CHARACTERIZATION OF THREE NOVEL POLYAMINE TRANSPORTERS IN PLANTS

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A Thesis
Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

May 2011

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ABSTRACT

Polyamines are low molecular weight aliphatic compounds which are ubiquitous in nature. In plants, polyamines engage in both cellular processes and defenses to environmental stress. In recent years, research on polyamines has focused on biosynthetic and degradative pathways, but no strategy to identify polyamine transport has been employed. In this study one rice gene AK105656 and two Arabidopsis genes At3G19553 and At3G13620 were hypothesized to function as polyamine transporters because of their homologous relationship with known polyamine transporters in several species. The transmembrane structures predicted by several computational tools suggested the probability for them to be membrane transporters. Full length cDNAs of AK105656, At3G19553, and At3G13620 were expressed in the yeast mutant AGP2Δ and analyzed via spot assays. The complementation assays supported the hypothesis that in yeast they were localized to the plasma membrane and functioned as polyamine transporters (importers). Since AK105656 shows close phylogenetic similarity with AtBAT1, which is a bidirectional amino acid transporter in Arabidopsis, AK105656 is also predicted to act as both importer and exporter of polyamines. Further research is still needed to test if AK105656 can also function as a polyamine exporter.
ACKNOWLEDGMENTS

I offer my sincerest gratitude to my supervisor, Dr. Paul Morris, who has supported and helped me a lot with my project in the past two years. I appreciate his encouragement and effort for my study and research. I would like to thank Dr. Vipa Phuntumart for giving me sufficient suggestions to help me complete the project. I also want to thank Gopala Vaishali Mulangi, a PhD student in our lab, who has completed the identification of several genes that was a reference of my research.
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INTRODUCTION

Polyamines are one of the oldest groups of substances known in biochemistry. The most common polyamines in higher plants are putrescine, spermidine, spermine, and cadaverine (Galston and Sawhney 1990) and their molecular formula and structures are shown in Table 1. More than 300 years ago, spermine was discovered in ageing human spermatozoa by Leeuwenhoek. Putrescine and cadaverine were characterized in putrefying cadavers more than 100 years ago (Alcazar et al. 2010). However, the biological functions of polyamines were not investigated by scientists until the 1960s, and the systematic research of plant polyamines has become an important area of research since the 1980s (Galston and Sawhney 1990). Previous research has indicated that polyamines play essential roles in many developmental processes in plants, including promoting the growth and enhancing resistance to environmental stresses (Evans and Malmberg 1989; Galston and Sawhney 1990; Martin-Tanguy 2001).

Table 1 Molecular formula and structures of four common polyamines.
Figure 1 Plant polyamine biosynthesis: ADC and ODC pathways in plants: Putrescine (PUT) is either synthesized via the arginine (Arg) decarboxylase (ADC, EC 4.1.1.9), or ornithine decarboxylase (ODC, EC 4.1.1.17) pathway, using Arg and ornithine as substrates, respectively. PUT is subsequently converted to Spermidine (SPD) via SPD synthase (EC 2.5.1.16) and SPD to Spermine (SPM) via SPM synthase (EC 2.5.1.22), by sequential addition of decarboxylated S-adenosyl-L-methionine (AdoMet) produced by AdoMet decarboxylase (EC 4.1.4.50).

Figure 2 Polyamine oxidation and degradation in plants: Polyamine degradation is carried out by diamine oxidases (DAO, EC 1.4.3.6) and polyamine oxidases (PAO, EC 1.5.3.3) in plants. DAO oxidizes putrescine and PAO degrades spermidine and spermine.
Polyamine Biosynthesis and Degradation

The pathways of polyamine biosynthesis are conserved in fungi and animals. The ornithine decarboxylase (ODC, EC 4.1.1.17) pathway has been demonstrated to be the main system of polyamine biosynthesis in fungi and mammals (Kusano et al. 2007). Two pathways have been found in plants for polyamine biosynthesis (Figure 1) and the starting compounds of them are both arginine. One of the pathways is the ODC pathway, which is similar to the corresponding pathway in mammals and fungi (Kusano et al. 2007). The plastid in plants contains a second biosynthetic pathway, the first step of which is the decarboxylation of arginine to produce agmatine, an important intermediate in this pathway. Agmatine can be converted into putrescine via agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase. Subsequently, putrescine can be transformed into spermidine by SPD synthase (EC 2.5.1.16), and then converted into spermine (SPM) by SPM synthase (EC 2.5.1.22) (Alcazar et al. 2010). Both of these enzymes are localized in the cytoplasm. Surprisingly the ODC pathway is not found in the model plant Arabidopsis and only ADC pathway is used to synthesize putrescine (Hanfrey et al. 2001). So far the same absence of the ODC pathway has only been discovered in the protozoan eukaryote, Trypanosoma cruzi.

Since polyamines play incomparable roles in plant development and responses to biotic and abiotic stresses, the intercellular concentrations of them should be stringently regulated. Some free polyamines in cells are conjugated to certain small molecules or very few large molecules to regulate their titers (Bagni and Tassoni 2001). Other free polyamines are induced to oxidative
degradation to reduce their titers. Polyamine degradation is carried out by diamine oxidases (DAO, EC 1.4.3.6) and polyamine oxidases (PAO, EC 1.5.3.3) in plants (Figure 2). DAO oxidizes diamines (putrescine and cadaverine) while PAO prefers spermidine and spermine (Moschou, Paschalidis, Roubelakis-Angelakis 2008). It has been proposed that polyamines could act through their degradation to produce molecules that may function as secondary messengers signaling developmental and stress adaptation processes (Moschou, Paschalidis, Roubelakis-Angelakis 2008). For example, in both degradation pathways, polyamine oxidation produces H₂O₂, which is considered to be a signal to activate defense pathways against pathogens (Kusano et al. 2007).

**Multiple Functions of Polyamine in Plants**

Polyamines are aliphatic cations so that they could bind other key bio-functional molecules, such as nucleic acids and proteins. These linkages might account for the pleiotropic effects of polyamines. Present evidences suggest that polyamines play regulatory roles in developmental processes (Galston and Sawhney 1990). Changes in polyamine levels can affect cell division, embryogenesis, and tissue development (Evans and Malmberg 1989). Felix & Harr (1987) concluded that polyamine contents increased with the germination of different organs of seedlings in different plant species (Felix and Harr 1987). Jarvis et al (1985) identified that polyamines are essential for root initiation and early growth in *Phaseolus* (Jarvis, Yasmin, Coleman 1985). If *ADC2*, one of ADC (arginine decarboxylase) -encoding genes in *Arabidopsis*, is overexpressed, it will cause dwarfism and late-flowering of *Arabidopsis* by affecting
gibberellins metabolism (Alcázar et al. 2005). In that case, putrescine concentration was observed to increase in response to environmental stresses. Exogenous application of polyamines was found to inhibit plant senescence; while conversely, decreasing polyamine concentration might stimulate senescence (Evans and Malmberg 1989).

Changes of polyamine titers influence the ability of plants to respond to environmental stresses. Increase of putrescine in response to low temperature was reported in Arabidopsis to control abscisic acid levels in order to acclimate to cold (Cuevas et al. 2008). The deficiency of potassium could induce the increase of putrescine titers in different plant species (Young and Galston 1984). The higher concentration of H⁺ ions in the tissue can also enhance the putrescine levels in plants (Santerre, Markiewicz, Villanueva 1990). Transgenic plants which overexpress certain polyamine biosynthetic genes could have higher tolerance to stress. As mentioned above, putrescine accumulated in the ADC2 overexpressor Arabidopsis (Alcázar et al. 2005). Transgenic rice plants which expressed Datura stramonium ADC gene showed tolerance to drought and the authors summarized that conversion of putrescine to spermidine and spermine is important in conferring drought tolerance to plants (Capell, Bassie, Christou 2004). On the other hand, loss-of-function experiment could also demonstrate the defensive function of polyamines. For instance, an insertion mutant of one of Arabidopsis ADC2 gene showed more sensitivity to salt stress than control plants and the sensitivity was relieved by adding exogenous putrescine (Urano et al. 2004).

The polyamine biosynthetic pathway in fungal pathogens is different from the corresponding pathway in plants (multiple pathways). Fungi are known to only use the ODC
pathway to synthesize putrescine. Therefore, it is possible to use this difference to control the fungal plant diseases by inhibiting special polyamine biosynthesis (Rajam and Galston 1985). For example, it was demonstrated by Rajam et al. that difluoromethylornithine (DFMO) could protect beans from the infection of the bean rust fungus (Rajam, Wwinstein, Galston 1985).

**Polyamine Transport**

Two polyamine uptake systems were reported in *E. coli*: PotABCD and PotFGHI (Igarashi and Kashiwagi 2010). Meanwhile, PotE & CadB were identified as polyamine exporters in *E. coli* (Igarashi and Kashiwagi 2010; Schiller et al. 2000). In eukaryotes, published reports of polyamine transporters have only been characterized in *Saccharomyces cerevisiae*, *Trypanosoma cruzii* (*TcPAT12*) (Carrillo et al. 2006), and *Leishmania major* (*LmPOT1*) (Hasne and Ullman 2005) at the cellular level. In yeast, four genes have been demonstrated to express polyamine transporters: *UGA4*, *AGP2*, *DUR3*, and *SAM3* (Uemura, Kashiwagi, Igarashi 2007). The UGA4 protein was proved to mediate the uptake of GABA and putrescine on the vacuolar membrane (Uemura et al. 2004). The AGP2 protein, which was first identified as a high affinity amino acid permease (Aouida et al. 2005) in yeast and later as a carnitine transporter, functions as one of the spermidine uptake transporters in yeast (Lee et al. 2002).

In plants, most research focused on polyamine biosynthesis and functions (Alcazar et al. 2010; Angelini et al. 2008; Berta et al. 1997; Mattoo et al. 2009), but polyamine transport has not been an area of emphasis (Igarashi and Kashiwagi 2010). Early research on the transport of polyamines was conducted in carrot protoplasts and vacuoles in the 1980s (Pistocchi et al. 1988).
So far, plant polyamine transport systems have been reported for vacuoles, protoplasts, and mitochondria (Pistocchi, Bagni, Creus 1987; Pistocchi et al. 1988). In plants, there are two major super families, APC (Acid – Polyamine – Organocation) and ATF (amino acid transporter family), that include characterized amino acid transporters (Wolf-Nicolas Fischera et al. 1998), most of which are not mono-functional. Uncharacterized transporters in these families are potential polyamine transporters. They could be related to the transport of different amino acids and other compounds. By measuring the uptake of polyamines, some multiple functional amino acid transporters are identified as putative polyamine transporters.

**Multiple sequence alignment of candidate polyamine transporters**

Sequences of 77 transporters including all the characterized polyamine transporters from six species: *Arabidopsis thaliana*, *Leishmania major*, *Oryza sativa*, *Phytophthora sojae*, *Saccharomyces cerevisiae*, and *Trypanosoma cruzi*, were retrieved from NCBI database. The alignment of these sequences was performed with ClustalX (Larkin et al. 2007) and the phylogenetic analysis was obtained using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford 2003) (Figure 3). In the clade which has the most yeast polyamine transporters, there is no plant ortholog. The only ortholog of the yeast polyamine transporter ScUGA4 is the rice gene (AK105656). Three other rice genes (OsPOT1, AK071314, and AK099986) were previously characterized as polyamine transporters by Gopala Vaishali Mulangi in our lab. So AK105656 was chosen as one of the target genes in my project to test if it can function as polyamine transporter. Two *Arabidopsis* genes (At3G19553 and At3G13620) were also selected
due to their homologous relationship with OsPOT1. All the three genes were hypothesized to act as polyamine importer but the rice gene was also predicted as polyamine exporter because of its homology with AtBAT1, a bidirectional amino acid transporter (Dündar and Bush 2009).

**Figure 3 Cladogram of selected polyamine transporters sequences** from *Arabidopsis thaliana* (At), *Leishmania major* (Lm), *Oryza sativa* (Os or AK), *Phytophthora sojae* (Ps), *Saccharomyces cerevisiae* (Sc), and *Trypanosoma cruzi* (Tc). Sequences were aligned using ClustalX and subjected to parsimony analysis using PAUP. Bootstrapped values are shown in nodes. The three genes of interest are highlighted in red boxes.

From the phylogenetic tree, it is clear to see the close homologous relationships among AK105656, ScUGA4, and AtBAT1. Additionally, the two *Arabidopsis* proteins At3G19553 and At3G13620 exist in the same branch as LmPOT1 and TcPAT12, as well as OsPOT1, the rice polyamine importer. Therefore, I presented partial alignments of these sequences from the two
branches separately. Dendrograms of these proteins are shown in Figure 4. Sequence alignment results are shown in Figure 5.

**Figure 4**  
A. Dendrogram of AK105656, AtBAT1, and ScUGA4. Overall identities are 76.13% for AK105656 and AtBAT1 and 30.98% for AK105656 and ScUGA4.  
B. Dendrogram of At3G13620 and At3g19663 with rice polyamine transporter OsPOT1, *Trypanosoma* polyamine transporter TcPAT12, and *Leishmania* polyamine transporter LmPOT1. Identities are 55.7% for At3G19553 and OsPOT1, 26.9% for At3G19553 and LmPOT1, 26.02% for At3G19553 and TcPAT12, 46.35% for At3G13620 and OsPOT1, 27.09% for At3G13620 and LmPOT1, and 27.44% for At3G13620 and TcPAT12.
Figure 5  A. Clustal alignment of conserved protein domains of AK105656, AtBAT1, and ScUGA4.  B. Clustal alignment of conserved protein domains of At3G19553, At3G13620, LmPOT1, TcPAT12, and OsPOT1. All the alignments were performed by ClustalX. The residue positions of respective proteins are shown by the numbers at both ends of each sequence.
As mentioned above, ScUGA4 was demonstrated to be a putrescine transporter on yeast vacuolar membrane (Uemura et al. 2004). AtBAT1 protein was classified as a member of the amino acid–polyamine–organocation (APC) superfamily (Dündar and Bush 2009). Moreover, AtBAT1 protein was confirmed to be a bidirectional amino acid transporter by Dündar and Bush (Dündar and Bush 2009). AtBAT1 functions as an alanine, lysine, glutamate, and arginine transporter, but it has not yet been tested as a polyamine transporter. However, according to Dündar’s GUS expression research, AtBAT1 expression was enhanced when the plant was exposed to different stresses (cold and wounding) (Dündar 2009). This characteristic coincides with an important feature of polyamine transporters which is high expression confronting stresses. For instance, H$_2$O$_2$, the production of polyamine oxidation respond to tolerance, is accumulated during wound healing processes and is thought to act as a signal to induce additional cell responsive processes or even programmed cell death. Extracellular production H$_2$O$_2$ that is produced by the export of polyamines contributes to lignosuberization of the cell wall (Angelini et al. 2008). Importantly Moschou et al. (2008) also showed that the export of polyamines conferred tolerance to bacterial and oomycete pathogens in tobacco (Moschou et al. 2008). Therefore, we hypothesized that AtBAT1’s homology AK105656 might function as a bidirectional polyamine transporter in rice.

**Yeast as a model system for functional analysis**

In yeast, there are at least two polyamine transport systems for the most common three polyamines (putrescine, spermidine, and spermine) (Igarashi and Kashiwagi 1999). As mentioned
above, four polyamine transporters were already identified in yeast, including AGP2, UGA4, DUR3, and SAM3, which act as importers of polyamines. Besides, there are five known polyamine exporters TPO1-5 in yeast as well (Tachihara et al. 2005; Tomitori et al. 1999; Tomitori et al. 2001). Therefore, yeast is a valuable model in the research of polyamine transport (Uemura, Kashiwagi, Igarashi 2007). It is shown in the phylogenetic tree above, ScDUR3 and ScUGA4 have potential orthologus with plants.

Since high concentrations of polyamine can inhibit the growth of yeast, wild type yeast which have functional polyamine importers cannot survive in the environment with toxic levels of polyamines. However, the polyamine importer knock out mutant yeast should grow well with polyamine. Therefore, wild type and AGP2 knockout mutant yeast are used as the system of functional analysis in my research. The toxicity of polyamines to the yeast strains is evidence of the expression of polyamine importers or exporters.
MATERIALS AND METHODS

Bacterial Transformation

*Escherichia coli* (*E. coli*) strains, DH5α and Top10 (Invitrogen Corp., Carlsbad, CA, US), were used for transformation and cloning. They were grown at 37°C in Luria-Bertani (LB) medium (0.5% yeast extract, 1% NaCl, and 1% Bacto-Tryptone, adjust pH to 7 with 1M NaOH, add 1.5% Bacto agar for solid media). The harvested cells were treated with CaCl₂ to become competent. Plasmids with target genes were transformed into competent *E. coli* cells with 30 sec heat shock at 42°C. Transformants were selected on the media with kanamycin (50 mg/L) and ampicillin (100 mg/L).

The rice gene AK105656 was transformed by two steps including entry cloning and LR reaction into the destination vector pDest52. The *Arabidopsis* genes At3G19553 and At3G13620 had already been in entry vector pDONR, therefore, the plasmids were transformed into pDest52 with LR reaction directly. The pENTR™/D-TOPO® Cloning Kit from Invitrogen Co. was used for the entry cloning and the transformants were selected with kanamycin. The LR reactions were achieved with LR clonase from Invitrogen Co. as well and the transformants were selected with ampicillin. The details of the transformations are shown in Figure 6.
Figure 6 A. pENTR™/D-TOPO® Cloning. The gene expressed cloning is selected by 50 µg/ml kanamycin. B. LR reaction. The transformants are selected by 100 µg/ml ampicillin. (The two reaction kits were adapted from Invitrogen, CA)

Yeast electroporation

Yeast strains *BY4741* (*MATa his3Δ leu2Δ met15Δ ura3Δ*) and *AGP2Δ* (*agp2Δ::kan*) were used in the project. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar for liquid media), YPG (1% yeast extract, 2% peptone, 2% galactose), or SC-ura-gal media (0.67% Yeast Nitrogen Base (w/o amino acids), 2% galactose, and 0.2% uracil, with the pH adjusted to 5.6).

Wildtype yeast strains *BY4741* and mutant *AGP2Δ* were transformed by electroporation using a BioRad Micropulser following manufacturers instructions. Briefly, wild type and *AGP2Δ* cells were cultured in YPD media at 30°C, harvested at OD$_{600}$=1.0, and washed with ice-cold 1M sorbital and ice-cold water to make electrocompetent cells (Becker and Guarente 1991).
Afterwards, isolated DNA and competent yeast cells were mixed in a 0.4cm cuvette and pulsed for 5 milliseconds. The electroporated cells were then transferred onto SC-ura-gal agar plates and incubated at 30°C for 48 hours.

**Spot assays**

Wildtype, $AGP2\Delta$, three transformed $AGP2\Delta$ strains (At3G19553-AGP2 Δ, At3G13620-AGP2 Δ, and AK105656-AGP2 Δ), and one transformed wildtype strain (AK105656-WT) were tested in the spot assays. Yeast cell cultures were grown in the SC medium (6.7g yeast nitrogen base w/o amino acids dissolved in 1L water) with 2% galactose to normalize the density at an OD600 of 0.6. Cell suspensions were then diluted to 4 gradient concentrations (1:1, 1:10, 1:50, 1:100, and 1:500). An aliquot of 3µl from each dilution was spotted onto YPG (1% yeast extract, 2% peptone, 2% galactose) agar plates containing 25 mM spermidine (SPD), 200 mM putrescine (PUT), or 1.5 mM paraquat (para). Paraquat is an herbicide which has two similar positively charged amino groups as putrescine (Hart et al. 1992). In addition, paraquat and putrescine exhibit related uptake features in animal systems (Gaudreault, Karl, Friedman 1984). Therefore, paraquat was also applied in the spot assay to investigate its toxicity to yeast cells. Pure YPG plates with cells growing were maintained to be controls. There were two duplications of each group. All the plates were incubated at 30°C for 48 hours.
Figure 7 Hydropathy plots of At3G13620, At3G19553, and AK195656 generated by TopPred II
RESULTS

Investigation of transmembrane domains of the three candidates

Hydropathy analysis and membrane topology predictions were used to identify the transmembrane domains of the three proteins. The results calculated by TMHMM online server are shown in Table 2 (Möller, Croning, Apweiler 2001). The plots of hydropathy analysis generated using TopPred II are shown in Figure 7 (Claros and Heijne 1994). All the three candidates exhibit essential characteristics to be transporters since their transmembrane domains are either 10 or 12.

Table 2 Prediction of transmembrane helices in proteins by TMHMM

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<tr>
<td>At3G19553</td>
<td>479 aa</td>
<td>12</td>
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<tr>
<td>At3G13620</td>
<td>478 aa</td>
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Polyamine spot assay

Since high concentrations of polyamine were known to inhibit yeast growth, wild type yeast with polyamine importers could not survive on the plates with high concentration of polyamines. However, the polyamine importer knock out mutant yeast $AGP2\Delta$ grew well with polyamines. It can be identified from the photographs of spot assay (Figure 8) that, elevated concentration of polyamines and herbicide paraquat are toxic to wildtype but the growth of yeast mutant $AGP2\Delta$ was not influenced by contraries. Transformed plant genes were expressed successfully in $AGP2\Delta$ under the control of the GAL1 promoter. The three transformants in $AGP2\Delta$ showed
more sensitivity than \textit{AGP2Δ} to exogenous polyamines and complemented the phenotype of wildtype in varying degrees. All the transformants showed more sensitivity to 25mM spermidine than the knockout but were less sensitive to spermidine than the wildtype strain. On the plate with 200 mM putrescine, the At3G19553 and At3G13620 transformants grew better than the rice transformants. It could be predicted that the rice gene might have better capability for putrescine transport. On the plates with herbicide paraquat, wildtype growth was entirely inhibited but the transformants endured to a certain extent. It could be interpreted that these genes had lower capability to transport paraquat. By comparison with the growth of wildtype and \textit{AGP2Δ}, the sensitivity of these transformants to elevated polyamines and paraquat proved the hypothesis that they act as transporters to uptake polyamines.

In order to investigate the polyamine exporting feature of AK105656, it was also expressed in wildtype with the inducement of GAL1 promoter. The growth of this transformant was inhibited by high concentrations of polyamines and paraquat but it was more tolerant to exogenous polyamines than the wildtype strain. These observations might be indicative of some polyamine export capability.
Figure 8 Spot Assay of wildtype (WT) BY4741, AGP2Δ, At3G19553-AGPΔ, At3G13620-AGPΔ, AK105656-WT, and AK105656-AGPΔ. Wildtype yeast is susceptible to overwhelming polyamines while the mutant AGP2Δ has resistance to polyamines because of lack of AGP2 polyamine importer. The transformants in AGP2Δ complement the phenotype of wildtype and present different growth status. Likewise, the transformant of the rice gene in wildtype expressed more resistance to polyamines than wildtype. The yeast cells were grown in SC media with galactose and the density of cell cultures were normalized to an OD600 of 0.6. Cell suspensions were gradually diluted as indicated and 3 μl of each were spotted onto plates of different media. Plates were photographed after 48 h of incubation at 30°C. The data is representative of two independent experiments. (YPG: yeast-peptone-galactose medium; SPD: YPG media with spermidine; PUT: YPG media with putrescine; para: YPG media with paraquat)
**DISCUSSION**

Polyamines are considered to be essential for cell viability. Polyamines play various roles in plant tissue development and differentiation. They frequently accumulate in response to abiotic and biotic stresses. Even though they can be synthesized inside cells, polyamines are still known to be taken up by plant cells and translocated between organs via the xylem and phloem tissues. In plants, polyamines are localized in the cytoplasm and organelles such as vacuoles, mitochondria and chloroplasts. High accumulations of polyamines are also known to be harmful to plant cells. Since polyamines are mobilized throughout the plant and localized within organelles, several types of transporters likely to be involved.

The objective of the study was to characterize putative polyamine transporters in rice and *Arabidopsis*. Using a bioinformatic approach, a clade of polyamine transporters has been identified with similarity to two known polyamine transporters of human pathogens (TcPAT12 and LmPOT1). The spot assay shows significantly lower viability of transformed *AGP2* strains than *AGP2Δ* strains which illustrates that these strains are acquiring exogenous polyamines (Figure 8). Nevertheless, the better growth of transformants than wildtype indicates that those transporters cannot completely complement the phenotype of wildtype.

Because the rice gene shares 77% identity with AtBAT1, AK105656 could potentially functions as a bidirectional transporter. AtBAT1 is the first reported plant transporter which mediates the transport of amino acids bidirectionally, importing arginine and alanine and exporting lysine and glutamic acid (Dündar and Bush 2009). As a result, we hypothesized that
AK105656 could also be an antiporter which probably comprises both polyamine and amino acid.

In order to demonstrate that AK195656 can operate as a polyamine exporter in the yeast assay system, it may be necessary to use an appropriate counter ion (such as arginine). Under conditions in which this experiment was conducted, there may have been insufficient amounts of amino acid outside the cell capable of exchanging for polyamines that were being imported. 3mM of amino acid was provided by Dündar (2009) in the transport assays (Dündar and Bush 2009). Depending on this information, certain amino acid could be added into the media to facilitate the export of polyamine. Due to the import of arginine and alanine of AtBAT1, both the amino acids could be used in future research. In addition, different pH values might also influence the export characteristic, especially with yeast. The yeast media we used were kept at about pH5.5-6.0, which was the optimal pH of yeast. However, it was reported that the export of polyamine in yeast happened in slightly alkaline environment (Igarashi and Kashiwagi 2010).
CONCLUSIONS AND FUTURE DIRECTIONS

The data presented here provide evidence for the characterization of polyamine transporters for the three genes AK105656, At3G19553, and At3G13620. The determination of At3G19553 and At3G13620 as polyamine transporters supplements information to the clade of OsPOT1 so that all the proteins in the clade have been examined as polyamine transporters. The whole clade of identified polyamine transporters enables us to develop a computational approach to predict potential polyamine transporters in crop plants. The future experimental work with these two proteins will be to confirm their subcellular localizations. At3G13620 is predicted to be likely localized to the plasma membrane but At3G19553 has no specific localization predicted in WoLF PSORT (Table 3) (Horton et al. 2007). In the prediction by a novel Arabidopsis Subcellular Localization Prediction Server (AtSubP), At3g19553 is indicated to be chloroplast localized protein (Kaundal, Saini, Zhao 2010). These potential localizations need to be testified in the future.

Table 3 Predicted subcellular localization of plant transporters (predicted by WoLF PSORT)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>plasma membrane</th>
<th>vacuolar membrane</th>
<th>chloroplast</th>
<th>Golgi apparatus</th>
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<td>AtBAT1-2</td>
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<td>11.0</td>
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</table>
The rice gene AK105656 has been demonstrated to be an importer of polyamine but the evidence that it also functions as a polyamine exporter is not powerful. Considering the fact that AtBAT1 mediates bidirectional transport of amino acids, we provide a new hypothesis: AK105656 might be an antiporter of both polyamines and amino acids. It means that the transporter could act as an exporter when cells are importing certain proteins. A few of independent exporters and importers in plants have been described to mediate the bidirectional transport of kinds of solutes across the plasma membrane (Bush and Briggs 1993). The bidirectional transport through one single facilitated transporter via different mechanisms has also been reported. However, AtBAT1 is the only reported bidirectional amino acid transporter to date, and little has been known about polyamine export in plants as well. Therefore, the mechanism of bidirectional movement of polyamines is unclear. In order to fully characterize the bidirectional transport of polyamines by this transporter, several additional experiments are needed. The future work related to this putative bidirectional polyamine transporter in rice will focus on the investigation of export and the antiport of amino acids and polyamines.

**Figure 9 Map detail image of two splice variants of AtBAT1 gene**

AtBAT1= AT2G01170.1 and AtBAT1-2=AT2G01170.2

The subcellular localization of AK105656 is also an issue that must be addressed. The *AtBAT1* gene has been predicted to appear in two alternative splicing types (*AtBAT1* with 516
amino acids and AtBAT1-2 with 437 amino acids) (Figure 9). The longer version AtBAT1 was partially characterized by Dündar and Bush (2009). Dündar and Bush (2009) stated that AtBAT1 was a bidirectional amino acid transporter on the plasma membrane. Surprisingly, this protein was predicted to be localized to vacuolar membrane but not plasma membrane by WoLF PSORT (Table 3). Comparatively, AtBAT1-2, the short version, was predicted to be mainly localized to the vacuolar membrane and lower possibility to the plasma membrane. Therefore, a model of the localization of both proteins was built to interpret the different localizations and functions of them in cross membrane transport (Figure 10). In the model, AtBAT1 was localized to the plasma membrane and AtBAT1-2 was localized to the vacuolar membrane. They performed the bidirectional transport of amino acid in and out of the cell or in and out of the vacuole, respectively. This model can also be applied to potential localization of AK105656. In the prediction by WoLF PSORT, it was shown that AK105656 might be localized to plasma membrane and vacuolar membrane equally. Additionally, considering the homology between AK105656 and ScUGA4, the yeast vacuolar polyamine transporter, it is reasonable for AK105656 to be a vacuolar transporter as well. In the case of polyamine transport, we can hypothesize that AK105656 can export redundant polyamines out of cell or import them to the vacuole for storage. All these hypotheses should be testified in future work.

Identification and characterization of plant polyamine transporters can complement the present research on plant polyamines. Importantly, the study of polyamine transport will enable us to determine whether these compounds play a role in long distance signaling. Additionally,
the research on plant polyamine transporters could also give experimental and theoretical information for polyamine transport in other eukaryotic organisms.

**Figure 10 Predicted model of subcellular localization of two alternative splicing types of AtBAT1** The shorter version AtBAT1-2 is localized to the vacuolar membrane and mediates the bidirectional transport of amino acids in and out of the vacuole. The longer version AtBAT1 is predicted to be localized to the plasma membrane and works as a bidirectional transporter of amino acids in and out of the cell.
REFERENCE


