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Functional Characterization of Two Putative Nucleobase Transporters in Arabidopsis Using Heterologous Complementation in Yeast

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FUNCTIONAL CHARACTERIZATION OF TWO PUTATIVE NUCLEOBASE TRANSPORTERS IN ARABIDOPSIS USING HETEROLOGOUS COMPLEMENTATION IN YEAST

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ABSTRACT

Miller, Sara E. M.S., Purdue University, August 2012. Functional Characterization of Two Putative Nucleobase Transporters in Arabidopsis Using Heterologous Complementation in Yeast. Major Professor: George S. Mourad.

To identify the substrate profile function for AtNAT5, one of the twelve members of the NAT/NCS2 gene family putatively identified to transport xanthine and/or uric acid, and to identify other functional aspects of transport for the already characterized AtNCS1 adenine-guanine-uracil transporter (Mourad et al., 2012) we used heterologous complementation techniques in *S. cerevisiae* mutant strains deficient in nucleobase transport. The ability of transformed yeast cells expressing the *AtNAT5* locus to grow in the presence of a panel of nucleobase toxic analogs revealed that the AtNAT5 protein is unable to transport adenine, guanine, cytosine, or uracil as expected by its previously defined putative function. Using uptake based assays, the ability of AtNAT5 to uptake [^{3}H]-nucleobases from the growth medium confirmed that using this particular methodology AtNAT5 does not transport xanthine. Heterologous complementation in yeast deficient in native ScNCS1 transporters provided additional evidence of transporter function for the previously characterized AtNCS1 transporter (Mourad et al., 2012). When AtNCS1 transformed strains were grown on media containing the toxic analogs 5-bromo-2-deoxyuridine and pyrithiamine, allantoin as the sole nitrogen source, and media containing various concentrations of pyridoxine (Vitamin B_6), AtNCS1 did not function to transport toxic analogs of uridine or thiamine, nor allantoin or pyridoxine.

CHAPTER 1: INTRODUCTION

Importance of Nucleobases

Purines and pyrimidines are nitrogenous bases that are essential components to all living cells. Nucleotides are required for growth, development and metabolism, they play an essential role in information storage and retrieval in dividing and elongating tissues by acting as building blocks of DNA in the nucleus or in DNA-synthesizing organelles and as components of transcripts (Zrenner et al., 2009). Nucleotide metabolism comprises an evolutionarily ancient and indispensible complex of metabolic pathways, which nucleobases and their metabolite derivatives are processed and utilized. These pathways include: *de novo* synthesis and salvage of DNA and RNA, the production of energy molecules and essential coenzymes such as ATP, NAD, and FAD, and biosynthesis of carbohydrates, glycoproteins, phospholipids and secondary metabolites such as cytokinins, therobromine and caffeine (Kafer et al., 2004; Zrenner et al., 2006).

Nucleotide metabolism can be divided into four broad categories including: *de novo* synthesis, nucleotide degradation, salvage pathways that recycle nucleosides as well as free bases, and phospho-transfer reactions that convert mono- and dinucleotides to the triphosphate form and equilibrate different pools of nucleotides. Nucleotides may be degraded to simple metabolites permitting cellular recycling of phosphate, nitrogen and carbon into central metabolic pools (Zrenner et al., 2006). *De novo* synthesis pathways utilize simple molecules or preformed nucleosides and nucleobases derived via salvage reactions; this type of synthesis is energetically expensive (Schmidt et al., 2006) but is essential at the whole plant level and in specific cells or tissues at different developmental stages. This interplay of synthesis and salvage relies upon an extensive network of intra- and inter-cellular transport mechanisms.

Cellular Compartmentalization and Membrane Transporters

Cells maintain biological functions by importing and exporting various substrates to maintain a constant supply of energy, nutrients, and an efflux of salts. Metabolites and ions are absolutely necessary for maintenance of bioactivity in both prokaryotic and eukaryotic cells (Nagata et al., 2008). Cellular compartmentalization allows for the optimization of enzymatic reactions by providing various sub-cellular pH environments favoring such reactions, while simultaneously permitting the independent operation of pathways competing for similar substrates. Compartmentalization eliminates futile cycles and confines toxic metabolic by-products to specific sub-cellular reaction spaces. These pathways are frequently interconnected across several compartments and depend on the supply of metabolic precursors from other parts of the cell (Linka and Weber, 2010). Nucleotides are frequently compartmentalized between the cytosol, plastids and mitochondria of plant cells; functional characterization of nucleobase transport proteins is providing deeper insight into pathways and inter- and intra-cellular localization and interactions of nucleotide metabolism (Zrenner et al., 2009).

This extensive compartmentalization of nucleobase metabolism suggests the existence of several nucleobase transporters with distinct specificities which function in different membranes and utilize different mechanisms of action (Argyrou et al., 2001). To date, 360 families of transporters comprising 5-20% of genes in all genomes studied have been found through biochemical and genomic analysis, highlighting the substantial importance of transport processes to cells (Koukaki et al., 2005). Membrane transporters are classified in three ways based on their mechanism of action. Primary active transporters use energy released from light reactions, redox reactions, or ATP hydrolysis to translocate substrates across membranes. Secondary active transporters use free energy stored in an ion gradient. The third class of membrane transporters utilizes facilitated diffusion without energy input for the movement of substrates across cell membranes (Weyand et al., 2001). The membrane transporters examined in this study are characterized as secondary active transporters which are putatively thought to use an H^+ ion gradient (Cecchetto et al., 2004).

Transporters comprise about 10% of the proteins encoded in an organism's genome and are studied for their agricultural, pharmacological and medical importance (de Koning and Diallinas, 2000). Purines and pyrimidines are commonly used as antimicrobial, antiviral or anticancer agents (Goudela et al., 2005). The toxic pyrimidine analog 5-fluorouracil was once

the most widely used anti-neoplastic drugs for the treatment of solid tumors, mainly due to its ability to be efficiently absorbed in the gut and subsequently reabsorbed by the kidney via high-affinity transporters (Desimone et al., 2002; Goudela et al., 2006). Understanding the differences among membrane transporters of prokaryotes and lower eukaryotes may allow for greater developments in agriculture, science and medicine.

Redundancy of Transporter Gene Families

Specific carrier-mediated transport of purine and pyrimidine nucleobases across cell membranes is a basic biological process observed in both prokaryotes and eukaryotes (Meintanis et al., 2000). Nucleobase transporters have been identified in bacteria, protozoa, fungi, algae, plants and mammals and are divided into three major groups; the Nucleobase-Ascorbate Transporters also known as the Nucleobase Cation-Symporter -2 family (NAT/NCS2) (Cecchetto et al., 2004), which occurs in Achaea, eubacteria, fungi, plants and metazoan, the Purine-Related Transporters (PRT) now known as the Nucleobase Cation-Symporter-1 family (NCS1) which occurs in prokaryotes and fungi, and the Purine related Permeases (PUPs) family found only in plants (de Koning and Diallinas, 2000). Analysis of the Arabidopsis genome indicates the presence of several gene families encoding for specific solute transporters. Within these gene families, 8,600 encoded proteins possessing at least one transmembrane domain have been identified and are therefore classified as membrane proteins (Hawkesford, 2003).

The six known families of nucleobase transporters are represented in the Arabidopsis genome. The NAT gene family contains twelve members which are specific for the transport of ascorbic acid, uric acid, xanthine, uracil and hypoxanthine (Maurino et al., 2006). Arabidopsis has only one member of the NCS1 gene family, responsible for the uptake of adenine, guanine and uracil (Mourad et al. 2012). The PUP family is represented by 20 members that function to transport cytosine, hypoxanthine and adenine (Pélissier et al., 2004). The Ureide Permeases (UPS) family has 5 members in Arabidopsis responsible for the uptake or uracil, uric acid, xanthine and allantoin. The Equilibrative Nucleoside Transporters (ENT) family is represented by eight members, specific for mostly nucleotides such as adenine and guanine and occasionally for some nucleobases such as xanthine and hypoxanthine (de Koning and Diallinas, 2000). Finally, the recently discovered AzgA-Like family has two members in *A. thaliana,* which function in the uptake of guanine, hypoxanthine and adenine (Mansfield, 2009). Due to overlap in the

substrate specificities of each gene family, which introduces the potential for functional redundancy between members, it has been difficult to isolate and definitively characterize individual genes.

Nucleobase-Ascorbate Transporter/Nucleobase Cation-Symporter-2 (NAT/NCS2) Gene Family

The largest and most conserved family of nucleobase transporters is the NAT/NCS2 family found in Achaea, eubacteria, fungi, plants and metazoans (de Koning and Diallinas, 2000). The NAT/NCS2 family was historically defined by the cloning and characterization of the UapA and UapC uric acid-xanthine permeases of *A. nidulans*, and the *UraA* and *PyrP* uracil permeases of *E. coli* and *B. subtilis*. Members of the NAT/NCS2 family function as transporters of oxidized purines, pyrimidines, and surprisingly in metazoans, the secondary metabolite ascorbic acid (vitamin C) (de Koning and Diallinas, 2000). This large gene family can be subdivided into three sub-families based on substrate specificity. The first sub-family occurs in bacteria, fungi and plants and is specific for the oxidized purines xanthine and/or uric acid. The second sub-family is found in bacteria and is specific for uracil, while the third sub-family is present in vertebrates and is specific for L-ascorbic acid. Bacterial, fungal and plant NATs have been shown to be H^+ symporters, while the mammalian sodium-dependent vitamin C transporter (SVCT) utilizes Na^+ symport (Gournas et al., 2008). Transporters dependent on different ion exchange mechanisms are known to coexist within some phylogenetic families (Cecchetto et al., 2004). The mammalian ascorbic acid NATs seem to have evolved from the uric acid/xanthine transporters of lower eukaryotes, since uric acid and xanthine are structurally unrelated to ascorbic acid (Goudela et al., 2005). To date, no NAT/NCS2 members of known function are specific for the salvageable purines adenine, guanine or hypoxanthine (Gournas et al., 2008).

NAT/NCS2 genes encode for distinct purine-pyrimidine carriers and have 25-65% identical amino acid sequences among members (Diallinas et al., 1998). Three criteria are used to classify an unknown transporter in the NAT/NCS2 family: a size ranging from 400-650 amino acid residues, the prediction of 10-14 putative transmembrane segments, and the presence and topology of two very highly conserved motifs known as the NAT signature motif [Q/E/P]-N-X-G-X-X-X-X-T-[R/K/G] where X is a hydrophobic amino acid and located in a region just upstream from the 9^{th} transmembrane segment, and the QH motif in the middle of the first transmembrane segment (Gournas et al., 2008). The most common predicted NAT protein

structure has twelve transmembrane segments with cytoplasmic N- and C-termini along with the presence of some nearly absolutely conserved single polar charged amino acids (Gournas et al., 2008).

The Arabidopsis NAT proteins are divided into 5 clades based on multisequence alignments. The proteins within each clade share patterns of organ and tissue specific expression during the whole life cycle of *A. thaliana*. Knock-out mutations have been produced for each of the twelve individual NAT proteins, but do not induce phenotypic alterations (Maurino et al., 2006). Absence of obvious mutant phenotypes in single, double, and even triple mutants suggests a high degree of functional redundancy among the *AtNAT* genes; however, it may also point to redundant functions of genes or pathways unrelated to the AtNATs. AtNAT proteins belonging to the same clade or sub-clade possess similar patterns of expression and are expected to serve similar functions (Maurino et al., 2006).

AtNAT5 (locus At5g49990) contains 528 amino acid residues with a genome coding sequence of 2760bp that contains 14 exons, and belongs to clade III which is divided into two sub-clades. The first sub-clade consists of *AtNAT5* and *AtNAT6* while the second contains *AtNAT7* and *AtNAT8*. *AtNAT6* is most similar to *AtNAT5* based on amino acid sequence and expression patterns. Maurino et al. observed tissue-specific expression of the *AtNATs* using RT-PCR and cDNAs from 6-week old leaves, stems, flowers and roots. In particular, AtNAT5 showed expression in all tissues with lower levels in the leaves (2006). Most *AtNAT* genes showed a pronounced expression in vascular tissues which may be an indication that the *AtNATs* functions are associated with long-distance transport processes (Maurino et al., 2006).

Only one NAT in the plant kingdom has been characterized at the molecular level. The *Zea maize* LPE1 was determined to be a high-affinity transporter of xanthine and uric acid via functional complementation of a purine transport deficient *A. nidulans* strain. LPE1 has also been shown to competitively bind to but not transport ascorbic acid (Argyrou et al., 2001). According to Schultes et al. *LPE1* encodes a polypeptide similar to pyrimidine and purine transport proteins, and loss of function *lpe1* mutants exhibit a defective chloroplast phenotype which preferentially affects bundle sheath chloroplast morphology (1996). Since none of the AtNATs cluster phylogenetically with *LPE1*, it is assumed they have different substrate specificities (Maurino et al., 2006).

Nucleobase Cation-Symporter-1 (NCS1) Gene Family

The NAT family is distantly related to two other nucleobase transporter families, the NCS1 family and the AzgA-like family, which are restricted to microorganisms and plants (Pantazopoulou and Diallinas, 2006). Over 200 sequenced proteins derived from gram-negative and gram-positive bacteria, Achaea, yeast, fungi and plants belong to the NCS1 family, and most known members are specific for purines and/or pyrimidines while some are also specific for other purine related compounds such as allantoin, hydantoin, thiamine, pyridoxal-based compounds and nicotinamide riboside (Diallinas and Gournas, 2008; Hamari et al., 2009). In general, all NCS1 transporters have been characterized to function as essential components of salvage pathways for nucleobases and related metabolites (Zrenner et al., 2006).

The Arabidopsis genome contains only one gene belonging to the NCS1 family; *AtNCS1* (At5g03555) encodes a protein with amino acid similarity to the FUR4 gene (Jund, et al., 1988; Mourad et al., 2012). *In planta* uptake of radio-labeled compounds and expression via heterologous complementation in *S. cerevisiae* performed by members of Dr. Mourad's Lab has revealed that AtNCS1 is specific for the transportation of adenine, guanine and uracil (Mourad et al., 2012). Although *FUR4* only encodes a transporter for uracil, the *S. cerevisiae* genome encodes several members of the NCS1 gene family. Another *S. cerevisiae* NCS1 gene known as *FCY2*, functions to transport adenine, guanine, cytosine and hypoxanthine (Wagner et al., 2001). AtNCS1 has been shown to transport similar solutes transported by at least two of the native *S. cerevisiae* NCS1 genes, *FUR4* and *FCY2* (Mourad et al., 2012).

Heterologous Complementation in *S. cerevisiae*

Most heterologous complementation studies can be informative by taking advantage of select yeast knock-out mutants or exploiting yeast transport deficiencies because yeast has several over-lapping transporters. Using *Saccharomyces cerevisiae* for heterologous complementation has two advantages. First, wild-type *S. cerevisiae* do not contain a NAT homolog (Diallinas and Gournas, 2008; Gournas et al., 2008) and lack the ability to transport oxidized purines but can still break down allantoin or allantoic acid to urea and ammonia (André, 1995), thus allowing for functional expression of eukaryotic NATs *in vivo* by direct measurement of uptake (Möhlmann et al., 2010). It is also the best studied simple eukaryotic system in respect to the ability to provide unique genetic tools for modifying and improving the

expression of a protein (André, 1995). Heterologous complementation of eukaryotic proteins has proven to be an efficient method to characterize their functions (André, 1995; Enjo et al., 1997). The use of bacterial and fungal model systems as an empty background lacking all other transporters allows for substrate interactions of eukaryotic proteins to be tested by reverse genetics (Gournas et al., 2008).

Objective

Individual function of each member of the Arabidopsis NATs remains unknown; however, they are putatively identified to function in the transport of xanthine, uric acid, and L-ascorbate based on multisequence alignments (Gournas et al., 2008). Using heterologous complementation with one of the twelve members of the AtNAT family, AtNAT5, expressed in *S. cerevisiae* mutant strains deficient in nucleobase transporters will allow for the identification of the unique solute specificity profile of this individual AtNAT5 gene locus. Growth-based assays using a concentration series of toxic purine and pyrimidine analogs of substrates not putatively identified to be transported by the AtNATs will allow for a qualitative analysis of the solute specificity of transport for AtNAT5. Due to AtNAT5 exhibiting its highest levels of expression in germinating seeds, testing its ability to transport nucleobases such as adenine, guanine, cytosine and uracil are relative due to the increased need for these substrates in seed development and tissue growth and elongation. [^{3}H]-xanthine uptake mediated by AtNAT5 will assess quantitatively whether or not AtNAT5 can function as a xanthine transporter, as putatively described by Gournas et al. (2008).

Although the *AtNCS1* gene locus has been characterized to transport adenine, guanine and uracil (Mourad et al., 2012), using heterologous complementation in nucleobase transport deficient yeast will allow us to identify aspects of transport for other secondary metabolites and nucleobase derivatives. Growth-based assays of allantoin and pyridoxine (Vitamin B_6) on media using allantoin as the sole nitrogen source and media containing various concentrations of pyridoxine, as well as, growth-based assays using the toxic analogs 5-bromo-2-deoxyuridine and pyrithiamine will identify other potential solutes of transport of the already characterized *AtNCS1* gene(Mourad et al., 2012).

CHAPTER 2: METHODS AND MATERIALS

Materials

The cDNA (255bp) clone U17917, containing the At5g49990 (*AtNAT5*) locus in the universal cloning vector generated by the SSP Consortium (Stanford, CT) PUNI51 in *E. coli* were obtained from The Arabidopsis Biological Resource Center (Columbus, OH). Plasmid DNA extractions were performed with Qiagen QIAprep Spin Miniprep kits. 8-Azaguanine (8-AZG), 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), 5-Bromo-2-deoxyuridine and Pyrithiamine were purchased from Sigma-Aldrich (St. Louis, MO). 8-Azaadenine (8-AZA) was purchased from Tokyo Kasei Gakuin (Tokyo, Japan). [8-^{3}H]-Xanthine (19.3Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA).

DNA Manipulations

Members of Dr. Neil Schultes' Lab from the Connecticut Agricultural Experiment Station (New Haven, CT) used coding regions derived from the At5g49990 locus and cloned them into a yeast expression vector pRG399 (Figure 1) [Mascorro-Gallardo and Goxiola, unpublished data – based upon a multicopy yeast shuttle vector pRS424 (Christianson et al., 1992) containing the high expression level promoter from *Saccharomyces cerevisiae* locus PMA1 and a MCS and CYC1 terminator as reported (Mascorro-Gallardo et al., 1996; Toki et al., 1992)]. Polymerase chain reaction (PCR) primers K9P8RTA 5' GGAAGTTCGGGGCAGTATTT 3' K9P8RTB 5' TGACCATGTCGTTGAACCAT 3' were used by Dr. Schultes' Lab to amplify a DNA fragment from the cDNA clone U17917 [Gb# BT008887]. The resulting DNA fragments were treated with restriction enzymes Xhol and Notl (New England Biolabs, Beverley MA) and cloned into pRG399 resulting in the pRH194 plasmid. The *AtNCS1* gene locus At5g03555 was obtained through PCR amplification from genomic DNA with primers At5g03555YEA and At5g03555YEB and cloned into the yeast expression vectors pRG402 and pRG399 resulting in the pNS467 and pRH369 plasmids respectively (Mourad et al., 2012). Genomic DNA was extracted using a Qiagen

(Germantown, MD) DNeasy Plant Mini Kit. The yeast expression vector pRG402 was derived from the same multicopy shuttle vector as pRG399, except instead of containing a gene required for leucine biosynthesis it contains a gene required for tryptophan biosynthesis.

Yeast Strains and Culture Medium

Yeast strains deficient in nucleobase transporters (Table 1) were used for [^{3}H]-xanthine uptake assays, growth assays on toxic analogs, media containing allantoin as the sole nitrogen source, or on medium containing various concentrations of pyridoxine. Parent strains were grown on YPD [1% yeast extract, 2% peptone, 2% glucose, 1.8% agar (solid media)] or on Synthetic Complete medium (SC) [0.67% yeast nitrogen base, 2% glucose, select amino acid mix, and 1.8% agar (solid media)] at 30˚C (Table 2).

Yeast Transformation

Yeast transformation was performed using the lithium acetate method as described by (Geitz and Woods, 2002). The yeast expression vector pRG399 complemented a lost gene for leucine biosynthesis in the RG191, NC122-sp6, ATCC#4006706, and W303-1A-derived yeast strains, thus leucine was subtracted from the media for transformed lines. The subtraction of leucine insured the selection of yeast cells carrying the pRG399 expression vector. The yeast expression vector pRG402 complemented a lost gene for tryptophan biosynthesis in the RW105dal4 and RW128 yeast strains. Tryptophan was subtracted from the media for transformed lines to insure the selection of yeast carrying the pRG402 expression vector (Table 3).

Growth-Based Assays of AtNAT5 Using Toxic Analogs

Sensitivity to toxic nucleobase analogs was assayed by the addition of filter-sterilized stock solutions of individual toxic analogs to the growth medium. The *fcy2* deficient parent strain RG191, its transformed strain containing the *AtNAT5* gene, an empty expression vector strain and a positive control strain were used for growth-based assays using the toxic analogs 8-AZA, 8-AZG, and 5-FC. The *fur4* deficient parent strain NC122-sp6, its respective transformed strain containing the *AtNAT5* gene, an empty vector strain and a positive control strain were used for growth-based assays using the toxic analog 5-FU. Yeast strains RG191 and NC122-sp6

containing pRH369 were used as a positive control for assays using the toxic analogs 8-AZA, 8-AZG, and 5-FU. The yeast strain FY1679-5C was used as a wild-type control for assays using the toxic analog 5-FC. Overnight cultures of yeast were serially diluted to 10,000, 1,000, 100, and 10cells/mL and plated drop-wise on solid media containing a concentration series of toxic analogs and grown for 2 days at 30˚C (Figures 2-6). All experiments were performed in triplicate.

[8-^{3}H]-Xanthine Uptake

Yeast from an overnight culture were diluted to an OD_{600} of 0.15 in 25mL of SC media and allowed to grow until they reached an OD_{600} of 0.6. Cells were harvested by centrifugation, washed and re-suspended in 100mM citrate buffer (pH 3.5) with 1% glucose at an OD_{600} of 4. The yeast suspension was pre-incubated for 5 minutes at 30˚C. Equal volumes (30µL) of yeast suspension and buffer containing 0.2µM [8-^{3}H]-xanthine (Moravek Biochemicals Brea, CA) were combined and mixed (Leung et al., 2010). The composition of the final solution was yeast at OD_{600} of 4 with 0.2µM [8-^{3}H]-xanthine in 100mM citrate buffer (pH 3.5) with 1% glucose. The mixture was incubated at 30˚C with samples taken at 0 and 2.5 minutes. Samples of 50µL were transferred to 4mL ice cold water to terminate the uptake of the radiolabeled compound. Samples were then filtered through a 0.45µm Metricel membrane filter (Pall Corporation, Ann Arbor, MI) and filters were washed with 8mL of water. Radioactivity was measured by scintillation counter (Beckmean Coulter, Fullerton, CA) in 3mL scintillation fluid (EcoLume, Costa Mesa, CA), and background values were subtracted from the uptake values of the parent strain, RG191. Error bars indicate the standard error of the mean of three independent experiments (Figure 6). Statistical analysis was performed using an independent paired t-test. Significance was measured at a P-value of 0.05 (Table 4).

Growth-Based Assays of AtNCS1 Using Toxic Analogs and Allantoin as the Sole Nitrogen Source and Varying Concentrations of Pyridoxine

Sensitivity to the toxic nucleobase analogs 5-bromo-2-deoxyuridine and pyrithiamine was assayed by the addition of filter-sterilized stock solutions of these toxic analogs to the growth medium. The *fui1* deletion strain RW128, its respective transformed strain containing the AtNCS1 gene, an empty vector and two control strains (RG191 and RW128/pRW45) were

used for growth based assays in media containing 5-bromo-2-deoxyuridine. The *thi7* deletion strain ACCT#4006706, ACCT#4006706 containing pRH369 and ACCT#4006706/pRG399 were used for growth based assays using the toxic analog pyrithiamine. Ability to transport allantoin was assayed by using allantoin as the sole nitrogen source of the growth medium. The *dal4* deletion mutant RW105dal4, RW105dal4 containing the *AtNCS1* gene (pNS467) and RW105dal4/pRG402 were used for allantoin growth-based assays. Ability to transport pyridoxine was assayed by adding various concentrations of pyridoxine to the growth medium. Since yeast nitrogen base (YNB) contains 2µg/L (1.93µM) of pyridoxine, a YNB without pyridoxine was synthesized for this experiment. The parent strain W303-1A, and W303-1A derivatives; snz1-sno1Δ strain and the *tpn1* deletion strain MVY30; a MVY30 transformed strain containing the *AtNCS1* gene (pNS467), and MVY30 containing an empty vector (pRG402) were used for growth-based assays on media containing various concentrations of pyridoxine. Overnight cultures of yeast were serially diluted to 10,000, 1,000, 100, and 10cells/mL and plated drop-wise on solid media containing a concentration series of toxic analogs, media containing allantoin as the sole nitrogen source or media containing various concentrations of pyridoxine then grown for 2 days at 30˚C (Figures 7-10). All experiments mentioned above were performed in triplicate.

Table 1. NCS1 deficient *S. cerevisiae* strains used in this study.

Yeast Strain	NCS1 gene	Solutes of transport	Screen or Assay used
RG191[a]	FCY2	adenine, guanine, cytosine, hypoxanthine	8-AZA, 8-AZG, 5-FC
NC122-sp6[b]	FUR4	uracil	5-FU
RW105dal4[c]	DAL4	allantoin	Allantoin
RW128[d]	FUI1	uridine	5-bromo-2-deoxyuridine
ACCT#4006706[e]	THI7	thiamine	Pyrithiamine
W303-1A[f]	TPN1	pyridoxine	Pyridoxine

[a] Jund and Lacroute, 1970; Jund et al., 1988
[b] Enjo et al., 1997; Jund and Lacroute, 1970
[c] Desimone et al., 2002; Péllisier et al., 2004; Wagner et al., 1998
[d] Hamari et al., 2009; Jund and Lacroute, 1970; Wagner et al., 1998
[e] Singleton, 1997
[f] Stolz and Vielreicher, 2003

Table 2. Yeast strain genotypes used in this study.

Yeast Strain	Genotype
RG191	*his3Δleu2Δura3Δmet15Δfcy2Δ*
NC122-sp6	*leu2Δfur4Δ*
FY1679-5C	*leu2Δhis3Δura3Δ*
RW105dal4	*fur4Δhis3Δtrp1Δdal4Δ*::HIS3
RW128	*fur4Δhis3Δtrp1Δfui1Δ*::HIS3
ATCC#4006706	*his3Δleu2Δmet15Δura3Δthi7Δ*
W303-1A	*leu2Δhis3Δtrp1Δade2Δura3Δcan1Δ*
Snz1-sno1Δ	W303-1A *snz1-sno1Δ::his5*$^{+}$
MVY30	W303-1A *snz1-sno1Δ::his5*$^{+}$ *tpn1Δ::kanMX4*

Table 3. Plasmids used for heterologous complementation.

Plasmid	Contents	Purpose
pRG399	2µ LEU2-PMA1-MCS-CYC1	Empty vector
pRG402	2µ TRP1-PMA1-MCS-CYC1	Empty vector
pRH194	At5g49990 in pRG399	Transformed AtNAT5
pRW45	2µ TRP1-FUI1	Control
pRH369	At5g03555-short version in pRG399	Transformed AtNCS1
pNS467	At5g03555-short version in pRG402	Transformed AtNCS1

Table 4. AtNAT5 mediated [^{3}H]-xanthine uptake P-values.

Time of reaction	t-value	P-value
0 minutes	1.7725	0.2183
2.5 minutes	1.6738	0.2361

Table 5. *AtNAT5* transformed yeast cells exhibit no significant resistance to toxic purine or pyrimidine analogs (- to +).

Toxic Analog	At5g49990 (*AtNAT5*)
8-Azaadenine	-
8-Azaguanine	-
5-Fluorocytosine	-
5-Fluorouracil	-

Table 6. *AtNCS1* transformed yeast cells exhibit no significant resistance to toxic analogs of uridine or thiamine and exhibit no ability to transport allantoin or pyridoxine (- to +).

Assay Used	At5g03555 (*AtNCS1*)
5-bromo-2-deoxyuridine	-
Pyrithiamine	-
Allantoin	-
Pyridoxine	-

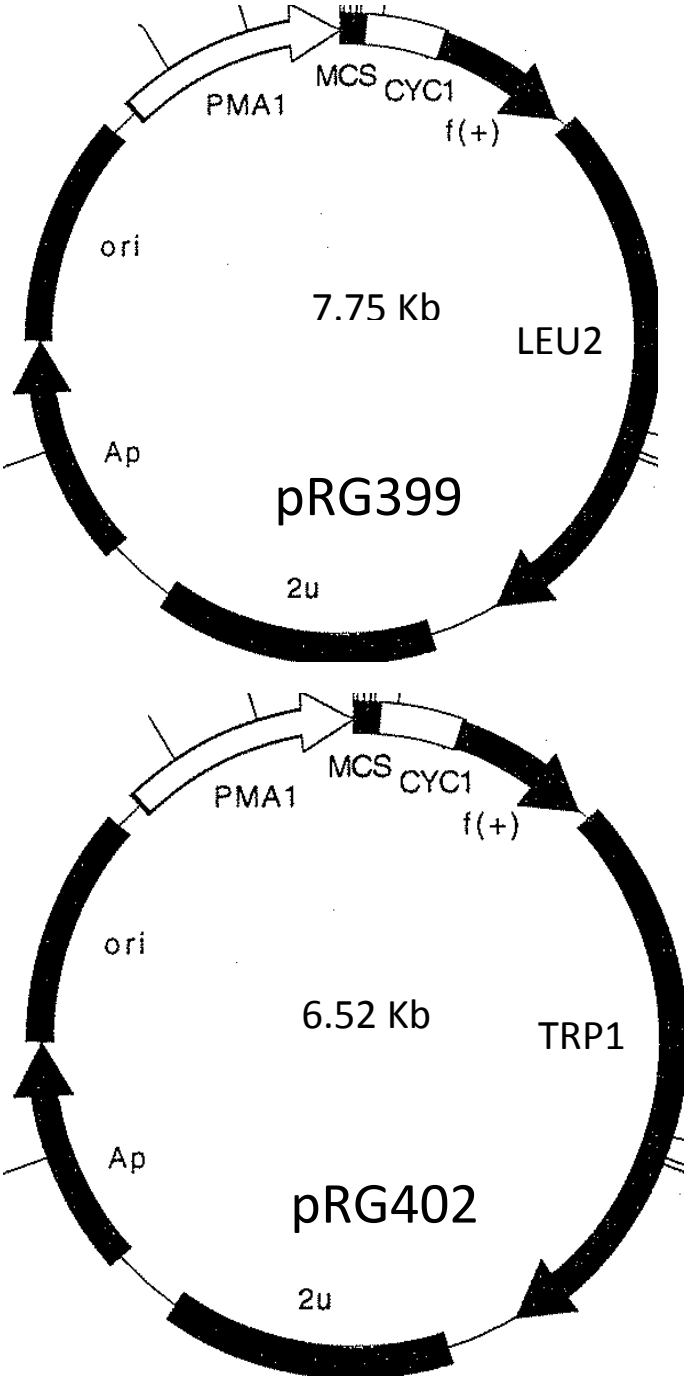

Figure 1. Yeast expression vectors pRG399 and pRG402. Contains the PMA1 constitutive yeast promoter and multiple cloning site (MCS) required for insertion of *AtNAT5* or *AtNCS1*. pRG399 contains a LEU2 gene, while pRG402 contains a TRP1 gene.

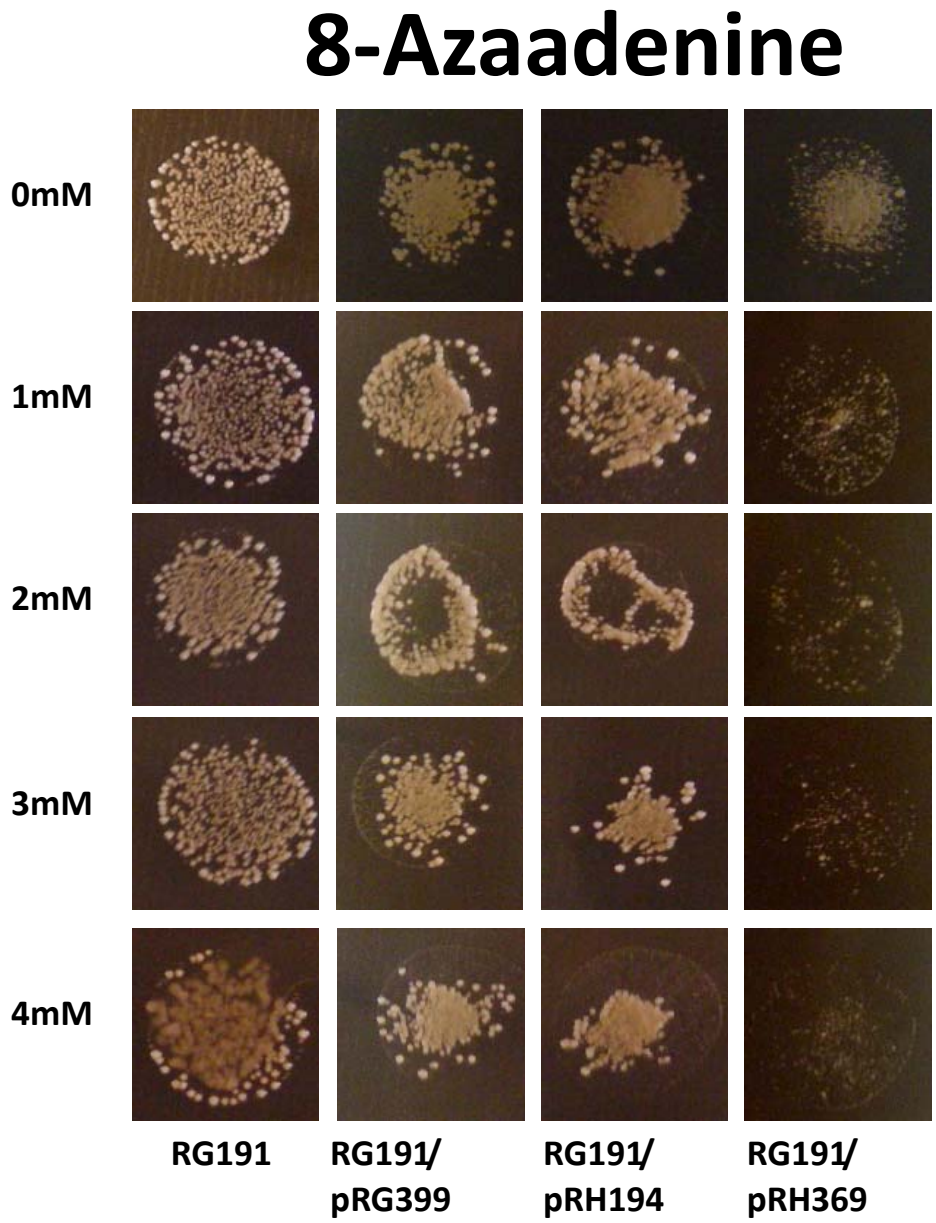

Figure 2. Growth of *S. cerevisiae* cells expressing *AtNAT5* on 8-Azaadenine. Strains RG191; RG191/pRG399; RG191/pRH194; and RG191/pRH369 were grown on SC media containing various concentrations of 8-AZA.

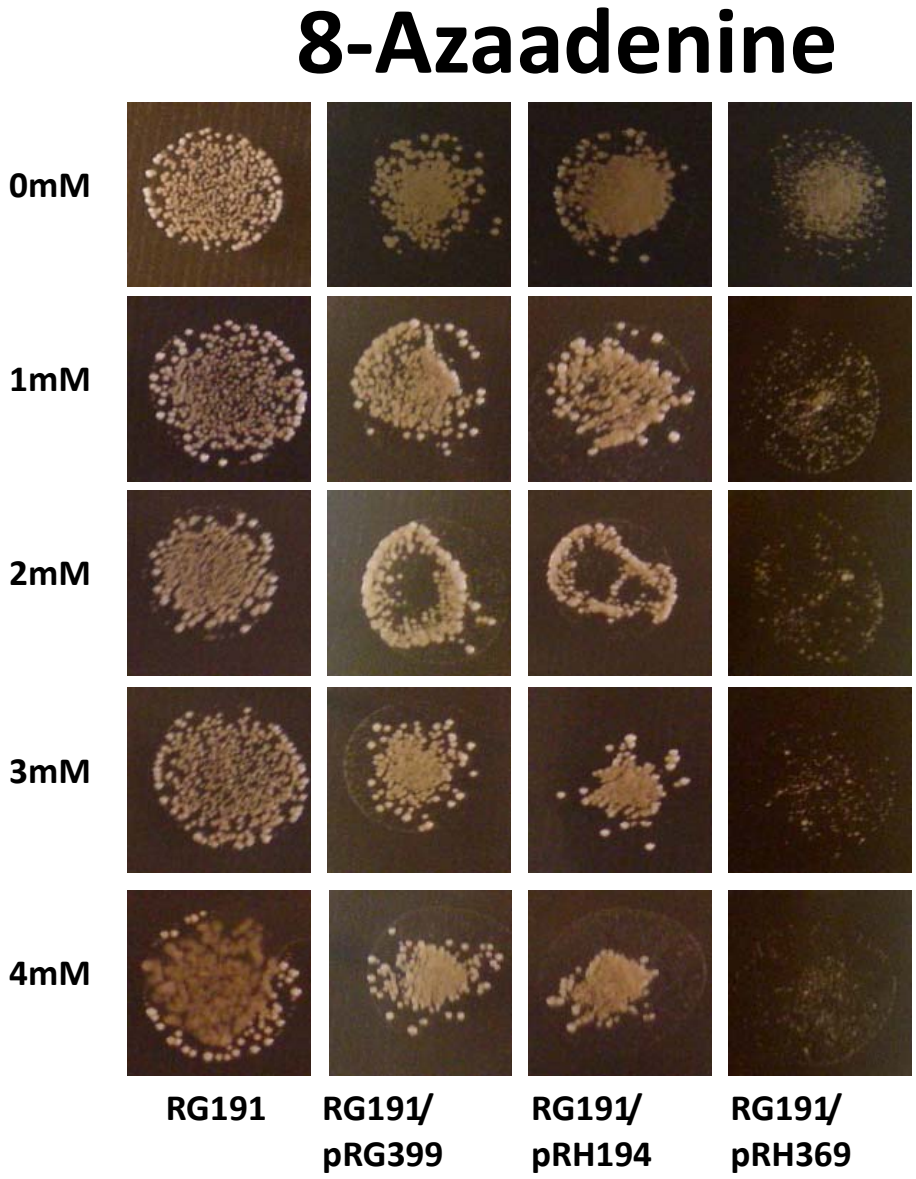

Figure 3. Growth of *S. cerevisiae* cells expressing *AtNAT5* on 8-Azaguanine. Strains RG191; RG191/pRG399; RG191/pRH194; and RG191/pRH369 were grown on SC media containing various concentrations of 8-AZG.

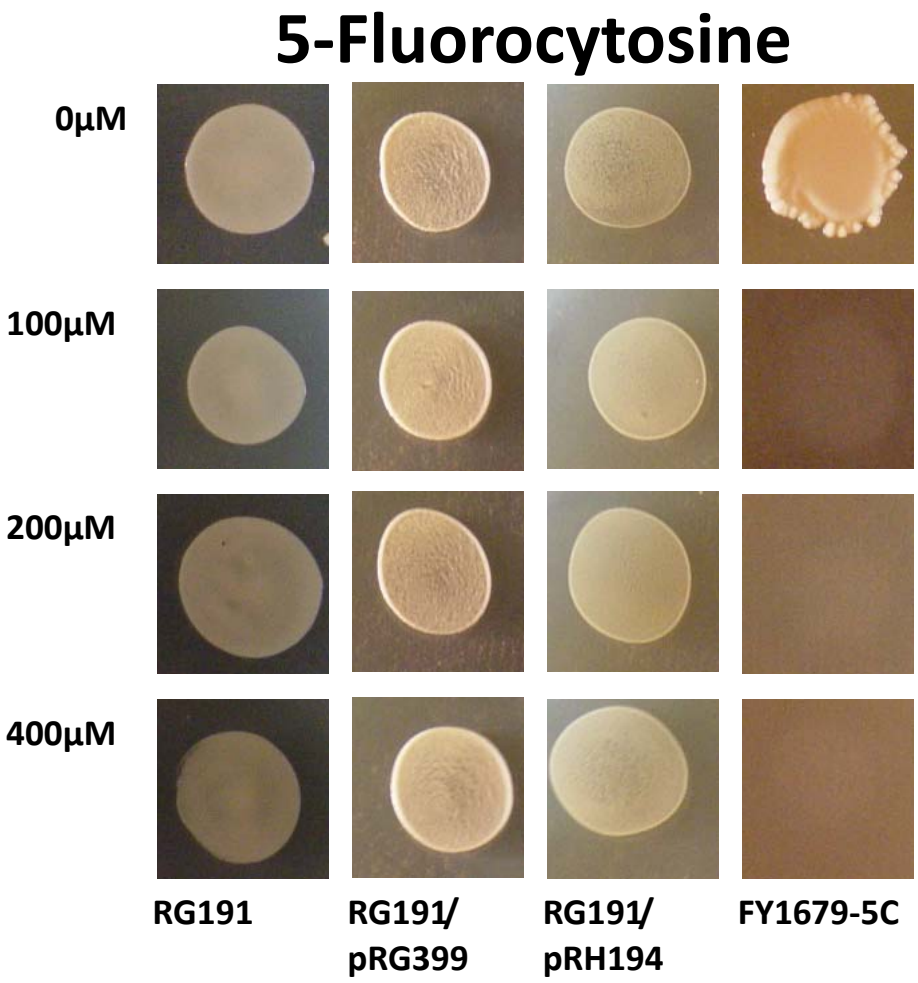

Figure 4. Growth of *S. cerevisiae* cells expressing *AtNAT5* on 5-fluorocytosine. Strains RG191; RG191/pRG399; RG191/pRH194; and FY1679-5C were grown on SC media supplemented with various concentrations of 5-fluorocytosine.

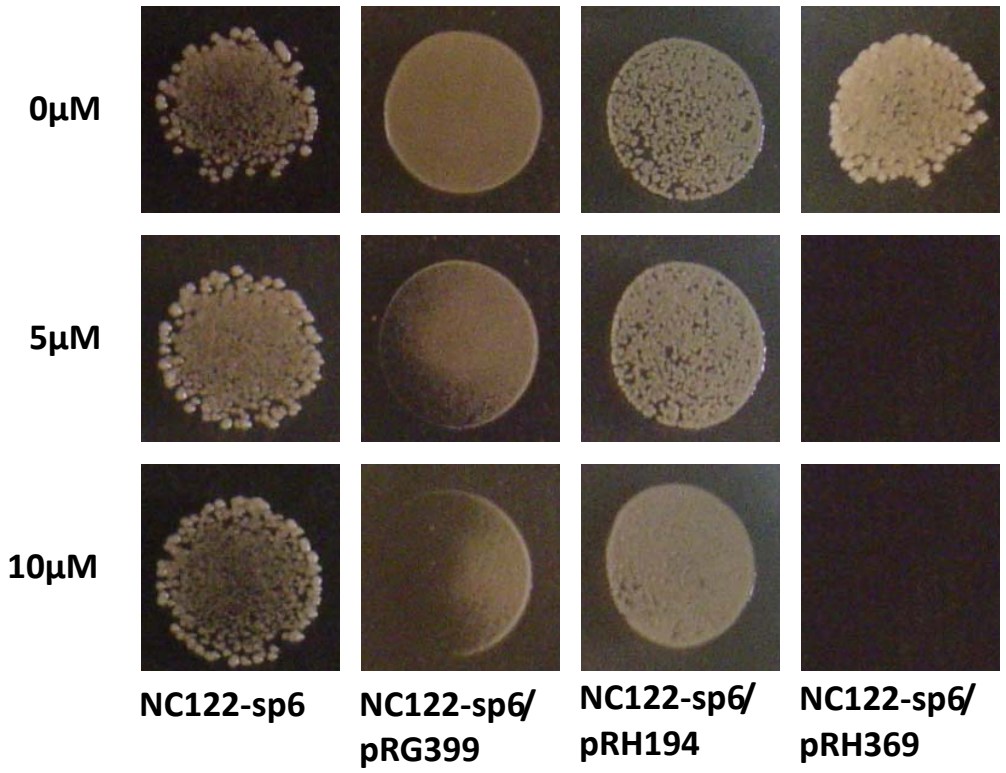

Figure 5. Growth of *S. cerevisiae* cells expressing *AtNAT5* on 5-fluorouracil. Strains NC122-sp6; NC122-sp6/pRG399; NC122-sp6/pRH194; and NC122-sp6/pRH369 were grown on SC media containing various concentrations of 5-fluorouracil.

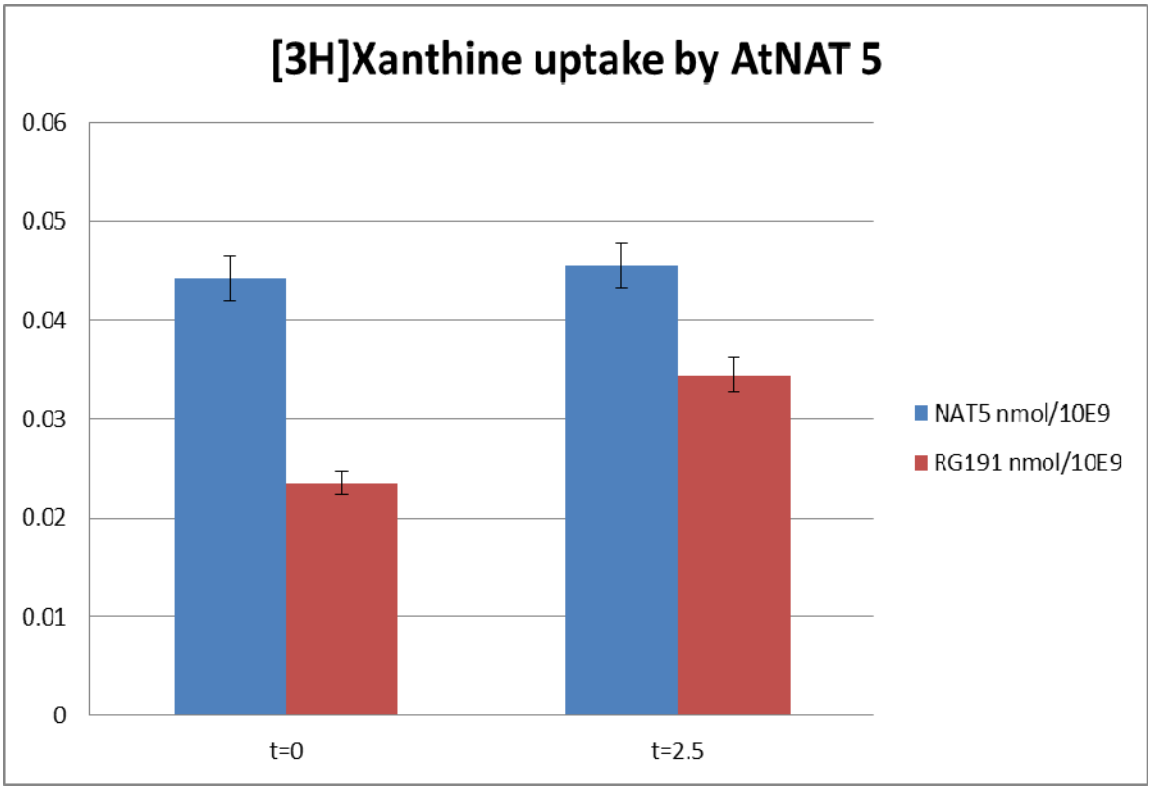

Figure 6: *AtNAT5* mediated uptake of [^{3}H]-xanthine. Strains RG191 and RG191/pRH194 were incubated with 0.2µM [^{3}H]-xanthine at 30°C. Back ground values were subtracted from the uptake values of RG191. Standard error bars represent the standard error of the mean of three independent experiments.

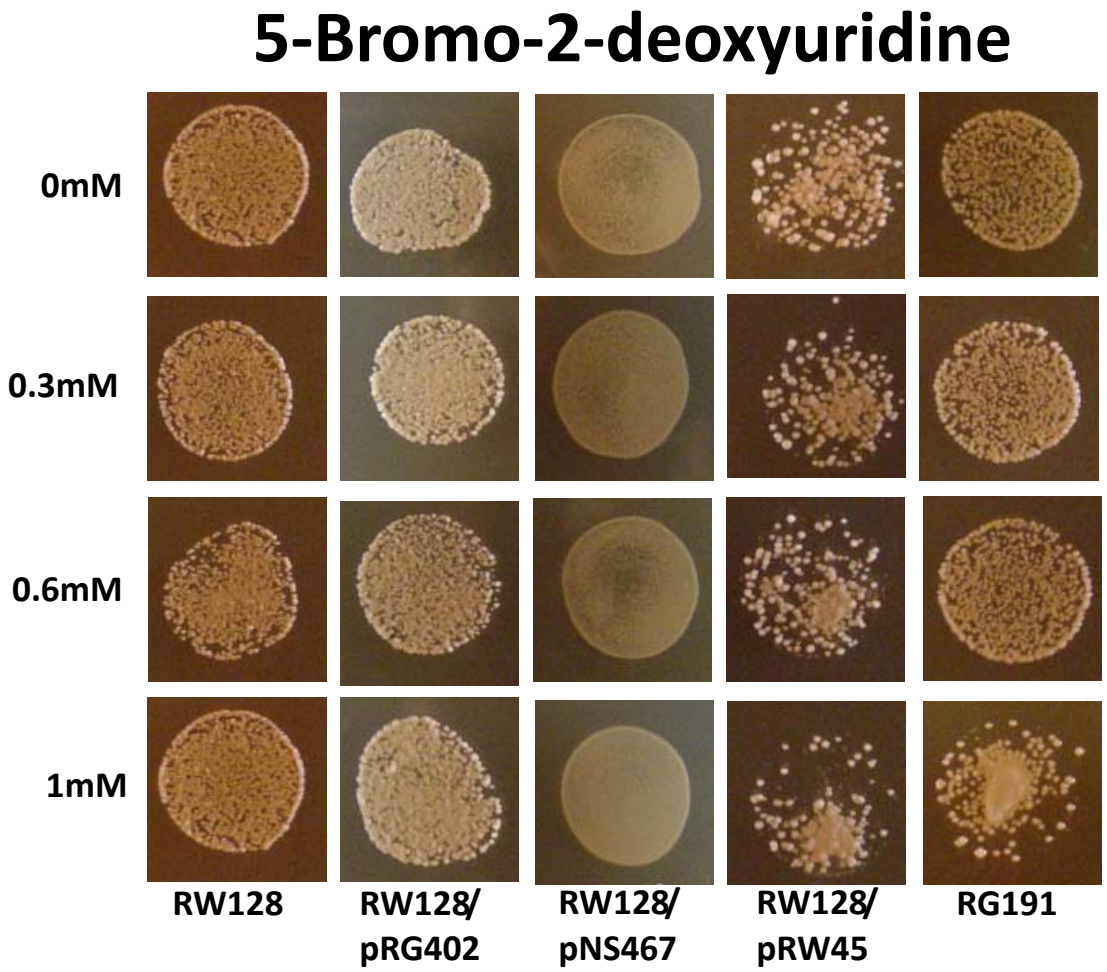

Figure 7. Growth of *S. cerevisiae* cells expressing *AtNCS1* on 5-bromo-2-deoxyuridine. Strains RG191; RW128; RW128/pRG402; RW128/pRW45; and RW128/pNS467 were grown on SC media containing various concentrations of 5-bromo-2-deoxyuridine.

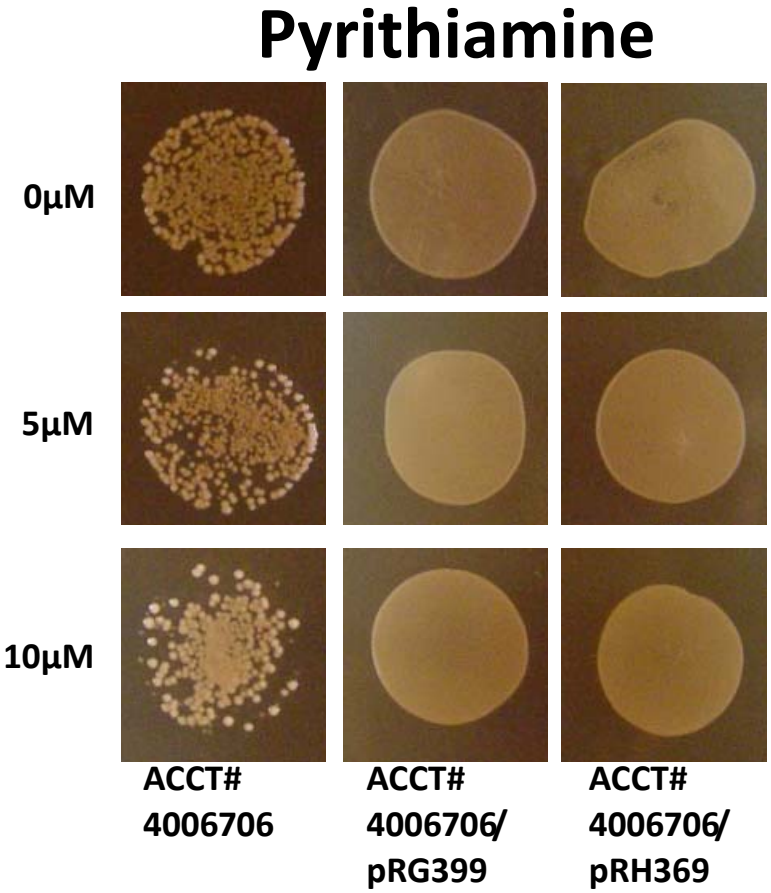

Figure 8. Growth of *S. cerevisiae* cells expressing *AtNCS1* on pyrithiamine. Strains ACCT#4006706; ACCT#4006706/pRG399; and ACCT#4006706/pRH369 were grown on SC media containing a concentration series of pyrithiamine.

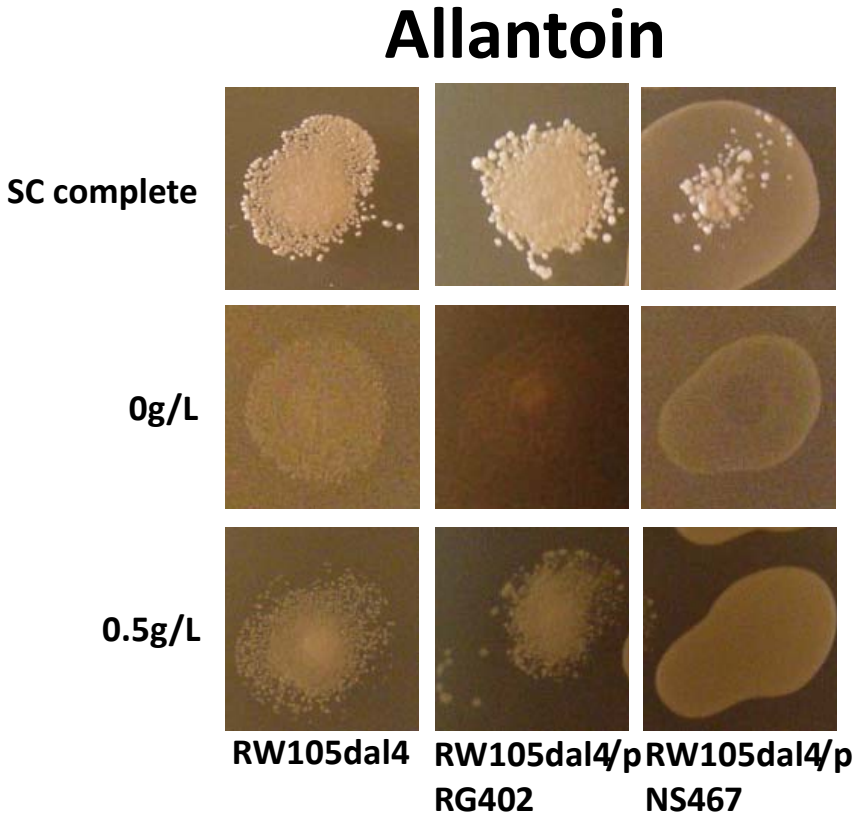

Figure 9. Growth of *S. cerevisiae* cells expressing *AtNCS1* on media containing allantoin as the sole nitrogen source. Strains RW105dal4; RW105dal4/pRG402; and RW105dal4/pNS467 were grown on SC medium supplemented with allantoin as the sole nitrogen source.

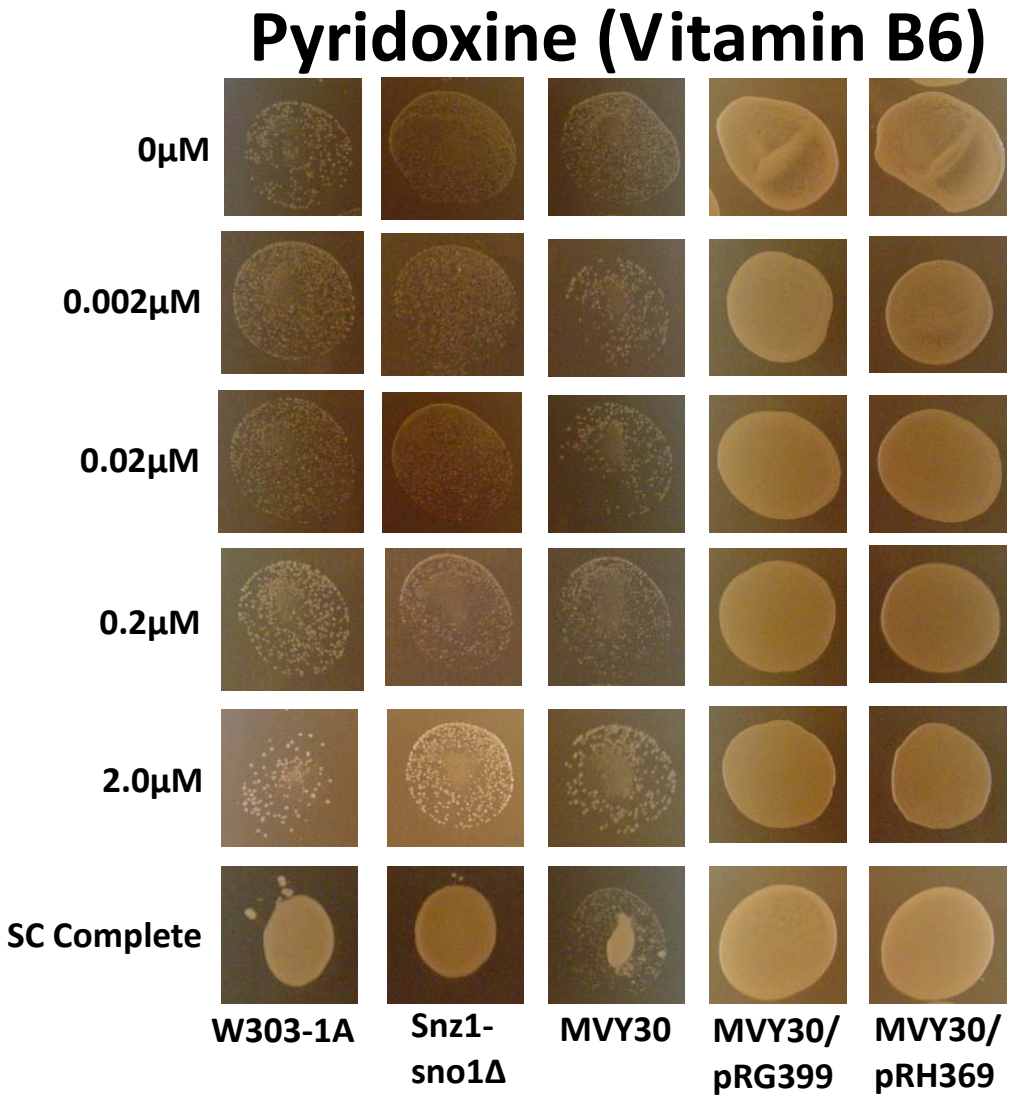

Figure 10. Growth of *S. cerevisiae* cells expressing *AtNCS1* on media containing various concentrations of pyridoxine. Strains W303-1A; sno-snz1Δ; MVY30; MVY30/pRG399, and MVY30/pRH369 were grown on SC medium containing a concentration series of pyridoxine.

CHAPTER 3: RESULTS

Growth-Based Assays of AtNAT5 Mediated Uptake of Toxic Purines and Pyrimidines

Yeast expression plasmids containing the gene locus At5g49990 encoding *AtNAT5* (pRH194) were transformed into *fcy2* (adenine-cytosine-guanine transport) or *fur4* (uracil transport) mutant strains then grown on Synthetic Complete media containing various concentrations of toxic analogs. *fcy2* mutants and transformed with *AtNAT5* were grown on media containing 8-AZA, 8-AZG and 5-FC (Figures 2-4). *fur4* mutants and transformed *AtNAT5* strains were grown on media containing 5-FU (Figure 5). Results show that yeast strains expressing *AtNAT5* exhibit no sensitivity to growth on media containing 8-AZA, 8-AZG, 5-FC or 5-FU suggesting that AtNAT5 does not transport adenine, guanine, cytosine or uracil. These results are consistent with the putative function assigned to the AtNATs, which are suggested to function as xanthine/uric-acid transporters (Diallinas and Gournas, 2008).

Uptake Based Assays of [8-^{3}H]-Xanthine by AtNAT5

AtNAT5 transformed yeast strains deficient in *fcy2* exhibit differences in [^{3}H]-xanthine uptake not statistically significant relative to RG191 ($t=1.7725$, $P=0.2183$); ($t=1.6738$, $P=0.2361$) at times 0 and 2.5 minutes respectively (Table 4). Results suggest that AtNAT5 does not function as a xanthine transporter when using this method of heterologous complementation in yeast (Figure 6). This result may be due to the lack of necessary genetic elements not present in the *S. cerevisiae* genome. Previous research of the AtNATs using heterologous complementation as well as single, double and triple knock-out mutants *in planta* attempted to elucidate the function of these putative nucleobase-ascorbate transporter genes has produced similar negative results (Maurino et al., 2006). Heterologous complementation was used to identify the function of a single AtNAT gene while controlling for functional redundancy of other nucleobase transporters; however, due to the high functional redundancy among the AtNAT

gene family in particular, elements required for proper protein function may be absent when expressed complementarily in yeast.

Growth-Based Assays of AtNCS1 Using Toxic Analogs

AtNCS1 was expressed using heterologous complementation in *S. cerevisiae* strains deficient in native ScNCS1 genes. To determine other solutes of transport by the recently characterized AtNCS1 (Mourad et al., 2012), yeast expression vectors containing *AtNCS1* (pRH369 and pNS467) were transformed into *fui1* (uridine transporter) or *thi7* (thiamine transporter) mutant strains and grown on media containing various concentrations of the toxic analogs 5-bromo-2-deoxyuridine and pyrithiamine respectively (Hamari et al., 2009; Wagner et al., 1998; Jund and Lacroute 1970; Singleton 1997). Results show *fui1* transformed *AtNCS1* strains show no sensitivity to growth on the toxic analog 5-bromo-2-deoxyuridine, suggesting that AtNCS1 does not transport uridine (Figure 7). Growth-based results of *thi7* transformed *AtNCS1* strains exhibit no sensitivity to growth on media containing pyrithiamine (Figure 8). These results suggest that AtNCS1 does not transport thiamine.

Growth-Based Assays of AtNCS1 Using Allantoin as the Sole Nitrogen Source

Heterologous complementation of *AtNCS1* in *dal4* (allantoin transporter) mutant strains was used to determine if AtNCS1 functions to transport allantoin when used as the sole nitrogen source. *dal4* transformed *AtNCS1* strains were grown on Synthetic Complete media containing allantoin as the sole nitrogen source (Desimone et al., 2002; Pélissier et al., 2004) (Figure 9). Results show that AtNCS1 is able to grow on media containing as little as 0g/L of allantoin as a sole nitrogen source; however, the growth of the transformed cells cannot be determined as statistically different than growth exhibited by the *dal4* strains using this particular methodology.

Growth-Based Assays of AtNCS1 Using Varying Concentrations of Pyridoxine (Vitamin B_6)

A *S. cerevisiae tpn1*Δ mutant (MVY30) deficient in the transport of pyridoxine (Vitamin B_6) was transformed with *AtNCS1* and tested for its ability to grow in media containing various concentrations of Vitamin B_6. The results obtained from this experiment are inconclusive and

inconsistent with the literature due to the apparent ability of the *tpn1* deletion strain to exhibit growth similar to that of the parent strain W303-1A (Stolz and Vielreicher, 2003) (Figure 10).

CHAPTER 4: DISCUSSION

Functional Redundancy Among Nucleobase Transporters

Transporter gene families comprising of genes with similar function may have evolved from gene duplication events with individual genes subsequently diverging (Gournas et al., 2008). The full relevance of heterogeneity among family members is unknown, but it has been proposed that in some cases characteristic subtypes have evolved. Preservation of similar subtypes in different species would argue for specialized function rather than redundancy. With increased homology between family members it is unclear whether members have individual functions or whether there is redundancy and therefore compensatory mechanisms to operate if any individual transporter system malfunctions. However, selection pressure would be unlikely to maintain the expression of such large gene families if redundancy were the only explanation for the presence of these multiple member gene families (Hawkesford, 2003). Nucleobase transporters have distinct yet overlapping solute specificities and their associated genes also have distinct yet overlapping functional expression in various tissues and developmental stages (Bürkle et al., 2003; Enjo et al., 1997; Linka and Weber, 2010; Maurino et al., 2006; Schmidt et al., 2006). Gene family member interactions, patterns of expression, and post-translational control are still being identified for nucleobase transporters, while their relative effects upon one another are still unknown.

Yeast Complementation

Heterologous complementation of eukaryotic transporters is in general very problematic; however, yeast provides one of the most promising systems for expression despite significant retention at the endoplasmic reticulum, a proportion of expressed transporters have been shown to find their way to the plasma membrane and exhibit detectible activity (Liu et al., 2007; Schultes et al., 1996). Using *S. cerevisiae* as a tool to characterize eukaryotic transporter functions has proven to be a viable method due to the ease in genetic manipulation as well as

the wealth of previous knowledge obtained through research of native biosynthetic pathways (Amillis et al., 2007; Gillissen et al., 2000; Goudela et al., 2005). Heterologous complementation was performed to test whether *AtNAT5* could function in a transport capacity outside of a plant, and to identify other potential solutes transported by the recently characterized *AtNCS1* (Mourad et al., 2012). The coding regions of both *AtNAT5* and *AtNCS1* were cloned into yeast expression vectors downstream from a constitutive yeast promoter (PMA1), by members of Dr. Schultes' Lab resulting in the plasmids pRH194 for *AtNAT5* and pRH369 as well as pNS467 for *AtNCS1*. While both vectors contained multiple cloning sites and a constitutive yeast promoter, expression vector pRG399 contained a complementary gene for leucine biosynthesis and pRG402 contained a complementary gene for tryptophan biosynthesis.

The *AtNAT5* plasmid pRH194 was transformed into two mutant strains deficient in either *fcy2* (adenine-guanine-cytosine-hypoxanthine transporter) or *fur4* (uracil transporter) and were grown on media containing 8-AZA, 8-AZG, 5-FC, or 5-FU. Results shown in Figures 2-5 indicate that yeast strains expressing *AtNAT5* do not transport adenine, guanine, cytosine, or uracil. Radio-labeled uptake of [^{3}H]-xanthine of an *AtNAT5* transformed *fur4* mutant strain was used to test whether AtNAT5 could function as a xanthine transporter outside of the plant. Results from this radio-labeled uptake experiment, seen in Figure 6, suggest that AtNAT5 does not transport xanthine when expressed in yeast.

Other alternative explanations may be applied to the results obtained using *AtNAT5*. For proper expression of a eukaryotic transporter gene within a yeast host genome specific 5' upstream sequences, a yeast specific promoter, correct 3' downstream termination sequences, as well as secretory sequences tagged to the polypeptide are required. To ensure AtNAT5 is being transcribed RT-PCR may be performed and gel electrophoresis used to determine if mRNA of *AtNAT5* is present. To determine if *AtNAT5* is translated from mRNA into a protein a Western-blot may be performed. Other post-translational modifications may occur using heterologous complementation so expression analysis of *AtNAT5* in yeast can be determined by the addition of a GFP-tag or by GUS-staining techniques. If any of the above techniques provide evidence to the improper functional expression of *AtNAT5* then manipulations to the heterologous complementation system can be applied and tested further.

Expression of eukaryotic membrane transporters using heterologous complementation does not necessarily ensure that the gene product will function in the same manner when

expressed in its native genome. Elements not present in the yeast host, may affect gene function, protein expression, and/or protein folding. Heterologous complementation is useful for reducing redundant functions of other transporter genes, but gene interactions between other family members or transcriptional regulators not present in the yeast genome may cause the gene to be expressed but not be functionally active. Alterations to the expression vector through the use of an inducible promoter instead of a constitutive promoter may provide a tool for protein induction, but a constitutive promoter is best used for toxic analog assays versus radiolabeled uptake assays. More information on gene member interactions and post-translational regulation and control are necessary to understand the requirements for proper gene function.

Heterologous complementation of *AtNCS1* in yeast has already provided functional characterization of its ability to transport adenine, guanine and uracil (Mourad et al., 2012). *AtNCS1* plasmid pRH369 was transformed into *thi7* (thiamine transporter) and *tpn1* (pyridoxine transporter) mutant strains then grown on media containing the toxic analog pyrithiamine or media containing various concentrations of pyridoxine respectively. Results from the pyrithiamine growth-based assay, shown in Figure 8, suggest that thiamine is not a substrate transported by *AtNCS1*. When transformed strains were grown on media containing various concentrations of pyridoxine, shown in Figure 10, the results prove to be inconsistent with literature describing *tpn1* deletion strains being unable to grow on media containing less than 0.02µM concentrations of pyridoxine (Stolz and Vielreicher, 2003). The ability of *AtNCS1* to transport pyridoxine remains unknown at this time.

AtNCS1 plasmid pNS467 was transformed *fui1* (uridine transporter) and *dal4* (allantoin transporter) mutant strains. Transformed strains were grown on media containing 5-bromo-2-deoxyuridine or on media containing allantoin as the sole nitrogen source, respectively. Results from growth-based assays using the toxic analog 5-bromo-2-deoxyuridine, as shown in figure 7, suggest that *AtNCS1* does not function to transport uridine. Media containing allantoin as the sole nitrogen source was used in growth-based assays to determine if AtNCS1 could function as an allantoin transporter. The results obtained from this assay, as shown in figure 9, suggest allantoin is not a substrate of transport by *AtNCS1*; however, the differences in growth between strains cannot be significantly identified using this particular assay. To date, AtNCS1 functions as an adenine-guanine-uracil transporter in Arabidopsis (Mourad et al., 2012).

Conclusion

In summary two basic types of experiments were performed to assay the solute specificity profiles of AtNAT5 and the recently characterized adenine-guanine-uracil transporter AtNCS1 (Mourad et al., 2012). First, growth-based studies using toxic analogs, allantoin as the sole nitrogen source and various concentrations of pyridoxine tested for the ability for both AtNAT5 and AtNCS1 to transport previously unidentified solutes. The results obtained from these growth-based assays provided evidence to support *AtNAT5*'s putatively defined xanthine/uric-acid transport function (Gournas et al., 2008). Results from growth-based assays using *AtNCS1* provided evidence that AtNCS1 does not function to transport the related nucleobase metabolites: allantoin, thiamin, pyridoxine, and uridine. Second, radio-labeled uptake experiments in functionally complemented yeast tested the ability of AtNAT5 to function in a transport capacity outside of the plant to transport [^{3}H]-xanthine (Figure 6). Results of the radio-labeled uptake experiment can be confirmed further by testing for the proper expression of AtNAT5 by the methods and techniques described above.

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