Kavanagh, & Bevan, 1987). After staining, chlorophyll was extracted from photosynthetic tissues with 70% ethanol. GUS staining was observed in young and old plants.

HPLC analysis of WT and transgenic plants

Reversed-phase HPLC was performed on Agilent technologies 1200 series HPLC and Applied Biosciences 980 programmable fluorescence detector (excitation at 340nm, emission at 500nm). Dansylated polyamines were separated on a Gemini® 5 μm C18 110 Å, 250 × 4.6 mm LC column.

Rosette leaves of WT and transgenic plants were collected at two, four and six weeks. Six replicates were made for wild type and each transgenic line. Sample preparation was carried out according to the method of Marce et al., (1994) with some modifications. Leaves of Arabidopsis plants were extracted in 5% cold perchloric acid (PCA)
(300 mg fresh weight/ml) in an ice bath. After extraction, the samples were centrifuged at 27,000 g for 10 minutes. The supernatant, containing the free polyamines, was stored at -20°C until dansylation. The pellet was re-suspended in 1 M NaOH (300 mg fresh weight/ml) and stored at -20°C until dansylation.

To determine the concentration of soluble and insoluble polyamine conjugates, a 300 μl aliquot of the supernatant or pellet was mixed with 300 μl of 12 M HCl into an injectible vial. The vial was then sealed with a flame and heated at 100°C for 20 hours. The resulting mixture was then transferred to an eppendorf tube and dried in a vacuum. The dried material was re-dissolved in 300 μl of 5% PCA and stored at -20°C until dansylation.

The polyamines were derivatised according to the method of Marcé et al., (1994) with some modifications. First, 200 μl of the perchloric acid extracts were mixed with 40 μl of 0.05mM diamino heptane (HTD) as the internal standard. Next, 200 μl of a saturated solution of sodium carbonate and 400 μl of dansyl chloride (10 mg/ml acetone) were added. The resulting mixture was vortexed briefly and incubated in the dark overnight. Then, 100 μl of a proline solution (100 mg/ml water) was added to react with excess dansyl chloride and vortexed briefly. The mixture was incubated in dark for 30 minutes. After incubation, the dansylated polyamines were extracted in toluene according to the method of Minocha et al., (2004) with some modifications. 350 μl of the aqueous solution was transferred to a separate microfuge tube and 500 μl of toluene was added. The tube was vortexed for 1 minute and centrifuged at 13 500 g for 1 minute. After transfer of the first 350 μl of the aqueous solution, the remaining portion was also extracted with 500 μl of toluene in the same way. This extraction was repeated two times. Then toluene was evaporated from each tube with a stream of air and the dry polyamines were dissolved in 500 μl of methanol. The tubes were vortexed for 1 minute to thoroughly dissolve the
Dansylated polyamines were extracted with toluene and transferred to a glass vial since in the aqueous phase they stick to the walls of the original microfuge tube in which the reaction was carried out and do not proportionately get transferred to the new tube. A blank sample was run using dansylated 5% PCA. 1 mM polyamine standard stock solutions were made in 5% PCA and they were also dansylated following the same procedure. Finally, the dansylated samples were filtered through a 0.45 μm pore size syringe filter and 50 μl were injected to the HPLC.

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. After that, the concentration of methanol was further increased to 95% over 17 minutes. These conditions were kept constant for 1 minute and then returned to initial conditions. After each cycle, the column was washed with 100% methanol for five minutes and re-equilibrated for five minutes.

Results

In our prior work, members of a diverse clade of transporters in the amino acid superfamily of plants were found to function as highly selective PUTs by heterologous expression in yeast mutants (Mulangi, Chibucos, Phuntumart, & Morris, 2012). While the yeast uptake experiments were consistent with the localization of this gene to the plasma membrane, full length constructs of OsPUT1-GFP that were transiently expressed in both onion epidermal cells and tobacco leaves showed it to be localized to vesicular compartments (Fig. 6). To determine whether the localization was a consequence of a mis-folding of the protein-GFP
complex we created half-transporter fusions of OsPUT1-GFp and examined their localization by agroinfiltration of \textit{N. benthamiana} leaves, but it was not possible to determine the subcellular localization of the protein (Fig. 7).

\textbf{Figure 6.} Localization of full-length OsPUT1. (A) 35S::\textit{OsPUT1::GFP} in onion epidermal cells, (B) BF image of onion epidermal cells, (C) 35S::\textit{OsPUT1::GFP} in tobacco leaf tissue, (D) 35S::\textit{GFP} in onion epidermal cells, (E) BF image of onion epidermal cells, (F) 35S::\textit{GFP} in tobacco leaf tissue. BF = Bright Field, GFP = Green Fluorescence Protein.
Figure 7. Localization of half OsPUT1-GFP fusion protein (750 bp from the N-terminal of OsPUT1 was fused with GFP). (A) 35S::halfOsPUT1::GFP in tobacco leaf tissue, (B) Autofluorescence of chlorophyll, (C) Composite of A, B and bright field.

In contrast, transient expression of AtPUT3–GFP fusions show that this gene is localized to the chloroplast (Fig. 8). This localization pattern supports software prediction programs that also indicate that this gene has a plastid targeting signal. However other work has shown that in transient expression of this gene in rice protoplasts indicate that the gene fusion is restricted to the Golgi (Li, Mu et al. 2013). In A thaliana, two PUTS were found to have predicted chloroplast transit peptides, and both were found to be localized to the plastid (Fig. 8).
To test the hypothesis that alterations in the activity of selective polyamine transporters would result in changes in the levels of polyamines and that these changes would alter the plant phenotype, we made stable transformants in *A. thaliana* using the *CaMV* constitutive promoter to drive the expression of OsPUT1 and OsPUT3. Overexpression of OsPUT1 was hypothesized to potentially limit the export of PA from leaf tissues by reimporting PA that had been exported into the apoplast by other transporters. Overexpression of OsPUT3 was hypothesized to potentially change cytoplasmic levels of polyamines by importing more PAs into the plastid. Overexpression of heterologous rather than native genes was used to avoid issues dealing with gene silencing.

Plants expressing OsPUT1 and OsPUT3 grew at the same rate as WT plants (Fig. 9A) butthere
was a delay in the transition to flowering (Fig. 9B). At six weeks we noted transgenic plants had large leaves, thicker stems and produced almost twice as many siliques. In contrast to WT plants, maturation and senescence of siliques occurred very slowly (Figs. 10-13).

**Figure 9.** Transgenic plants overexpressing OsPUT1 and OsPUT3 are delayed in flowering. (A) Wildtype (WT) and transgenic plants at 3 weeks, (B) WT and transgenic plants at 4 weeks.
Figure 10. Transgenic plants with increased expression of OsPUT1 and OsPUT3 had larger leaves. (A) Photograph of WT and transgenic leaves, (B) Leaf area in cm²

Figure 11. Transgenic plants with increased expression of OsPUT3 had thicker stems. Stem thickness of WT and transgenic plants expressing 35S::OsPUT3 was measured using Vernier caliper. Plants were 4-5 weeks old.
Figure 12. Transgenic plants with increased expression of OsPUT3 had increased yield. Number of siliques in WT and transgenic plants expressing 35S::OsPUT3 were counted in 6 week old plants (n=4).
Figure 13. Transgenic plants with increased expression of OsPUT1 and OsPUT3 were delayed in senescence. (A) Arrows show green siliques in transgenic plants, (B) Leaf senescence was determined by measuring Fv/Fm values from leaves of ten WT and transgenic plants.

Since the transition to flowering is controlled by transport of metabolite and floral signals from the leaf to the shoot apical meristem, we hypothesized that altered expression of a PUT that was primarily expressed in the leaf might also affect the transition to flowering. Microarray analysis of At3g19553 (AtPUT5) indicates that this gene shows highest expression in mature leaves, the cauline leaf, and the stem (Winter, Vinegar et al. 2007). To determine how increased expression of a PUT in these tissues might affect plant development, we fused the upstream promoter sequence of At3g19553 to GUS and OsPUT1 and transformed these two constructs into A. thaliana. Independent transformants were examined by GUS staining. The highest level
of expression was observed in leaves, vascular tissue and developing flowers (Fig. 14). To determine whether altered expression of PUT transporters affects flowering, transformants were grown alongside WT and homozygous SALK mutants in both continuous light and long days. FluorPen measurements indicated no significant difference in growth rate of WT, KO or transformants expressing OsPUT1. However the transition to flowering occurred earliest in KOs, followed by WT, and plants expressing \textit{AtPUT5::OsPUT1}. The transgenic plants were also considerably taller, had thicker stems, increased number of siliques and larger leaves relative to the WT plant (Figs. 15-19).

\textbf{Figure 14.} Gus expression of promoter region of \textit{AtPUT5} in \textit{A. thaliana}

Polyamine levels of WT and transgenic plants were assessed at three intervals during the growth of plants at two weeks, four weeks and six weeks. At two weeks the wild type plants were at the early stages of development with no floral stem and at four weeks the plants were at the flowering stage with excessive flowering while the transgenics were at the beginning of the flowering stage with little flowering. At six weeks the wild type plants were in their seeds stage.
with a large number of dried siliques, while the transgenics had fresh siliques.

**Figure 15.** Plants expressing *AtPUT5::OsPUT1* were delayed in flowering.
**Figure 16.** Plants expressing *AtPUT5::OsPUT1* were taller compared to WT and CS869507.

**Figure 17.** Plants expressing *AtPUT5::OsPUT1* had larger leaves compared to WT and CS859607.
Figure 18. Expression of *AtPUT5::OsPUT1* in *A. thaliana* resulted in increased silique production.

Figure 19. Plants expressing *AtPUT5::OsPUT1* had thicker stems as compared to WT and CS859607.
Polyamine levels in leaves fluctuated during different developmental stages of the plant. Polyamine titers decreased during the flowering and seed stage in all plants except in OsPUT1 over expressing plants (Fig. 20).

![Graphs showing polyamine levels](image)

**Figure 20.** Levels of polyamines in WT, OsPUT1, and OsPUT3 plants before, during, and after flowering under continuous light. (A) Polyamine levels in WT plants at two, four and six weeks, (B) Polyamine levels in OsPUT1 plants at two, four and six weeks, (C) Polyamine levels in OsPUT3 plants at two, four and six weeks.

Higher levels of polyamines, especially spermidine was observed in all the transgenic plants that showed a delay in flowering. During the early stages of development, OsPUT1 over expressing plants showed significantly higher levels of soluble (S) and hydrolysable soluble (HS spermidine compared to the wild type plants. During flowering and seed stages, all polyamine fractions remained higher except for those recovered by hydrolyzing the pellet. OsPUT3 over expressing plants showed significantly higher levels of (HS) HS-spermidine throughout the life span and HS-spermine during the seeds stage compared to the wild type plants (Fig. 20).
Transgenic plants over expressing OsPUT1 under the control of AtPUT5 promoter showed significantly higher levels of S-putrescine, S-spermidine, HS-spermidine, soluble and soluble conjugated spermine during early stages of development compared to the wild type and mutant plants. During the flowering stage, the transgenic plants showed significantly higher levels of HS-spermidine compared to the mutants. During the seeds stage, the transgenics had significantly higher levels of HP-spermidine compared to wild type plants. Overall, CS859607, mutant of OsPUT5 showed lower levels of polyamines compared to the over expressing plants and wild type plants (Figure 21).

**Figure 21.** Polyamine levels in CS859607, WT, and OX 19553 were assessed at two, four and six weeks.
Discussion

In this study we have shown that constitutive expression of a PUT transporter that is localized either to the chloroplast or the plasma membrane results in a delay of flowering. Polyamines that are synthesized in the leaves are transported to the shoot apical meristem (Caffaro, Antognoni et al. 1994). In a similar manner we have used a SALK KO line of AtPUT5 and a promoter fusion of this gene with OsPUT1 to show that altered expression of a leaf PUT also results in a delay of flowering. HPLC analysis indicated that prior to the onset of flowering, levels of spermidine and spermidine conjugates fell in the leaves. In contrast, we noted that when flowering was delayed, levels of spermidine and spermidine conjugates remained high.

In prior work, the inhibition of spermidine synthesis resulted in a delay of flowering in both in plants transitioning from short days (SD) to long days (LD) and under continuous LD (Applewhite, Kaur-Sawhney et al. 2000). Since polyamine levels in the flowers were substantially higher than in other organs, the delay of flowering was attributed in part to the association of high levels of PAs in tissues undergoing high rates of mitotic activity. The addition of spermidine to the media promoted the rate of flowering under non-permissive conditions in both the Colombia ecotype and a late flowering mutant. In contrast under permissive LD conditions, the addition of spermidine to the media delayed the flowering of the Columbia ecotype. Flowering was also delayed in transgenic lines overexpressing AtADC2 (Alcazar, Garcia-Martinez et al. 2005). 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 reported and was rescued by gibberellin treatment. The inhibition of flowering that we described in the three different constructs is
correlated with the accumulation of spermidine and spermidine conjugates in the rosette leaves may also act by inhibiting the gibberellin response pathway in leaves, although other mechanisms are possible.

Polyamines have been previously shown to inhibit ethylene biosynthesis in a variety of plant tissues (Mattoo and Handa 2008). S-adenosylmethionine (SAM) is both a precursor for the synthesis of higher order polyamines as well as the entry substrate for ethylene biosynthesis (Mehta, Cassol et al. 2002). Spermidine synthesis makes use of the substrates putrescine and the aminopropyl group produced by SAM decarboxylase to form spermidine and methylthioadenosine (MTA). Spermine synthase in turn uses the aminopropyl group and spermidine to form spermine and MTA. Ethylene synthesis is a two-step pathway that first uses ACC synthase to convert SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and MTA. In the second step of the pathway ACC oxidase converts ACC to ethylene. Thus both pathways require SAM and produce the same byproduct MTA to be recycled. When the yeast SAMdc was introduced into tomato plants under the control of E8 a ripening specific promoter, the resulting plants had higher lycopene levels and prolonged shelf life as ripe tomatoes (Mehta, Cassol et al. 2002). Because the transgenic fruits produced more ethylene than the non-transgenics during the ripening of the fruit the authors concluded that the availability of SAM was not limiting for either pathway. Over expression of the yeast spermidine synthase gene under the control of a CaMv35S and the E8 fruit ripening promoter has also been used to alter polyamine levels in developing fruit of tomatoes (Nambeesan, Datsenka et al. 2010). In the CaMv35S lines constitutive expression of spermidine synthesis resulted in a delay of whole plant senescence. The delay in senescence caused by increased levels of spermidine is similar to the pattern we observed in plants with constitutive expression of a PUT transporter. Importantly, the delay in
whole plant senescence in our transgenic lines was not observed when the *AtPUT5* promoter was used to drive the expression of the rice *OsPUT1*.

*A. thaliana* and other plant species display a striking diversity of phenotypes that enable them to optimally adapt to different habitats across a wide geographical range. These changes in plant growth rates and architecture are the consequences of substantial changes in gene expression. Such changes must be mediated by different transcription factors. Developmental processes such as the timing of flowering and vascular development can be modulated by several interconnected regulatory pathways. Because of the complexity of these pathways our first assumption was that mutational changes in the expression of several key genes including transcription factors would be needed to enable a species to expand its geographical range. If several mutational changes were need to enable a species to successfully occupy a new environmental niche, then the expansion of geographical range by that species would likely be slow. The transgenic plants described in our experiments mirror phenotypes such as delayed flowering and larger size, which are seen in native populations of *A. thaliana* and other species. While the phenotypic changes are due to reprogramming of several regulatory pathways, we did so here by altering the expression of polyamine transporters. In the simplest case, *AtPUT5* driven overexpression of the rice gene *OSPUT1* is equivalent to mutations occurring naturally that result in increased expression of *AtPUT5*. Because changes in polyamine levels appear to mediate their effects by activation of hormone responsive pathways (Marco, Alcazar et al. 2011, Tiburcio, Altabella et al. 2014), we suggest that altered expression of polyamine transporters may be one of the strategies that different plants have used to delay flowering. How cells perceive a change in spermidine levels and how those changes result in changes in gene expression remains an open question. Thus far we have shown that we can generate significant
changes in plant phenotypes by altering the expression of different members one class of polyamine transporters. The number of different types of polyamine transporters is presently unknown, but in unpublished work in our lab, we have characterized three different classes of polyamine transporters.


"Putrescine accumulation in Arabidopsis thaliana transgenic lines enhances tolerance to dehydration and freezing stress." *Plant Signal Behav* 2:278-286(2).


CHAPTER 3: OVEREXPRESSION OF OSPUT3 CONFER DROUGHT TOLERANCE IN ARABIDOPSIS THALIANA

Introduction

Crops in field environments experience a variety of biotic and abiotic stresses during their development that limit their productivity. When plants experience unfavorable growth conditions, there is a yield gap, which results in yields that are significantly lower than the maximum yield potential (Lobell, Cassman, & Field, 2009). In the main growing areas of the world, the yield gaps for three major cereals maize, rice, and wheat are 30, 75 and 40% respectively (Fischer, Byerlee, & Edmeades, 2009). Abiotic stresses are responsible for almost 50% of crop yield reductions each year (Wang, Vinocur, & Altman, 2003). Drought stress is one of the most economically important abiotic stresses that affects the performance and yield of plants (Fujita et al., 2006). The change in the hydrological cycle will result in reduced plant productivity because of drought and reduced soil moisture in the drier regions (Schmidhuber & Tubiello, 2007). Thus, there is an urgent need for developing plants that are resistant to drought stress while maintaining high yield.

Stress tolerance in plants depends on the genetic make-up of a plant and their ability to reprogram gene expression and metabolism (Fujita et al., 2006). Recent studies suggest that amino acid and polyamine (PA) metabolism play key roles in plant response to abiotic stresses (G. Liu et al., 2010; Marina et al., 2008; Moschou et al., 2008; Zeier, 2013). Plants exposed to environmental stresses such as drought, salinity, chilling, heat, hypoxia, ozone, UV, and heavy metals display increased levels of PA (Alcazar et al., 2010; Gill & Tuteja, 2010). Changes in PA levels are mainly produced by alterations in PA homeostasis by change in PA biosynthesis, PA oxidation, and/or crosstalk between other pathways in response to stress (Tiburcio et al., 2014).
Differential regulation of PA biosynthetic genes under abiotic stress has been revealed by transcriptomic studies (Alcázar et al., 2006). Overexpression of PA biosynthetic genes from different organisms in rice, tobacco and tomato induced tolerance to many stresses, and the enhanced tolerance to these stresses was correlated with increases in PA concentration (Alcázar et al., 2010; Gill & Tuteja, 2010). Increase in PA levels during stress conditions is often accompanied by an increase in PA oxidation (Tiburcio et al., 2014). Arabidopsis AtCuAO1 mutants are hypo sensitive to osmotic stress (Wimalasekera, 2011). These observations suggest that PA homeostasis plays a significant role in plant stress responses.

The role of PAs in protection against osmotic or drought stress has been demonstrated in many plant species (Kusano, Yamaguchi, Berberich, & Takahashi, 2007; Takahashi & Kakehi, 2010a; Urano et al., 2004). Overexpression of ADC2, a PA biosynthesis enzyme confers drought stress tolerance in Arabidopsis, these plants accumulated putrescine but spermidine and spermine levels were not changed (Alcazar et al., 2010). A loss of function mutation in Arabidopsis of ADC2 resulted in plants that were hypersensitive to osmotic stress (Urano et al., 2004). Increased putrescine levels and drought tolerance was also observed in rice plants overexpressing ADC (Capell, Bassie, & Christou, 2004). In a study that measured the change of free, conjugated and bound PAs in vetiver grass adaptation in response to PEG induced water stress it was observed that vetiver grass accumulated more PAs in leaves in response to drought stress (Zhou & Yu, 2010). Alcázar et al. (2011) discovered that in contrast to Arabidopsis, Craterostigma plantagineum accumulates high spermine levels which associate with drought tolerance. Together these results indicate that increased levels of PAs correlate with drought stress tolerance.
PAs are considered as plant growth regulators and secondary messengers in signaling pathways (Davies, 1995; Kusano, Berberich, Tateda, & Takahashi, 2008a; J. Liu et al., 2007). Apart from plant stress responses, PAs play a major role in plant growth and development (Tiburcio et al., 2014). Exogenous treatment of maize genotype Bashaier with PAs, along with drought stress, resulted in increased leaf area and number of grains per ear (Shaddad, El-Samad, & Mohammed, 2011). Determination of PA contents at different stages of plant growth and development indicates that there is a correlation between PAs and flowering, cell division, senescence and other developmental processes. There are multiple reports of crosstalk between PAs and hormones such as ABA, auxins, brassinosteroids, cytokinins, gibberelins, jasmonates, salicylic acid, and ethylene but the intrinsic mechanisms underlying such interactions are not completely understood (Anwar, Mattoo, & Handa, 2015; Marco, Alcázar, Tiburcio, & Carrasco, 2011). PAs play a major role in plant growth and development, but the mechanism by which they do so has not been unraveled yet.

Transgenic approaches in recent years have been mainly focused on changing PA biosynthesis or catabolism, but PA transport in plants have not been exploited as a strategy to alter PA homeostasis. As a matter of fact, knowledge of PA transport between plant tissues is very limited. Recently, a rice gene, OsPUT1, was identified as the first polyamine transporter, by our lab (Mulangi, Phuntumart, Aouida, Ramotar, & Morris, 2012b). Five additional transporters from Arabidopsis and rice that cluster in the same clade as OsPUT1 have also been reported (Mulangi et al., 2012). An Arabidopsis L-type amino acid transporter (LAT) family transporter, named RMV1 was also identified as a polyamine/paraquat transporter (Fujita et al., 2012). In this study the effects of overexpression of OsPUT3, one of the rice Polyamine Uptake Transporters (PUTs), on growth, development and drought stress response in Arabidopsis were evaluated.
Materials and Methods

Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used for gene transformations and phenotypic analysis. Seeds were surface sterilized with 70% alcohol for 2 minutes and 10% bleach solution for 10 minutes, rinsed five times with sterile distilled water and sown onto ½ MS plates for germination or planted on soil directly. Plants were grown in a growth chamber at 22°C with relative humidity of 55% under long-day conditions (16 h light/8 h dark).

Subcellular localization analysis

Rice clone *OsPUT3* was obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/index.html). Full-length cDNA of *OsPUT3* was amplified (see Appendix for primer sequences) and cloned into pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, USA) according to the supplier’s protocol. Subsequently, the target gene was transferred to a destination/expression vector, pGWB6 by LR recombination reaction (Invitrogen, Carlsbad, USA) to generate a construct (pGwB6-*OsPUT3*) containing the cDNA flanked with N-terminal GFP under the control of CaMV 35S promoter. pGwB6-*OsPUT3* was then transformed into *Agrobacterium* strain *GV3101* and infiltrated into tobacco (*Nicotiana benthamiana*) leaves (Sparkes et al., 2006). The GFP fluorescence was visualized and photographed with a laser scanning confocal microscope (TCS SP5 multi-photon laser scanning confocal microscope - Leica Microsystems). Overlay of GFP and chlorophyll autofluorescence was created using *ImageJ* (Rasband, 2008).
Generation of transgenic *Arabidopsis* plants

The full-length coding sequence of *OsPUT3* was cloned into pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, USA) according to the supplier’s protocol. The resulting plasmid was used to mobilize *OsPUT3* into plant expression vector, pGWB2, to generate pGWB2-*OsPUT3* by LR recombination reaction (Invitrogen, Carlsbad, USA). pGWB2-*OsPUT3* was transformed into *Agrobacterium* strain GV3101 and then transformed into *Arabidopsis* Col-0 plants using the floral dip method (X. Zhang et al., 2006). Transgenic plants were screened by germination of seeds on ½ MS plates containing 100μg/ml of kanamycin. T3 homozygous lines were used for further analysis.

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). Leaf chlorophyll content was measured by a Flourpen FP 100 (Photon System Instruments) (Woo et al., 2008). Leaf area was measured by taking pictures of leaves from 4 week old plants and using *ImageJ* (Rasband, 2008).

Drought stress tolerance assay

For drought stress tolerance assay, *Arabidopsis* Col-0 and *OsPUT3/OsPUT1* transgenic plants were planted in soil in same pots (Verslues, Agarwal, Katiyar-Agarwal, Zhu, & Zhu, 2006). Plants were grown in a growth chamber at 22°C and a relative humidity of 55% for four weeks. Water was withheld until the plants displayed evident drought-stresses phenotypes, then the plants were re-watered. Leaf chlorophyll content was measured before withholding the water
and after the drought treatment by using a Flourpen FP 100 (Photon System Instruments) (Woo et al., 2008).

Results

Subcellular localization of OSPUT3

Prior to our work, the subcellular localization of OspUT3 had not been established. An attempt to localize OspUT3 showed that when expressed in rice protoplasts OspUT3-GFP fusion was trapped in the golgi (J. Li et al., 2013). However, TargetP (Emanuelsson, Nielsen, Brunak, & von Heijne, 2000) an online subcellular localization tool, predicts that OspUT3 contains a chloroplast transit peptide. To confirm that OspUT3 is localized to the chloroplast, 35S::OspUT3::GFP fusion protein was transiently expressed in tobacco leaves. Confocal analysis of the tobacco leaves showed that OspUT3 is localized to the chloroplast (Fig. 25). Samples were observed at early, and late stages of transient expression, we did not observe the evidence of the protein being trapped in the golgi of tobacco leaf tissue.

Overexpression of OspUT3 in Arabidopsis increases leaf and stem size

PAs are involved in regulating plant growth and development, and many of these effects are because of a change in PA homeostasis. To test the hypothesis that a change in expression of PA transporters can alter plant growth and development, OspUT3 was overexpressed in Arabidopsis. Plants expressing OspUT3 under the control of CaMV 35S promoter were taller, with larger leaves and had thicker stems compared to the Arabidopsis wild type plants (Chapter II). Plants overexpressing OspUT3 had twice the leaf area and three times thicker stems as compared to the wild type Arabidopsis plants. In addition, plants overexpressing OspUT3 were visibly bigger, with more and bigger leaves compared to the wild type (Chapter II).
In addition, to increase in size, *Arabidopsis* plants overexpressing *OsPUT3* showed a significant increase in number of siliques compared to the wild type. At the end of the growth cycle, there was a 200% increase in the number of siliques in plants overexpressing *OsPUT3* compared to the wild type. However, maturation and senescence of siliques occurred very slowly in plants overexpressing *OsPUT3*. Additionally, leaf and stem senescence were also delayed along with a delay in flowering (Chapter II).

Overexpression of *OsPUT3* in *Arabidopsis* enhances drought stress tolerance along with an increase in yield and biomass.

Accumulation of PAs in plants by either exogenous application or increase in PA biosynthesis results in enhanced stress tolerance (Shi & Chan, 2014b). *OsPUT3* plants accumulated more soluble and soluble conjugated spermidine as compared to wild type (Ariyaratne, 2014). To test the hypothesis that *OsPUT3* plants would show enhanced drought stress tolerance, plants overexpressing *OsPUT1*, *OsPUT3* and wild type *Arabidopsis* plants were grown for four weeks and exposed to drought stress by withholding water for five days. After five days of drought stress *OsPUT3* plants were greenest and displayed the highest ratio of variable (Fv) to maximum fluorescence (Fm), followed by *OsPUT1* and WT. Fv/FM ratio has been widely used for assessing plant physiological status and the state of Photosystem II (PSII) (Muilu-Mäkelä et al., 2015). After five days of drought stress, the Fv/Fm ratio in *OsPUT3* plants was almost the same as before drought stress, while in WT the Fv/Fm ratio was reduced to 0.2 compared to 0.8 before drought stress (Fig. 22).

We prolonged the drought stress to seven days and observed the response of plants after rehydrating them for seven days. For this assay, only WT and *OsPUT3* plants were used, water was withheld for seven days and the plants were allowed to recover for seven days. After seven
days of drought stress, the OsPUT3 plants were greener as compared to WT plants (Fig. 23. B). Post recovery fresh weight, number of siliques, number of branches bearing inflorescences, number of rosette leaves and length of plants was measured. OsPUT3 plants were ~17cms taller than the WT plants. OsPUT3 plants had 576% higher fresh weight and had 521% more yield compared to WT plants. The number of rosette leaves was higher by 244% and the number of branches bearing inflorescences was higher by 285% in OsPUT3 compared to WT plants. A slight increase in weight and length of siliques was also observed in OsPUT3 plants compared to WT plants (Fig. 24).

**Figure 22.** Overexpression of OsPUT3 in Arabidopsis enhances drought stress tolerance
Four weeks old wild type (WT), and plants overexpressing OsPUT3 and OsPUT1 were exposed to drought stress. Water was withheld for five days, Fv/Fm was measured using FluorPen FP 100 (n=3). A photograph of all three plants before drought is shown in (A). A picture of all three plants after drought is shown in (B). Fv/Fm measurements for OsPUT3 and WT are shown in (C).
Figure 23. Plants overexpressing OsPUT3 are taller as compared to WT and have increased silique length.

Four weeks old wild type and OsPUT3 plants were exposed to drought stress. Water was withheld for seven days and plants were rehydrated for seven days after the drought treatment. Pictures of WT and OsPUT3 before drought stress, after drought stress and after recovery are shown in A, B and C respectively. The plant on the left is OsPUT3 and the one on the right is WT. Fig. 23. D depicts a picture of WT (right) and OsPUT3 (left) after recovery; plants were clipped from the root-shoot junction. Siliques of WT (top) and OsPUT3 (bottom) after recovery are depicted in (E).
Figure 24. Overexpression of OsPUT3 in Arabidopsis leads to 5 fold increase in yield as compared to wild type.

Length of plants in cm (A). Weight of 30 siliques in mg (B). Number of branches bearing inflorescences (C). Number of siliques per plant (D). Fresh weight of plants in gm (E). Number of rosette leaves (F). Length of siliques in cm (G). All measurements were done in plants that were rehydrated for seven days after drought stress. (n=2).
Figure 25. *OsPUT3* is localized to the chloroplast. BF = Bright Field, GFP = Green fluorescence protein, CHL = Chlorophyll Autofluorescence, Merge = Overlay of bright field, GFP and CHL. *OsPUT3*-GFP fusion protein was transiently expressed in tobacco leaves and observed by confocal microscopy. The GFP signal was co-localized with chlorophyll autofluorescence.
Discussion

Drought has a profound effect on plant physiology, and adaptation of plants to water stress can influence the survival and yield of plants. PAs play important roles in plant development and stress responses (Tiburcio et al., 2014). Overexpression of \textit{ADC2}, polyamine biosynthesis enzyme, in \textit{Arabidopsis} results in drought tolerance but induces dwarfism in the transgenic plants (Alcázar et al., 2005). Drought tolerance was also observed in \textit{Arabidopsis} plants overexpressing spermidine synthase from \textit{Cucurbita ficifolia} (Kasukabe et al., 2004). This study shows that overexpression of \textit{OsPUT3}, a spermidine uptake transporter, results in enhanced drought stress tolerance and increased yield.

\textit{OsPUT3} is a spermidine uptake transporter (Vaishali Mulangi, 2011) and plants overexpressing \textit{OsPUT3} accumulate higher amounts of soluble as well as soluble conjugated spermidine as compared to wild type \textit{Arabidopsis} plants (Ariyaratne, 2014). Plants overexpressing \textit{OsPUT3} display increased drought stress tolerance (Fig. 22). Exogenous application of spermidine results in increased concentrations of endogenous PAs and confers drought tolerance in creeping bentgrass (Z. Li et al., ). \textit{Arabidopsis} mutant plant that is deficient in producing spermine was hypersensitive to drought stress (Yamaguchi et al., 2007b). Spermidine plays a role as intermediate by indirectly maintaining high spermine levels under abiotic stress, but accumulated spermidine might also get converted to putrescine in plants (Peremarti, Bassie, Christou, & Capell, 2009; Roychoudhury, Basu, & Sengupta, 2011). Putrescine can alter the partitioning of proton motive force in \textit{Arabidopsis}, optimizing the balance between energy transduction and dissipation under a variety of stress conditions (Ioannidis, Cruz, Kotzabasis, & Kramer, 2012). Since \textit{OsPUT3} is localized to the chloroplast and
is highly specific for spermidine uptake, the plants overexpressing OsPUT3 may be able to tolerate drought stress by compartmentation and conversion of spermidine.

In addition to enhanced drought tolerance plants overexpressing OsPUT3 also exhibit a significant increase in plant height, number of siliques per plant, number of branches bearing inflorescences per plant, fresh weight and a slight increase in silique length and weight compared to wild type (Figs. 23&24). The results shown in our experiments parallel observations that others have made, in which PA levels have been increased by modifying PA homeostasis or exogenous applications. Exogenous application of spermidine (100mg/l) during the vegetative stage of chamomile plants improved plant height, number of branches and fresh weight (Abd EA, 2004). External application of 0.75mM spermidine and putrescine increased biomass and enhanced growth in Beta vulgaris (Beet) and Tagetes patula (French marigold) (Bais et al., 2000). Plants that were sprayed for seven days with 1mM spermidine before being exposed to salt stress displayed a 62% increase in yield in Oryza sativa L. ssp. ‘indica’ as compared to control plants (Saleethong, Sanitchon, Kong-Ngern, & Theerakulpisut, 2013). Exogenous application of spermidine on 12-15 years old Citrus sinensis L. Osbeck (‘Blood Red” sweet orange) significantly increased initial fruit set, yield/tree, and production of grade-l fruit (Saleem, Malik, Anwar, & and Farooq, 2008). Yang et al. (2010) reported that exogenous application of polyamines enhance growth in Nepeta cataria. Spraying of putrescine on Comice pear increased yield and improved fruit set, crop density, fruit size, and seed content (Sugar, 1988). Application of spermine and spermidine mitigated the deleterious effects of waste water stress in wheat and resulted in increased kernel weight, grain yield/plant, and straw yield/plant (Aldesuquy, Haroun, Abo-Hamed, & El-Saied, 2014). Thus, the phenotypic changes observed by overexpression of
OsPUT3 in *Arabidopsis* are similar to the ones that can be achieved by an increase in PA concentrations via exogenous applications or modifying PA homeostasis.

In conclusion, this study confirmed that accumulation of spermidine in *Arabidopsis* can enhance drought stress tolerance. It is not necessary to alter PA biosynthesis directly to change PA concentrations, change in expression of a PA transporter is sufficient. Increased expression of *OsPUT3* also resulted in increased yield and biomass. This study also provides a marker (expression levels of polyamine transporters) to identify potential drought stress tolerant plants. However, further research is necessary to understand the underlying mechanisms of the stress response and phenotypic changes.
References


SUMMARY

This study set out to explore the role of polyamine transport on growth, development and stress responses in *Arabidopsis thaliana*. The approach taken in this study was to localize key enzymes known to be associated with polyamine biosynthesis and transport, as well as test the importance of polyamine transport in polyamine homeostasis. The importance of polyamine transport was evaluated by generating transgenic plants overexpressing polyamine uptake transport localized to the chloroplast or plasma membrane.

In *Arabidopsis thaliana* it has been assumed that there is only one pathway for putrescine biosynthesis although there are two genes in *A. thaliana* that code for arginine decarboxylase (ADC). Our research shows that one of the ADCs (ADC2) is localized to the chloroplast and can synthesize putrescine in conjunction with agmatinase, an enzyme that we have localized to the chloroplast. We have also shown that this pathway exists in *Glycine max* (soybeans). Soybeans also have a second ADC and agmatinase. Furthermore, conservation of key residues in arginases from soybean, Arabidopsis, and poplar indicates that arginases from these plants may be able to function as agmatinases if they have access to the right substrate (agmatine). There are several studies that preceded our work in which they manipulated biosynthesis of polyamines and observed altered plant stress responses. Recognition of the agmatinase pathway in plants provides insights that will be helpful in designing future experiments where polyamine levels are manipulated.

Our research has shown for the first time that polyamine transport is critical for polyamine homeostasis in plants. Change in expression of polyamine transport changed phenotype in *Arabidopsis*. This change may be due to crosstalk between polyamines and plant hormones.
Auxins are involved in stem elongation, gibberellins are involved in flowering, cytokinins play a role in seed development and ethylene is involved in plant ripening. We observed a delay in flowering, increase in plant height, increased yield, delayed senescence and enhanced drought tolerance in our transgenic plants. Comparison of common and specific gene networks that are affected by increased levels of polyamines have suggested that polyamines participate in drought stress signaling via crosstalk with abscisic acid. The phenotypes that we observed in our transgenic plants establish a role for polyamines as signaling compounds that control plant developmental processes. Our constructs provide a tool to help identify how polyamine levels are sensed and served to activate pathways involved in plant development and stress responses.