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Biochemical and Kinetic Analysis of Arabidopsis Nucleobase Transport

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BIOCHEMICAL AND KINETIC ANALYSIS OF *Arabidopsis* NUCLEOBASE TRANSPORT

A Thesis

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ABSTRACT

Gicheru, Yvonne W. M.S., Purdue University, August 2012. Biochemical and Kinetic Analysis of *Arabidopsis* Nucleobase Transport. Major Professor: George S. Mourad.

Due to its relevance in plant growth, reproduction and development, inter- and intracellular transport of nucleobases remains to be an important area of basic research. In this work, two nucleobase transporters of *Arabidopsis thaliana* from two distinct families were partially characterized with the goal of determining their solute transport specificity. The first transporter studied is nucleobase ascorbate transporter 2 (At*NAT2*), one of a 12-member putative gene family predicted to function as a nucleobase-ascorbate transporters. The coding sequence of *AtNAT2* was cloned into a yeast expression vector driven by a yeast constitutive promoter. Yeast expressing *AtNAT2* were tested through growth studies utilizing toxic purine/pyrimidine analogues as well as through radiolabel uptake studies. We found evidence that AtNAT2 does not transport adenine, guanine, cytosine, uracil and xanthine**.** The second transporter studied was the nucleobase cation sympoter 1 of *A. thaliana* (*AtNCS1*). Here kinetic analysis of the recently functionally characterized *A. thaliana* nucleobase cation symporter 1 (*AtNCS1*) was undertaken through radiolabeled uptake assays utilizing yeast strains expressing the *AtNCS1* gene. Substrate saturation kinetics was approached by gradually increasing the substrate concentration, and the apparent K_m and V_{max} values were determined.

CHAPTER 1. INTRODUCTION

Inter- and Intra- Cellular Transport

The metabolic activities of cells are maintained by transport of various metabolites and ions into and out of the cells; this transport may occur across short or relatively long distances. Because unicellular and multicellular organisms differ with regard to regulation of biochemicals, energy requirements and homeostatic ion concentration, differences in transport systems between these organisms are expected (Nagata et al., 2008). The need for transport of cell sustaining substrates across the plasma membrane makes it imperative that cells have well defined transport systems. These transport systems consist of transmembrane proteins which have been shown to account for approximately 10% of all proteins in a cell. Most transporters demonstrate a high degree of specificity, meaning that different cell types require different transporters, and or different relative frequencies of certain types of transporters (de Koning & Diallinas, 2000).

Importance of Nucleobases

Nucleobase transport in cells is vital for DNA and RNA synthesis, coenzyme ($FAD^{2+,}$ NAD^{+}, $NADP^{+}$) function, assembly of signaling molecules (cAMP) and as a source of nitrogen for many microorganisms (Gournas et al., 2008; Diallinas & Gournas, 2008). Nucleobases also contribute to the biochemistry of glycoproteins and carbohydrates, and to the biosynthesis of secondary metabolites like cytokinins and caffeine (Zrenner et al., 2006). Pharmacologically, nucleobase analogues are used as antimetabolites in the treatment of many diseases. In essence these drugs are similar to the normal

nucleobases needed for DNA replication and when incorporated into the growing DNA strand, strand extension is inhibited halting cell growth and division. These nucleobase analogues include antiviral drugs (acyclovir, ganciclovir), antibiotics, antitumor drugs, immunosuppressants, leukemia chemotherapy (5-Fluorouracil, thioguanine) and drugs for treating parasitic diseases (allopurinol, pyrimethamine) (de Koning & Diallinas, 2000; Diallinas & Gournas, 2008; Pantazopoulou & Diallinas, 2007). In addition, plants utilize nucleobase derivatives like xanthine, hypoxanthine and uric acid as a source of nitrogen for growth (Gournas at al., 2008).

Many nucleobase transporters have been identified and characterized biochemically and functionally; in fungi and bacteria some of these transporters have been cloned and studied in depth. The nucleobase families described below are categorized based on their degree of homology with previously characterized transporters. This homology may be molecular or physiological. Research on nucleobase transporters commonly strives to discover the basis of nucleobase specificity, as well as appropriate models for cloning and studying nucleobase transporters with relevance to medicine, agriculture and pharmacology (de Koning & Diallinas, 2000).

Nucleotide Synthesis and Metabolism

Two primary pathways are employed for the synthesis of nucleotides: the *de novo* pathway, in which enzymes build purine/pyrimidine nucleotides from simple molecules like amino acids, carbon dioxide and tetrahydrofolate; and the salvage pathway, in which the by-products of cellular metabolism or catabolism of nucleic acids are recycled to make new nucleotides or bases as needed. The *de novo* pathway is believed to be plastidic while the salvage pathway may occur in more than one compartment (Moffat & Ashihara, 2002). Nucleotide *de novo* synthesis pathways have high energy requirements compared to salvage pathways and are therefore regulated by the availability of carbon, nitrogen and phosphate sources. Cells present in growing tissues have high demands for

nucleotides, and will use salvage pathways for the generation of nucleotides necessary for processes like flowering and pollen tube formation for example (Schmidt et al. 2006). In plant embryos, successful germination is dependent on the availability of nucleotides during imbibition. Cellular requirements necessitate a balance between the salvage and *de novo* pathways at germination. For instance, during seed germination, the first stage of growth relies heavily on the pyrimidine salvage reactions. These reactions are later replaced by *de novo* synthesis as development progresses (Stasolla et al., 2003). As nucleotide degradation rarely occurs directly at tissues where nucleotides are in high demand, transport systems between cells, and or into and out of storage tissues are needed to maintain cellular function (Schmidt et al., 2006).

Nucleoside/Nucleobase Transporters

Nucleobase transporters can be classified into four distinct families based on substrate specificity: the AzgA family , the Equilibrative Nucleoside Transporter family (ENT), the Nucleobase Cation Symporter family 1 (NCS1) or Purine Related Transporter family (PRT), and the Nucleobase-Ascorbate Transporter family (NAT) or Nucleobase Cation Symporter family 2 (NCS2) (Diallinas & Gournas, 2008).

Members of the AzgA family are specific for adenine and guanine, and utilize H^+ symport for function. This family is represented by the AtAzg1 and AtAzg2 transporters in *A. thaliana*. Another transporter of the family is the *A. nidulans* transporter AzgA, which transports adenine, guanine and hypoxanthine. About 10-12 transmembrane regions are predicted for the AzgA family compared to the 12-14 transmembrane regions predicted for the NAT family. There is some sequence homology between transmembrane region 1 of NATs and transmembrane region 2 of the AzgA family; transmembrane region 2 is highly conserved in all proteins of the AzgA family. However, the AzgA family lacks the signature motif ([Q/E/P]-N-X-G-X-X-X-X-T-[R/K/G]) present in all NATs. The similarities between the two families indicate a relatedness but perhaps not

enough to be classified under NATs (Diallinas & Gournas, 2008; Goudela, 2005; Mansfield et al., 2008; Cecchetto et al., 2004).

Equilibrative Nucleoside Transporters (ENTs) are conserved transmembrane proteins present in protozoans, plants and mammals and are especially specific for nucleosides and nucleoside analogues like adenosine, uridine and thymidine (Chen et al., 2006; Diallinas & Gournas, 2008; Hyde et al., 2001; Mohlmann et al., 2001; de Koning & Diallinas, 2000). These transporters are especially important for the synthesis of nucleotides by salvage pathways in cells that lack de novo purine biosynthetic pathways such as protozoan parasites and some mammalian cells like enterocytes and bone marrow cells (Hyde et al., 2001). *A. thaliana* has eight isoforms of this transporter protein with only two having been characterized on the molecular level. These transport adenosine, guanosine and cytidine (Wormit et al., 2007).

Two transporter families that occur in plants do not seem to share any similarities with other nucleobase transporters: Purine Permeases (PUP) and Ureide Permease (UPS) are H^+ symporters and are specific for adenine, cytosine, uracil, uric acid and purine related metabolites like allantoin, caffeine, xanthine and cytokinin (Gillissen et al. 2000; Diallinas & Gournas, 2008; Desimone et al., 2002).

The NCS1/PRT family uses a proton symport mechanism to transport purines, allantoin, cytosine or thiamine. Members of the family are found in prokaryotes, plants and fungi. This family is predicted to have 12 transmembrane regions and shows no sequence similarity to the nucleobase ascorbate family. The NCS1 genes in *S. cerevisiae* and *S. pombe* are FUR4 which encode for a uracil transporter. The FCY2 transporter of *S. cerevisiae* is specific for adenine, guanine, cytosine and hypoxanthine and in *E. coli*, CodB transports cytosine (de Koning & Diallinas, 2000; Goudela et al., 2005). In plants the *A. thaliana* locus At5g03555 encodes a protein that has significant amino acid similarity (20-22% amino acid identity and 52-54% amino acid similarity) to the *S. cerevisiae* NCS1 FUR4 (Figure 1) (Mansfield et al., 2008; Mourad et al.,

2012). A detailed relationship between the AtNCS1 amino acid sequence and putative orthologs in the plant and bacterial kingdoms is shown in the phylogenetic tree below (Figure2) (Mourad et al., 2012). Greatest degree of similarity is seen with the dicots (*Vitis* and *Ricinus*) followed by the monocots (*Zea* and *Oryza*) and to a lesser extent but still significant with the bacteria (*Bacillus* and *Escherichia*) and yeast (*S. cerevisiae*). However, the NCS1 proteins are all grouped together. Separate from the NCS1 proteins are the *A. thaliana* nucleobase transporters including AtNAT2 that do not appear to have sequence similarity to the NCS1 family. Recently, the solute specificity for the At5g03555 locus was demonstrated for adenine, guanine and uracil through *in planta* and yeast heterologous complementation studies (Mourad et al., 2012).

The NAT family includes H^+ symporters specific for xanthine, uric acid and uracil that occur in fungi, plants and bacteria, as well as the mammalian L-ascorbic acid/Na^+ symporters. Mammalian ascorbate transporters are classified under NATs based on their sequence despite the fact that ascorbate is structurally unrelated to nucleobases (Gournas et al., 2008; Diallinas & Gournas, 2008; de Koning &Diallinas, 2000). The NAT family was determined from characterization of the uric acid-xanthine permeases of *Aspergillus nidulans* (UapA and UapC) and the uracil permeases UraA of *E.coli* and the PyrP of *Bacillus subtilis* (Gournas at al., 2008; Diallinas & Gournas, 2008). The, the UapA permease is the most extensively and best studied NAT and is therefore used as the model for understanding the family (Leung et al., 2010; Kosti et al., 2010; Papageorgiou et al., 2008). To date, only the *Zea mays* plant NAT has been functionally characterized in the transport of uric acid and xanthine (Argyrou et al., 2001). A study by Maurino et al. (2006) classified the AtNAT proteins into clades along with the NAT proteins of other eukaryotes. According to the multiple sequence alignment conducted therein, all AtNAT proteins share 35-92% amino acid identity with each other and 20% amino acid identity with *A. nidulans* NATs. The *Zea mays* NAT (LPE1) was not classified with any of the

AtNATs, perhaps indicating a difference in substrate specificity between these two members of the NAT family.

Thesis Objective

The first objective of this study was to determine the solute transport specificity of the *A. thaliana* nucleobase-ascorbate transporter 2 (AtNAT2). Therefore, we hypothesized that AtNAT2 is a putative nucleobase-xanthine transporter based on two premises: the need for nucleic acids during germination based on the expression of the AtNAT2 in *A. thaliana* seeds and homology with the *Aspergillus nidulans* UapA xanthine transporter (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Cecchetto et al., 2004). To accomplish this objective *AtNAT2* was first expressed in *Saccharomyces cerevisiae* mutant strains deficient for the transport of nucleobases. The yeast expressing *AtNAT2* was then grown on media containing purine/pyrimidine toxic analogues. Restored sensitivity to any of the toxic analogues was taken to indicate a specificity for that compound by the transporter protein encoded by AtNAT2. As AtNAT2 is homologous to the *A. nidulans* permease UapA which transports uric acid and xanthine, a test for radiolabeled xanthine uptake was also done. The second objective of the study was to perform kinetic analysis of the *A. thaliana* nucleobase cation symporter 1 (*AtNCS1*), which was recently shown to transport adenine, guanine and uracil (Mourad et al., 2012). This was done with an assay that utilized radiolabeled purines/pyrimidines.

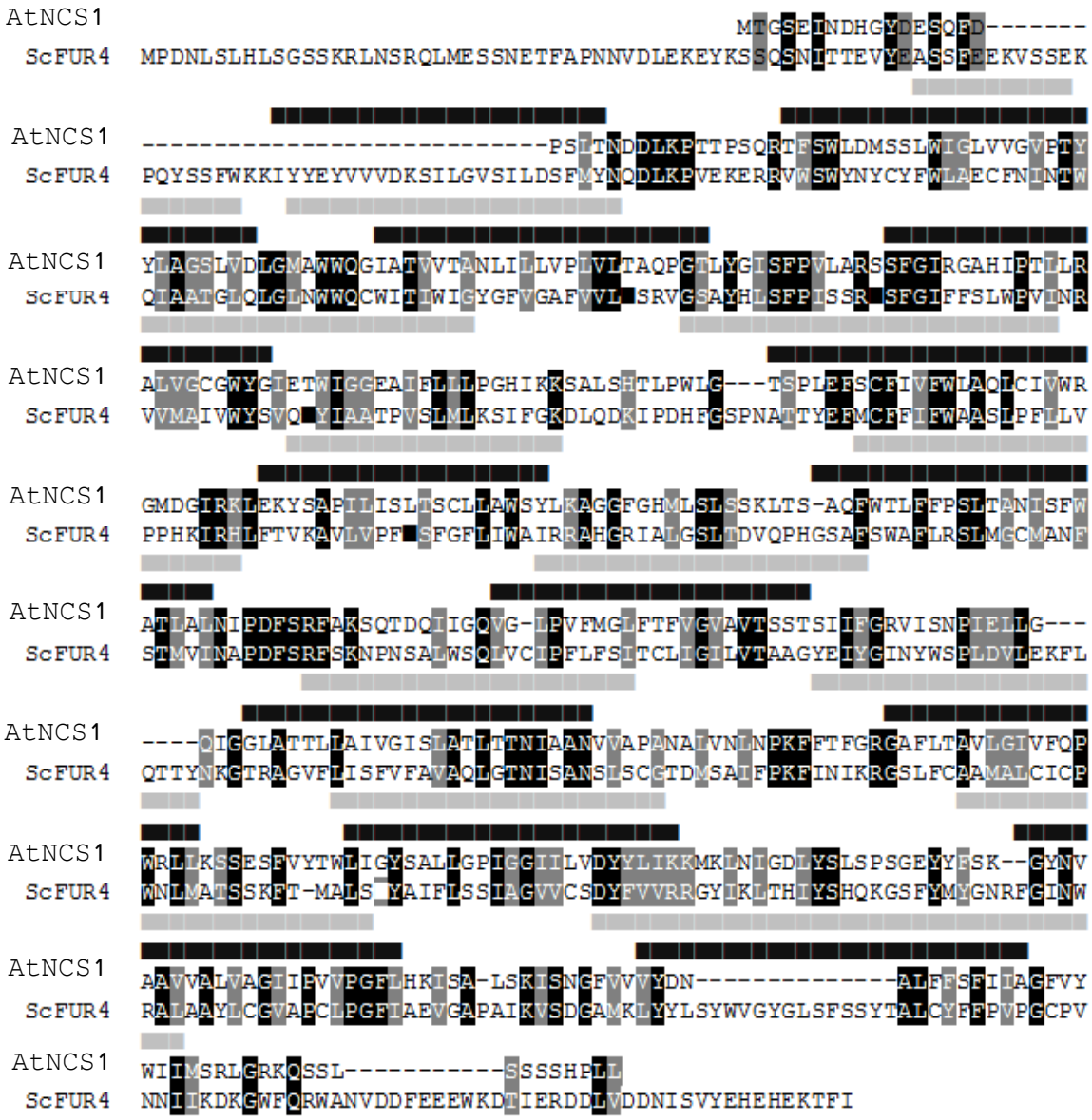

Figure 1. Amino acid alignment of AtNCS1and the *Saccharomyces cerevisiae* uracil transporter FUR4 by clustal W (Thompson et al., 1994). Black boxes represent amino acid identity while grey boxes indicate amino acid similarity. Predicted transmembrane spanning domains are indicated for AtNCS1 (light grey bars) and ScFUR4 (black bars) using TMHMM (Krogh et al., 2001; Mourad et al., 2012).

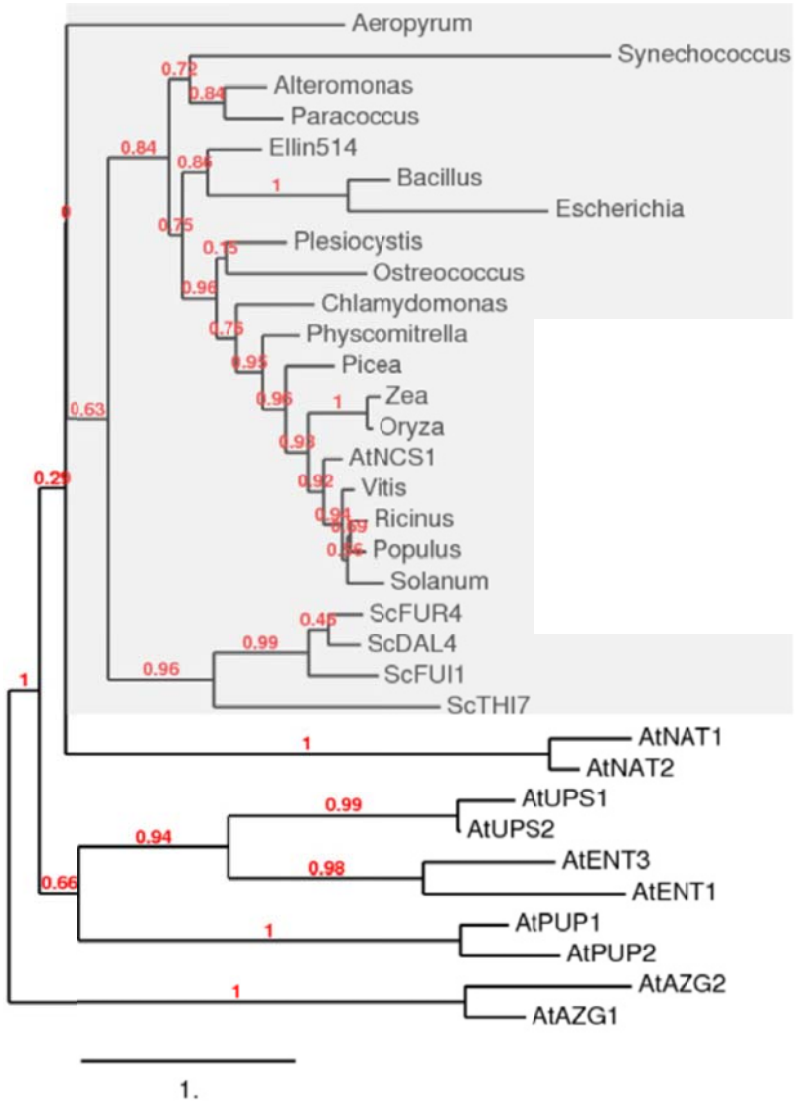

Figure 2. Phylogenetic tree showing the detailed relationship of AtNCS1 amino acid sequence to orthologous proteins from other organisms (Mourad et al., 2012).

CHAPTER 2. MATERIALS AND METHODS

Plasmid Generation

The plasmids pRH163 and pRH369 were generated by cloning the coding regions from loci At2g34190 (AtNAT2) and At5g03555 (AtNCS1) respectively into the multiple cloning site of the yeast expression vector pRG399 (2µ LEU2 PMA1-MCS-CYC). The cDNA (C104691) from the At2g34190 locus was amplified using primers C104691A and pUNI51REV and the resulting DNA fragment was treated with *Xho* I and *Not* I and ligated to the like cleaved sites on pRG399. Amplification of the coding region from the At5g03555 locus was done with genomic DNA and using primers At5g03555YEA and At5g03555YEB and the fragment was treated with *Xho* I and *Sph* I (Mourad at al., 2012). Another plasmid pRH183 carrying the coding region of the *Zea mays* NAT(*LPE1*) was generated by amplifying a DNA fragment from pNS175 with primers LPE1R5A and LPE1R5B, restricting with *Sal I* and *Not I* and cloning into yeast expression vector pRG399 treated with *Xho I* and *Not I*. This was used as a positive control for the radiolabel uptake assay. The plasmids were generated by Dr. Neil Schultes. Plasmids were extracted from *E. coli* grown overnight on LB media containing 50 mg/mL ampicillin for 18 hours at 37°C. The cultures were pelleted and DNA extracted using a Spin MiniPrep Kit (Qiagen). 50 µL of extract was acquired and quantified using the Nanodrop Spectrophotometer to ensure that DNA amount was above 100µg/uL. The extracted DNA was used to transform yeast strains by the lithium acetate method (Gietz & Woods, 2002).

Yeast Strains and Transformation

Three *Saccharomyces cerevisiae* strains RG191 [Mat *α, fcy2Δ:: kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*], NC122-sp6 [Mat *α leu2 fur4Δ*] and FY21679-5C [MAT *α leu2Δ1 his3Δ200 ura3-52*] (Mourad et al., 2012) were grown on Synthetic Complete medium (SC) (0.68% Yeast Nitrogen Base without amino acids, 2% glucose, 0.002% histidine, tryptophan methionine, leucine, 0.003% uracil , 2.2% agar (solid media) at 28-30°C and pH 5.6. Plasmids pRH163 and pRH369 were used to transform yeast strains RG191 and NC122-sp6. Due to the *fcy2* and *fur4* gene deletions, RG191 is unable to transport adenine, guanine and cytosine and NC122-sp6 cannot transport uracil respectively. FY1679-5C represents the *S. cerevisiae* wild type and has the ability to transport adenine, guanine, cytosine and uracil. The yeast expression vector pRG399 harbors a LEU2 selection marker that complements the deleted leucine gene in the mutant strains, restoring the ability to synthesize leucine. Cloned genes in the multicopy plasmid were placed under the constitutive promoter PMA1 and CYC terminator (Serrano & Villalba, 1995). Successfully transformed strains were thus able to grow on media without leucine.

AtNAT2 Toxic Analogue Growth Experiments

Purine and pyrimidine uptake by *AtNAT2* was assayed using the toxic analogues: 8-Azaadenine, 8-Azaguanine, 5-Fluorocytosine and 5-Fluorouracil. Overnight cultures of transformed and parental mutant strains were grown for 18 hours and then serially diluted to 10000, 1000, 100 and 10 cells/µL. Parent strains were grown on SC media while transformed strains were grown on SC media without leucine. Stock solutions of the toxic analogues were added to the media at the following concentrations: 0-4 mM 8-AzA and 8-AzG, 0-10 µM 5-FU and 0-100 µM 5-FC. A 30 µL drop of cells at each dilution was plated and growth was compared after 48 hours at 30°C between the strains containing *AtNAT2* gene and the control strains. The same experiment was done for the *AtNCS1* gene by Kevin A. Hunt, a student in the lab of Dr. Mourad.

Radiolabeled Uptake by Yeast Expressing *AtNAT2* and *AtNCS1*

Time course for the uptake of 0.5 µM [8-^{3}H]xanthine (Moravek Biochemicals, Brea, CA) was performed with yeast cells expressing *AtNAT2* and *Lpe1* (pRH183), a known xanthine and uric acid transporter in maize (Argyrou et al., 2001). Time course for the uptake of 0.5 µM [5,6-^{3}H]uracil (12.33 kBq ml_1) (Moravek Biochemicals, Brea, CA) was performed with yeast expressing *AtNCS1*(Mourad at al., 2012). Overnight cultures of yeast strains were incubated for 12 hours in 5 mL of SC media without leucine. Cultures were then transferred into a total volume of 30 mL of media and incubated for another 18 hours. Cells were harvested by centrifugation, washed and re-suspended with 100 mM Citrate buffer, pH 3.5 with 1% glucose at an OD_{600} = 4 and stored on ice. Yeast suspensions were pre-incubated at 30 °C for 2 minutes and equal volumes (50 µL) of yeast suspension and buffer containing radiolabeled compound were mixed. Final radiolabeled compound concentration in the samples was 0.5 µM. The mixture was incubated at 30 °C for 2 minutes with 50 µL aliquots taken at time 0 up to 10 minutes at two minute intervals. Samples were then transferred into 4 mL of ice cold water to stop the uptake reaction. Samples were filtered through a 0.45 µm Metricel membrane filter (Gelman Scientific, Ann Arbor MI) and then washed with 8 mL of water. Filters were placed in scintillation tubes and 3 mL scintillation fluid (EcoLume) was added. A liquid scintillation counter was used to measure radioactivity (Beckman Coulter, Fullerton, CA). Substrate saturation kinetics was done for varying concentrations of [5,6-^{3}H]uracil (0-10 µM) and [2,8-^{3}H]adenine (3.7 kBq ml_1) (0-20µM) at 2.5 minutes using yeast cells expressing *AtNCS1* in the same manner as described above. Statistical analysis of at least three independent experiments was done using a student's unpaired t-test and significance was measured at P = 0.05.

Table 1. Yeast strains and plasmids used in this study.

Yeast strain or plasmid	**Genotype**	**Substrate**	**Screen/assay**
RG191	MAT *α, fcy2Δ:: kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Adenine, guanine, cytosine	8-Aza, 8-Azg, 5-FC
NC122-sp6	MAT *α leu2 fur4Δ*	Uracil	5-FU
FY1679-5C	MAT *α leu2Δ1 his3Δ200 ura3-52*	Adenine, guanine, cytosine, uracil	8-Aza, 8-Azg, 5-FC
pRG399	2µ LEU2 PMA1-MCS-CYC (empty expression vector)	Adenine, guanine, cytosine, uracil	8-Aza, 8-Azg, 5-FC
pRH163	At2g34190 (*AtNAT2*) in expression vector	Adenine, guanine, cytosine, uracil	8-Aza, 8-Azg, 5-FC [^{3}H]xanthine
pRH369	At5g03555 (*AtNCS1*) in expression vector	Adenine, guanine, cytosine, uracil	8-Aza, 8-Azg, 5-FC [^{3}H]-adenine, guanine, uracil
pRH183	*Lpe1* gene in expression vector		[^{3}H]xanthine

CHAPTER 3. RESULTS

Toxic Analogue Experiments

Heterologous complementation studies were performed in *S. cerevisiae* in an attempt to establish the solute transport specificity of *AtNAT2* at locus At2g34190. The purine/pyrimidine toxic analogues 8-Aza, 8-Azg, 5-FC and 5-FU were added to SC media for control strains and SC media without leucine for experimental strains. Strain RG191/pRH163 (*AtNAT2*) was able to grow well on all the toxic analogues indicating that this gene does not mediate transport of these specific purines/pyrimidines (Figures 3-6). The positive control strain FY1679-5C showed increasing sensitivity with increase in concentrations of 8-Aza, 8-Azg and 5-FC while the negative control strains RG191 and RG191/pRG399 (empty vector) showed comparable growth to yeast expressing *AtNAT2*. Strain NC122-sp6/pRH369 (*AtNCS1*) showed heightened sensitivity to 5-FU indicating that this gene mediates uracil transport (Figure 6). In addition, yeast expressing *AtNCS1* showed sensitivity to 8-Azaadenine and 8-Azaguanine indicating that the transporter mediates these substrates as well (See Appendix). Work on the *AtNCS1* gene was completed by Kevin A. Hunt, a student in the lab of Dr. Mourad.

Radiolabel Uptake by Yeast Expressing *AtNAT2*

A time course experiment for the uptake of 0.5 µM [8-^{3}H]xanthine was done in three yeast strains: RG191 (negative control), Lpe1 (positive control known to transport xanthine) and RG191/pRH163 (AtNAT2). There was no significant difference in uptake between AtNAT2 and the two controls at P=0.05

(Figure 7). This indicates that AtNAT2 does not mediate xanthine transport in *A. thaliana*.

Radiolabel Uptake by Yeast Expressing *AtNCS1*

Radiolabeled adenine, guanine and uracil were used to perform uptake experiments on yeast expressing *AtNCS1*. Results show significant uptake in yeast expressing the transporter gene, as compared to the mutant control strains deficient in the *fcy2* (RG191) and *fur4* (NC122-sp6) transporters matching the toxic analogue growth experiments (see appendix). This work was completed by Julie Crosby, a student in the lab of Dr. Mourad.

Transport Kinetics of Yeast Expressing *AtNCS1*

Yeast cells expressing *AtNCS1* were used in a time course reaction for the uptake of [5,6-^{3}H]uracil. The results showed a linear relationship up to 10 minutes (Figure 8). The graph suggests that this linear relationship could continue for a longer time interval before plateauing. Substrate saturation for [5,6-^{3}H]uracil was approached at 10 µM concentrations (Figure 9) and a Lineweaver-Burk plot established an apparent K_m of 7.0 µM and a V_{max} of 0.30 nmol / 10^{9} cells / min (Figure 10). For [2,8-^{3}H]adenine substrate saturation was approached at 30 µM concentrations (Figure 11) and the Lineweaver-Burk plot showed an apparent K_m of 7.9 µM and a V_{max} of 3.3 nmol / 10^{9} cells / min (Figure 12).

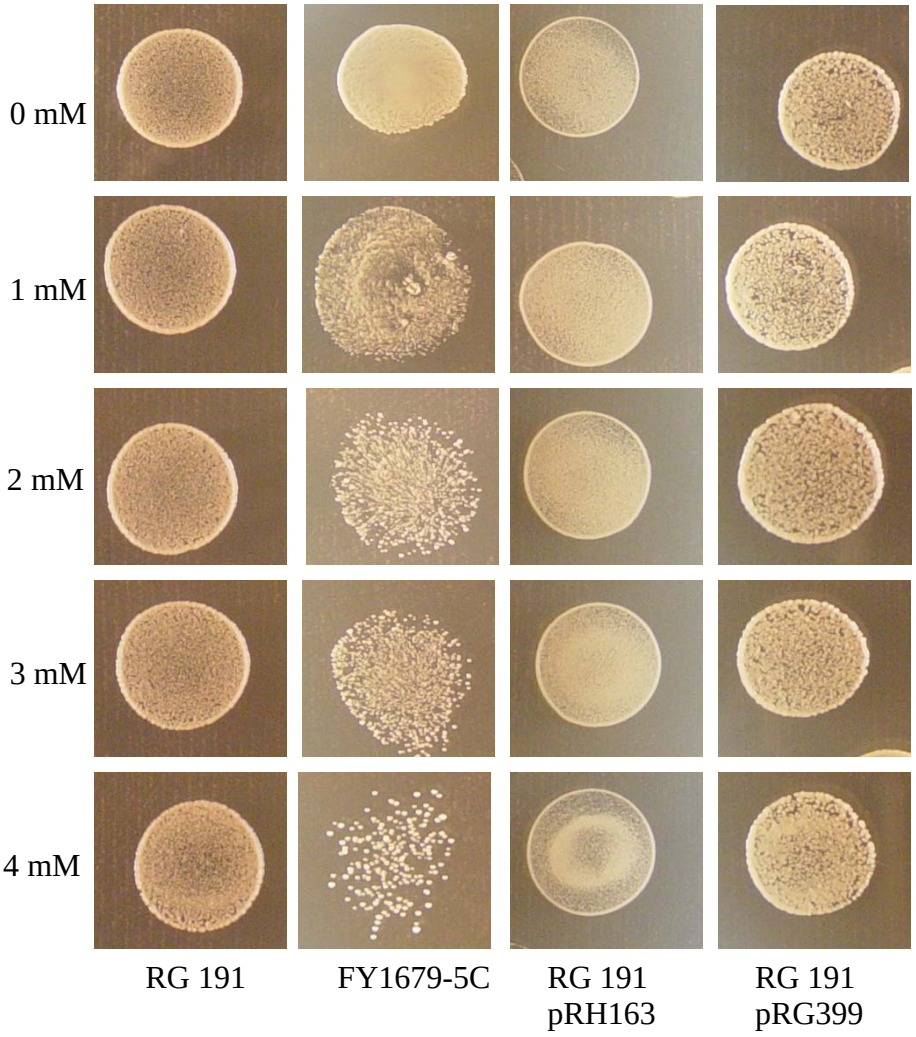

Figure 3. Growth of *Saccharomyces cerevisiae* expressing *AtNAT2* on 8-Azaadenine. *fcy2* mutant deficient for adenine, guanine and cytosine transport (RG191); *fur4* mutant deficient for uracil transport (NC122-sp6); wild type for *FCY2* and *FUR4* (FY1679-5C); empty expression vector (pRG399); expression vector with *AtNAT2* coding region placed under constitutive transcriptional control (pRH163). Strains grown on increasing concentrations (0-4mM) 8-Azaadenine.

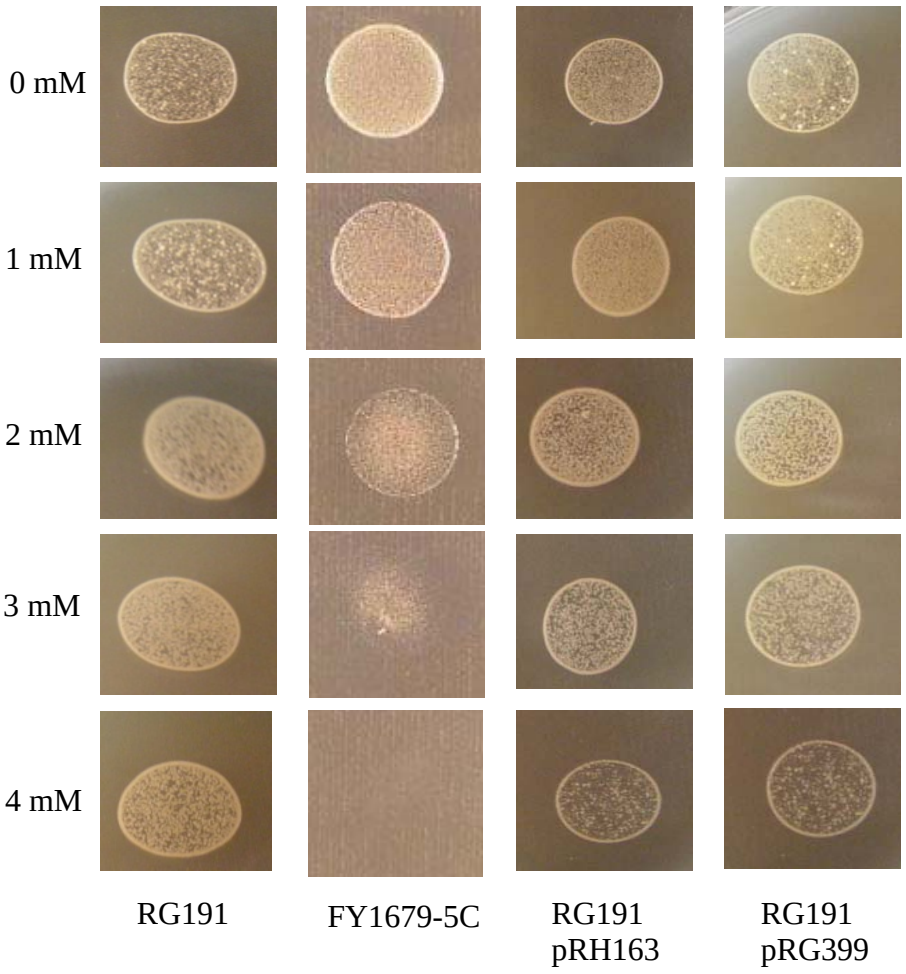

Figure 4. Growth of *Saccharomyces cerevisiae* expressing *AtNAT2* on 8-Azaguanine. *fcy2* mutant deficient for adenine, guanine and cytosine transport (RG191); *fur4* mutant deficient for uracil transport (NC122-sp6); wild type for *FCY2* and *FUR4* (FY1679-5C); empty expression vector (pRG399); expression vector with *AtNAT2* coding region placed under constitutive transcriptional control (pRH163). Strains grown on increasing concentrations (0-4mM) 8-Azaguanine.

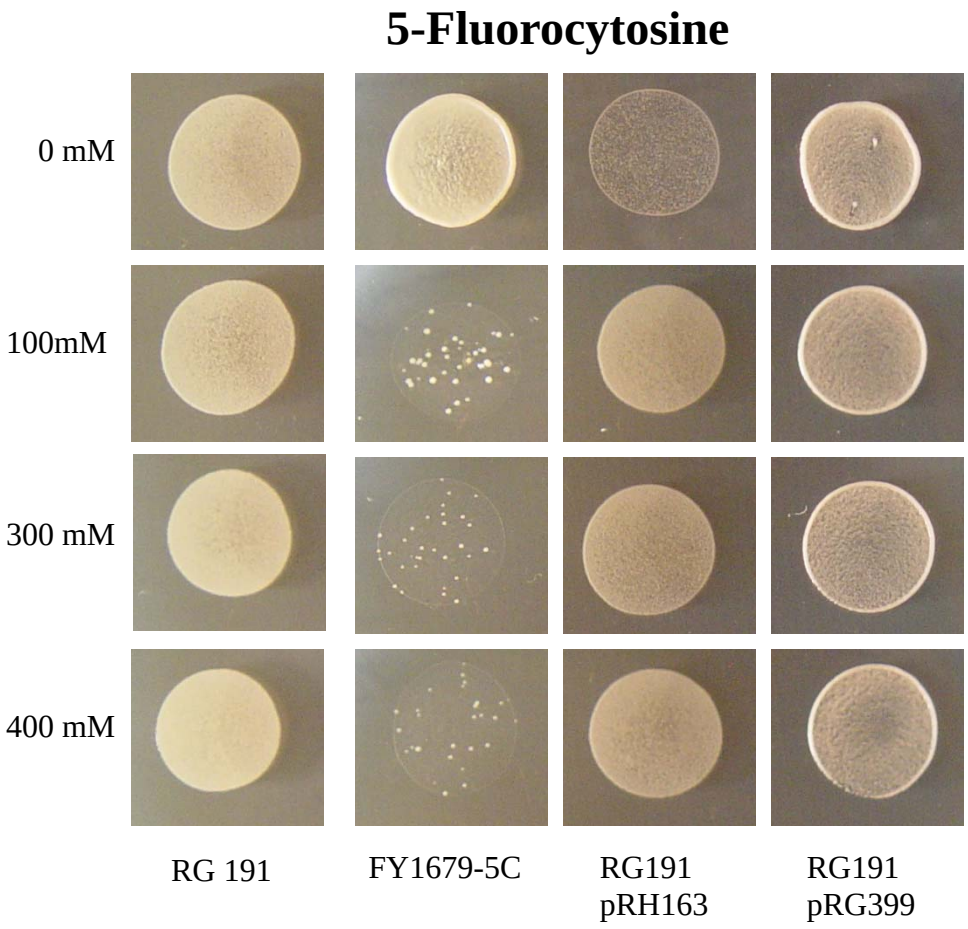

Figure 5. Growth of *Saccharomyces cerevisiae* expressing *AtNAT2* on 5-Fluorocytosine. *fcy2* mutant deficient for adenine, guanine and cytosine transport (RG191); *fur4* mutant deficient for uracil transport (NC122-sp6); wild type for *FCY2* and *FUR4* (FY1679-5C); empty expression vector (pRG399); expression vector with *AtNAT2* coding region placed under constitutive transcriptional control (pRH163). Strains grown on increasing concentrations (0-400mM) 5-Fluorocytosine.

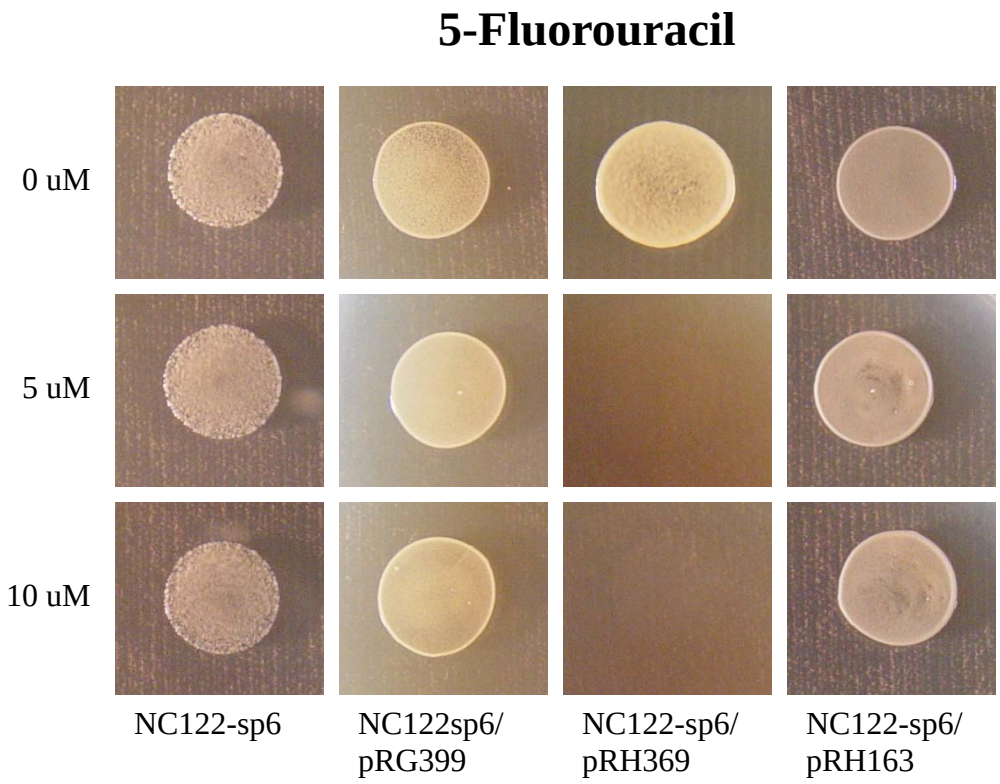

Figure 6. Growth of *Saccharomyces cerevisiae* expressing *AtNAT2* and *AtNCS1* on 5-Fluorouracil. *fcy2* mutant deficient for adenine, guanine and cytosine transport (RG191); *fur4* mutant deficient for uracil transport (NC122-sp6); wild type for *FCY2* and *FUR4* (FY1679-5C); empty expression vector (pRG399); expression vector with *AtNAT2* coding region placed under constitutive transcriptional control (pRH163); expression vector with *AtNCS1* coding region placed under constitutive transcriptional control (pRH369). Strains grown on increasing concentrations (0-10mM) 5-Fluorouracil.

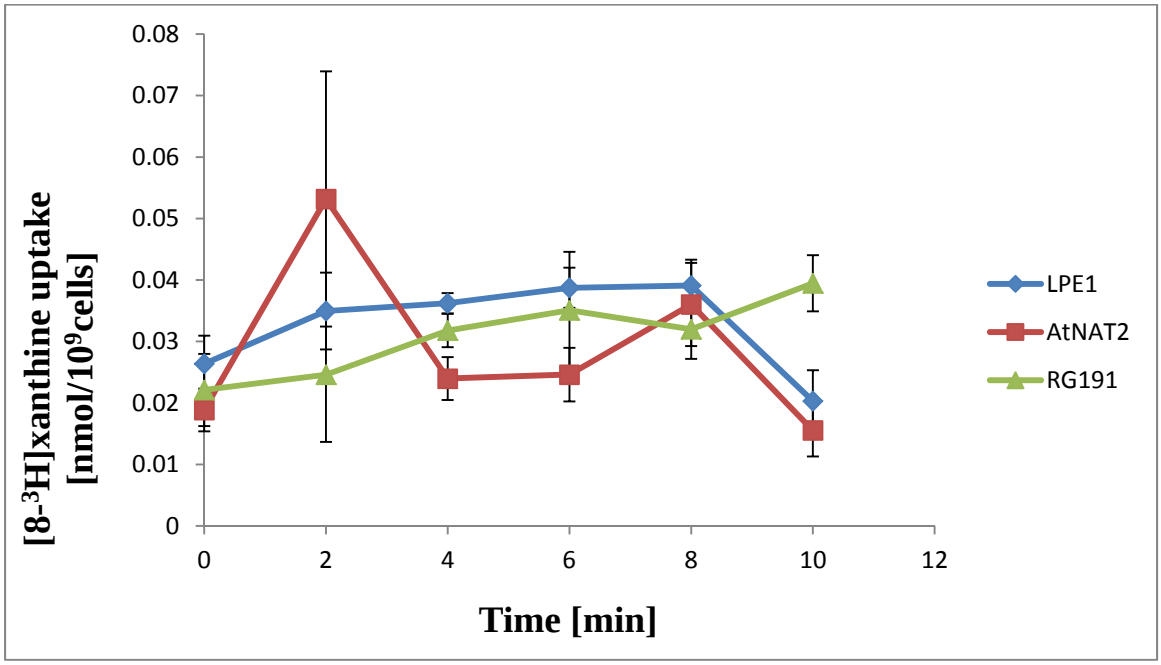

Figure 7. Uptake of [8-^{3}H]xanthine in *Saccharomyces cerevisiae* expressing *AtNAT2*. RG191 (*fcy2* mutant deficient in adenine, guanine and cytosine transport); RG191 expressing *AtNAT2* (pRH163 cloned into RG191); *Lpe1* (*Zea mays* xanthine-uric acid transporter cloned into RG191). There was no significant difference in uptake between AtNAT2 and the controls.

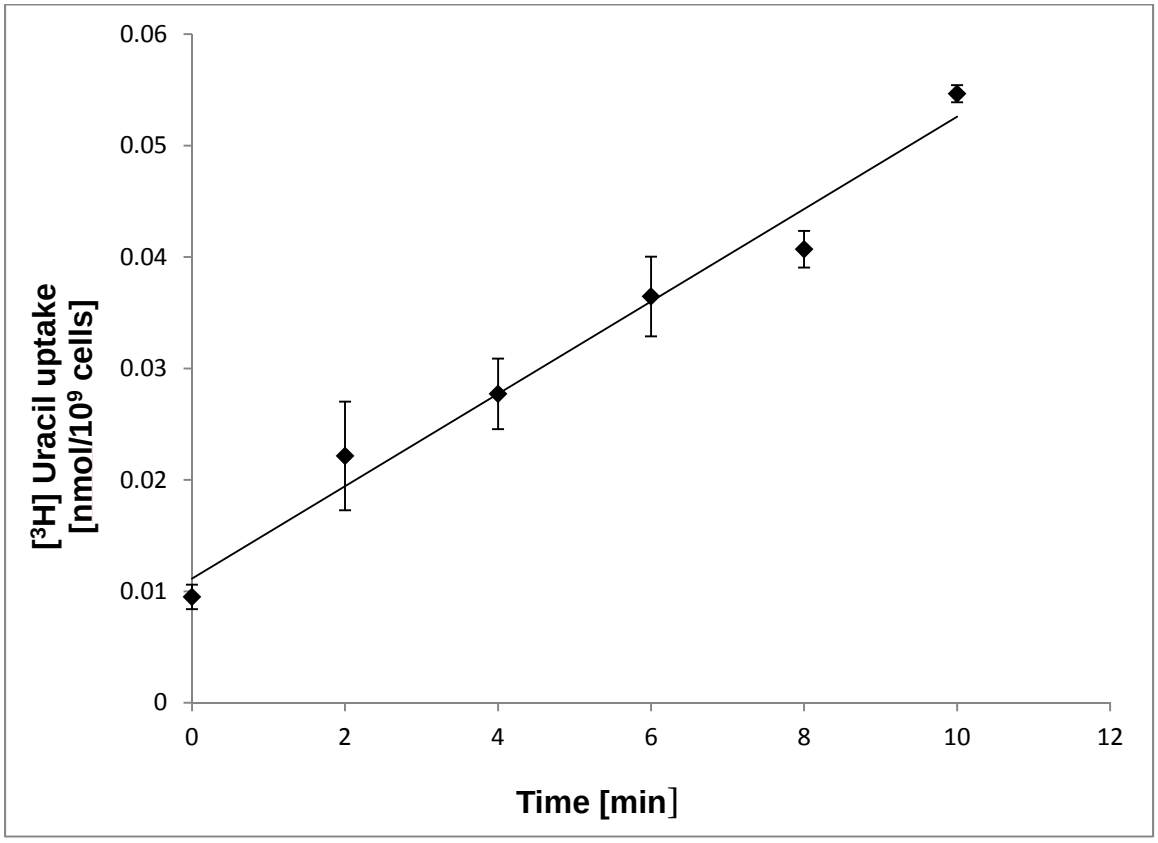

Figure 8. Time dependence of *AtNCS1* mediated [5,6-^{3}H]uracil uptake in *Saccharomyces cerevisiae*. Uptake rate remained constant during the first 10 minutes.

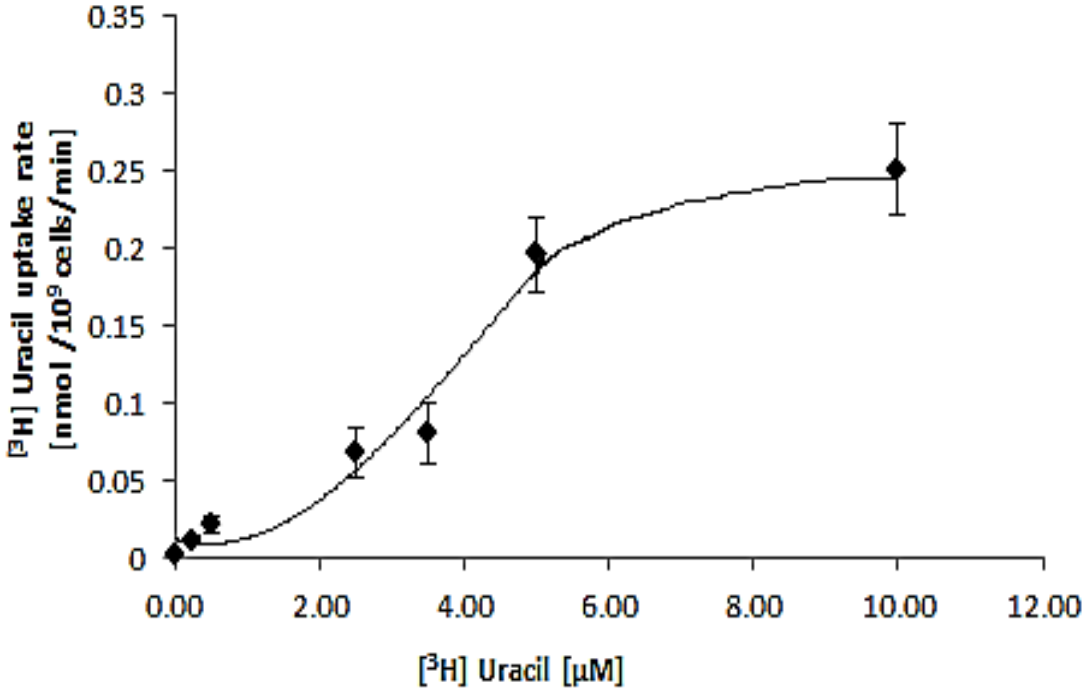

Figure 9. Substrate saturation with varying concentrations of [5,6-^{3}H]uracil. Yeast expressing *AtNCS1* approach saturation with increasing concentrations.

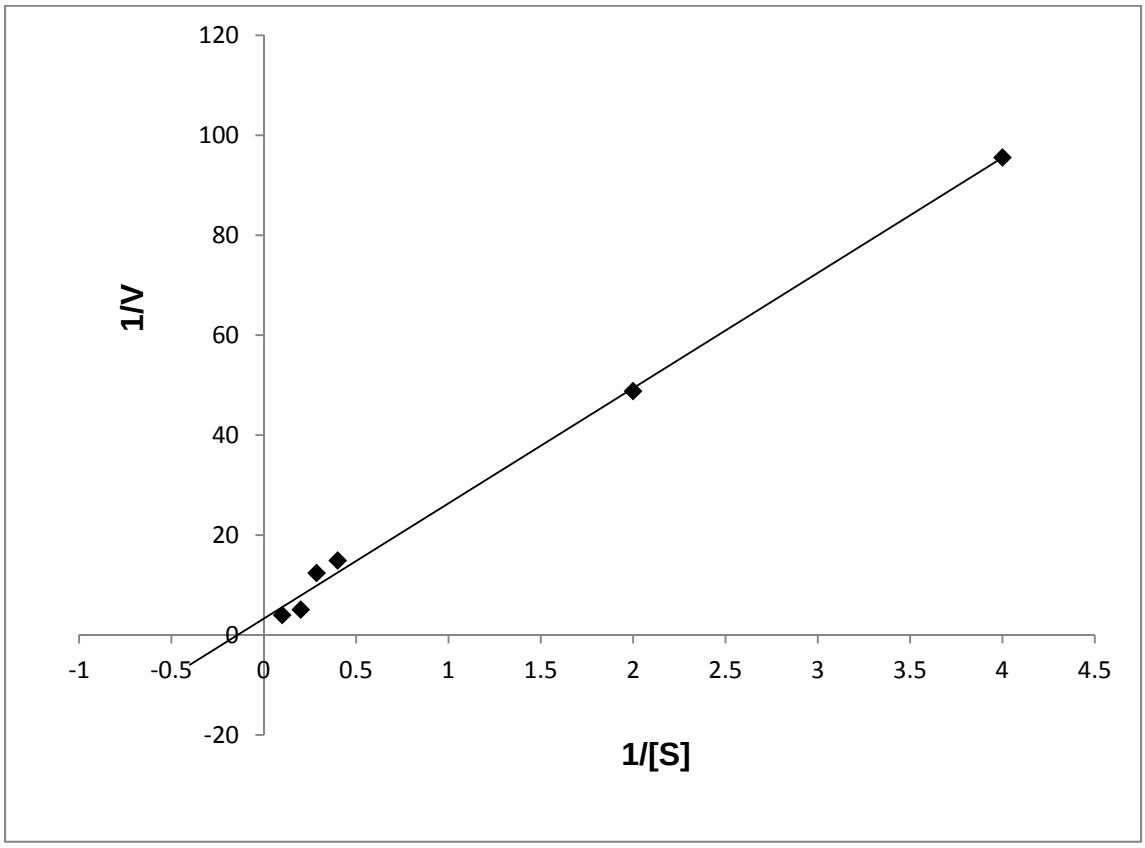

Figure 10. Lineweaver-Burk plot for [5,6-^{3}H]uracil in yeast expressing *AtNCS1* reveals a K_m apparent of 7.0 µM and a V_{max} of 0.304 nmol / 10^{9} cells / min.

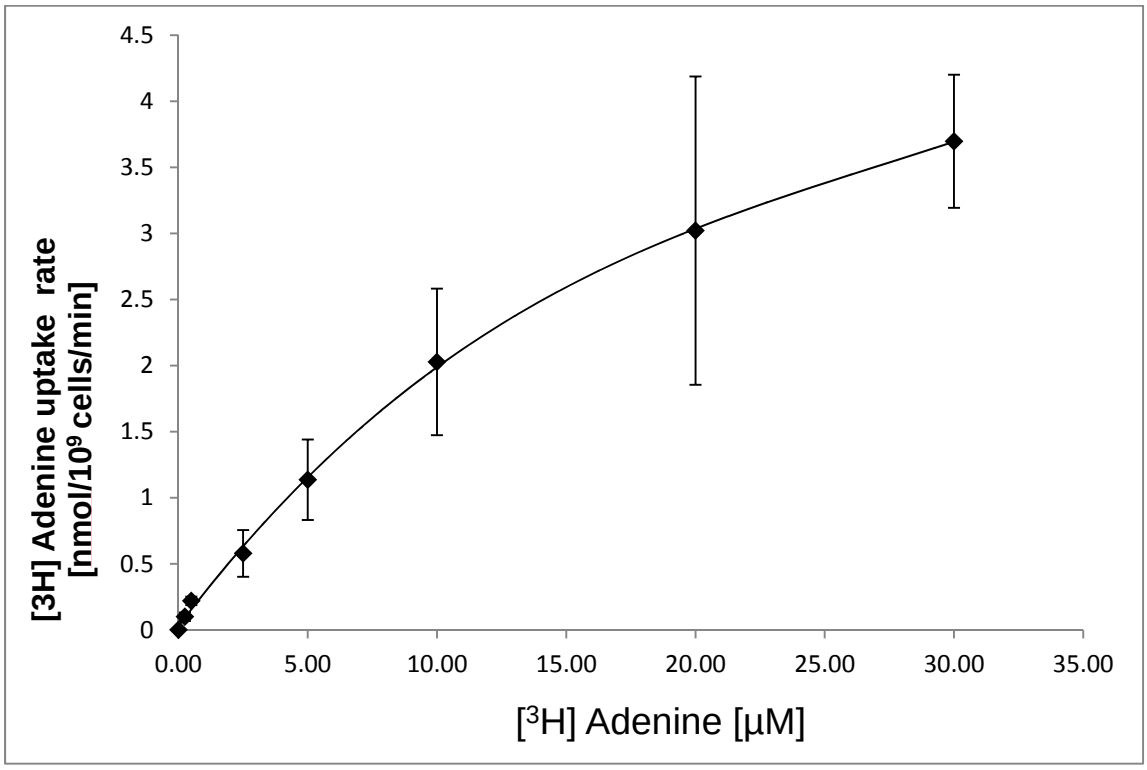

Figure 11. Substrate saturation with varying concentrations of [2,8-^{3}H]adenine . Yeast expressing *AtNCS1* approach saturation with increasing concentrations.

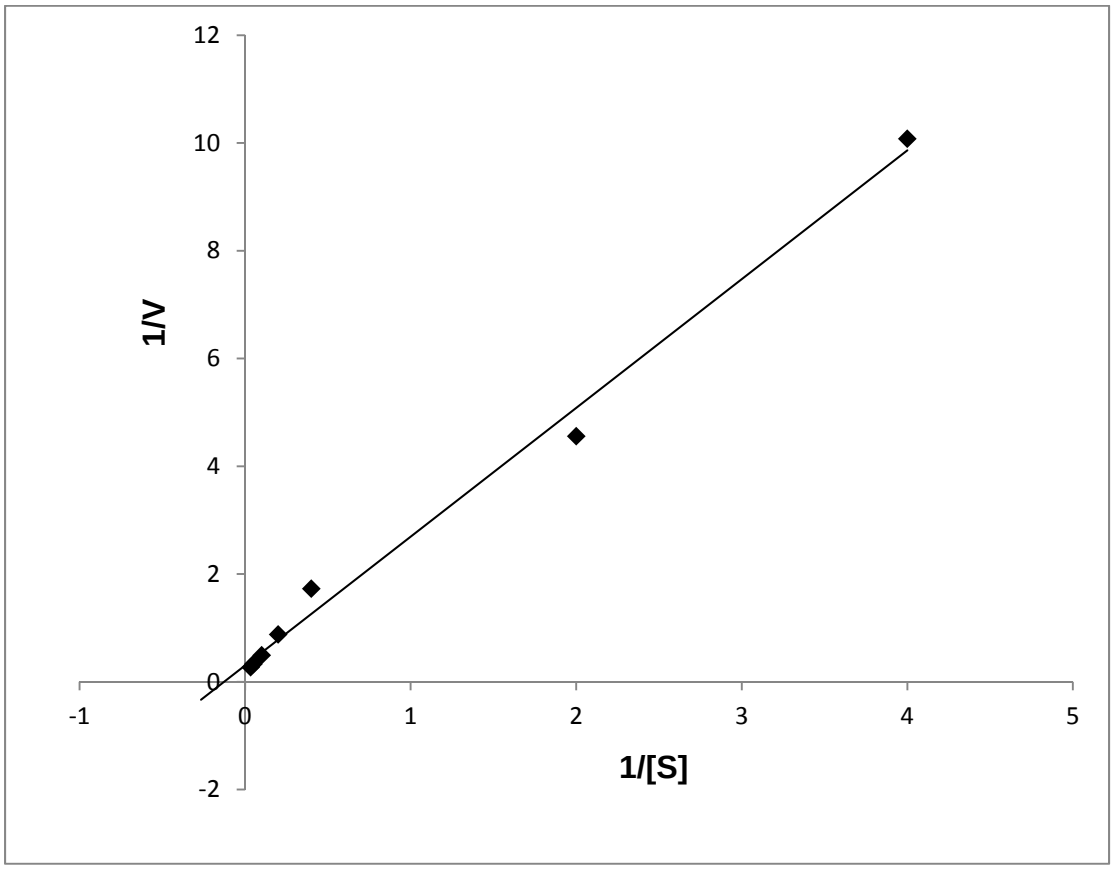

Figure 12. Lineweaver-Burk plot for [2,8-^{3}H]adenine in yeast expressing *AtNCS1* reveals a K_m apparent of 7.9 µM and a V_{max} of 3.3 nmol / 10^9 cells / min.

CHAPTER 4. DISCUSSION

Biochemical Analysis of AtNAT2

Saccharomyces cerevisiae mutant strains RG191 (*fcy2Δ*) and NC122-sp6 (*fur4Δ)* were transformed with plasmid pRH163 in order to elucidate the solute transport specificity of *AtNAT2*. The transformations were successful as verified by growth on SC media without leucine. A gel electrophoresis image (Figure 13) of a restriction digest of plasmid pRH163 with *Xho* I and *Not* I correctly showed the expression vector band at 7.75kb and the *AtNAT2* band at 1.66 kb. Yeast heterologous complementation studies are crucial for a definitive functional characterization of transporter genes in *A. thaliana*. This is due to the presence of at least six multigene families in *A. thaliana*, which creates the possibility of transport redundancy, necessitating the use of another host. Additionally, manipulating plant cells is difficult which substantially decreases their usefulness in primary transport studies. Finally, *S. cerevisiae* represents a well characterized, simple eukaryote, making it an ideal model organism for the expression and study of transporters that occur in higher organisms (Serrano & Villalba, 1995). Even more importantly, *S. cerevisiae* does not possess any NAT homologues (Diallinas & Gournas, 2008).

AtNAT2 is strongly expressed during germination of *A. thaliana* seeds. Using histochemical analysis of GUS (beta-glucuronidase reporter gene) activity the expression pattern of *AtNAT2* was visualized in the major and minor veins of *A. thaliana* young leaves and cotyledons at 4 days after imbibition. This pattern was also evident in mature leaves, pediceles and root tips (Maurino et al., 2006). Seeds imbibe water during germination, allowing them to swell, and supporting an increase in metabolic activity necessary to produce energy for germination.

In addition, during the early phases of imbibition seeds need an ample supply of purine and pyrimidine nucleotides for nucleic acid synthesis (Stasolla et al., 2003; Schmidt et al., 2004). The expression pattern and localization of *AtNAT2* in the vasculature of young leaves and cotyledons makes it a likely candidate for being a transporter of these nucleobases. Results of this study (Figures 3-6) however, did not support this supposition. Yeast expressing *AtNAT2* did not show sensitivity to any of the toxic analogues used in the heterologous complementation assays indicating that the transporter encoded by this gene does not mediate transport of purines/pyrimidines.

The UapA and UapC uric acid/xanthine transporters of *A. nidulans* are two of the most well characterized NAT proteins and have thus been used to define the NAT family. AtNAT2 has about 20% homology with the *A. nidulans* transporters belonging to the NAT family, prompting our group to test it for xanthine transport (Gournas et al., 2008). Results of the study (Figure 7) showed that yeast expressing *AtNAT2* does not mediate xanthine transport. *S. cerevisiae* does not have transporters for the oxidized purines xanthine and uric acid, allowing for a clear analysis of the mediation of these solutes by AtNAT2 (Argyrou et al., 2001).

Lack of Uptake by AtNAT2

A. thaliana possesses six distinct multigene families which show specific yet overwhelming overlap in terms of solutes transported. *AtNAT2* is one of 12 members of the NAT family and is classified in clade I together with *AtNAT1* and *3* because expression of these genes is seen in the same tissues (Maurino et al., 2006). There is strong expression of the 12 NAT genes in embryos, young leaves, roots and developing seeds, and high degree of functional redundancy occurs within this family. In addition, other gene families like the purine permease (PUP) family have members that transport adenine, cytosine and secondary compounds including caffeine and cytokinins (Gillissen et al., 2000; Burkle et al., 2003). The ureide permease (UPS) family has members that

mediate allantoin, uracil, thymine and xanthine transport (Desimone et al., 2002; Schmidt et al., 2004 & 2006). Finally, the *AtAzgA* gene transports adenine and guanine (Mansfield et al., 2009). The functional redundancy within and across gene families in *A. thaliana* could explain why there was no solute uptake demonstrated when *AtNAT2* was isolated for testing.

Using yeast to express a plant gene does not necessarily mirror transport as it occurs naturally in plants. Heterologous proteins are often poorly localized in yeast and many are retained within the endoplasmic reticulum. Because of this challenge, some specific *S. cerevisiae* strains have been developed to optimize expression of eukaryotic transporters at the plasma membrane. *Npi1* is a mutation in the Rsp5 gene, which encodes a ubiquitin ligase that targets proteins for degradation (Serrano& Villalba, 1995; Leung et al., 2010). In addition, the glycosylation process in yeast differs from that in higher organisms meaning that the resulting protein may possess long outer carbohydrate chains, which may render it inactive or differently active from the natural plant protein (Serrano& Villalba, 1995). These are possibilities that could have taken place when *AtNAT2* was being expressed. There were no assays conducted to check what portion of the expressed protein found its way to the plasma membrane of the yeast. However, *AtNAT2* was placed under constitutive control, meaning that there was continuous transcription of the gene, thus continuous production of membrane protein, increasing the likelihood that some proteins did reach the plasma membrane.

It is possible that *AtNAT2* only functions under certain physiological conditions. It is not known whether *AtNAT2* is constitutively expressed *in planta*, for example; changes that occur during expression in yeast may disrupt the environment required for *in vivo* function of this gene. In addition, the toxic analogues themselves may have contributed to the inconclusive results. Changes to the purines/pyrimidines may have caused steric hindrance thereby blocking their own uptake. For example, the additional nitrogen at position ‘8’ in 8-azaadenine may have caused steric hindrance blocking the site for its uptake;

using a variety of toxic analogues with the same general structure may establish affinity or recognition by the transporter that was not observed in this study (Leung et al., 2010).

Kinetic Analysis of AtNCS1

Recently, Mourad et al. (2012) successfully showed that AtNCS1 mediates adenine, guanine and uracil transport. The study included *in planta* radiolabel nucleobase uptake and yeast heterologous complementation studies where yeast expressing *AtNCS1* was grown on media containing toxic purine/pyrimidine analogues. Radionucleobase uptake studies were also done (This work was completed by Julie Crosby and Kevin Ann Hunt, students in the lab of Dr. Mourad). Yeast expressing *AtNCS1* showed sensitivity to toxic purine/pyrimidine analogues compared to the control strains, indicating transport of these nucleobases. The finding stands in stark contrast to the canonical NCS transport pattern seen in FUR4 and FCY2 of *S. cerevisiae*. These two genes mediate the transport of adenine, guanine cytosine or uracil while AtNCS1 transports adenine, guanine and uracil. A difference is also evident when comparison is made between the *AtNCS1* gene and the *A. nidulans furD* gene, which transports uracil but has no affinity for adenine, guanine or cytosine (Amilis et al., 2007). Interesting to note are the similar transport specificities seen in the distantly related AtAZG1 and AtAZG2 families which mediate adenine and guanine (Mansfield et al., 2009). Experiments to determine the kinetics of this gene were performed and a constant uptake rate of [5,6-^{3}H]uracil was evident during the first 10 minutes. Substrate saturation was approached in both [5,6-^{3}H]uracil and [2,8-^{3}H]adenine with increasing concentrations of both compounds. Line weaver-Burk plots of uracil and adenine established an apparent K_m of 7.0 µM and 7.9 µM and V_{max} of 0.3 nmol / 10^{9} cells / min and 3.3 nmol / 10^{9} cells / min respectively. It is interesting to note the ten-fold increase evident in the transport capacity of adenine compared to uracil. This may be because profiles of various plant materials have shown that the adenine nucleotide pool is always

the largest and also because of its more general role as a cofactor in metabolism and synthesis of nucleic acids (Stasolla et al., 2003; Zrenner et al., 2006). Up to date locus At5g03555 codes for the only known nucleobase cation sympoter 1 in *A. thaliana*.

Conclusion

AtNAT2 showed no uptake of purines or pyrimidines (Figures 3-6) though many variables may have affected these results. *AtNAT2* was placed under control of a constitutive promoter allowing for continuous protein production but perhaps not enough to show a significant difference in the uptake of substrates. A different approach would be to use an inducible promoter to increase the levels of production and attempt to identify optimal expression levels of this transport protein (Leung et al., 2010). Testing for the sub-cellular expression and localization of AtNAT2 would increase confidence in the function of the heterologous system used in this study. Various methods such as northern blotting or a green fluorescent protein (GFP) tag would verify expression of the plant membrane proteins in yeast.

Functional redundancy among multigene families present in *A. thaliana* pose a challenge in attempting to establish the solute specificities of genes whose functions are still unknown. Triple homozygous knockout AtNAT mutants in *A. thaliana* plants do not show phenotypic differences compared to the wild type but perhaps an in depth analysis of these mutants may offer deeper insight into the functions of these genes (Maurino et al., 2006). As research continues within the AtNAT family it is worth noting that sequence homology may not always be extended to functional conservation. An example is the mammalian ascorbate transporter classified under the NAT family based on sequence homology with plants and fungi while their solute specificities are structurally unrelated (de Koning & Diallinas, 2000).

AtNCS1 has been shown to effectively transport adenine, guanine and uracil in *A. thaliana* (see Appendix), though this is a departure from the canonical

NCS1 pattern of transport seen in *S. cerevisiae* FUR4 and FCY2. The finding contributes to the understanding of the multigene families of *Arabidopsis* (Mourad et al., 2012).

Table 2. Resistance of yeast expressing *AtNAT2* to a panel of toxic purine and pyrimidine analogues incorporated in the growth medium (+= sensitive; - = resistance).

Toxic analogue	**AtNAT2**
8-Azaadenine	-
8-Azaguanine	-
5-Fluorocytosine	-
5-Fluorouracil	-

Table 3. Resistance of yeast expressing *AtNCS1* to a panel of toxic purine and pyrimidine analogues incorporated in the growth medium (+= sensitive; - = resistance). Toxic analogues were tested by Kevin Ann Hunt, a student in Dr. Mourad's lab.

Toxic analogue	**AtNCS1**
8-Azaadenine	+
8-Azaguanine	+
5-Fluorocytosine	-
5-Fluorouracil	+

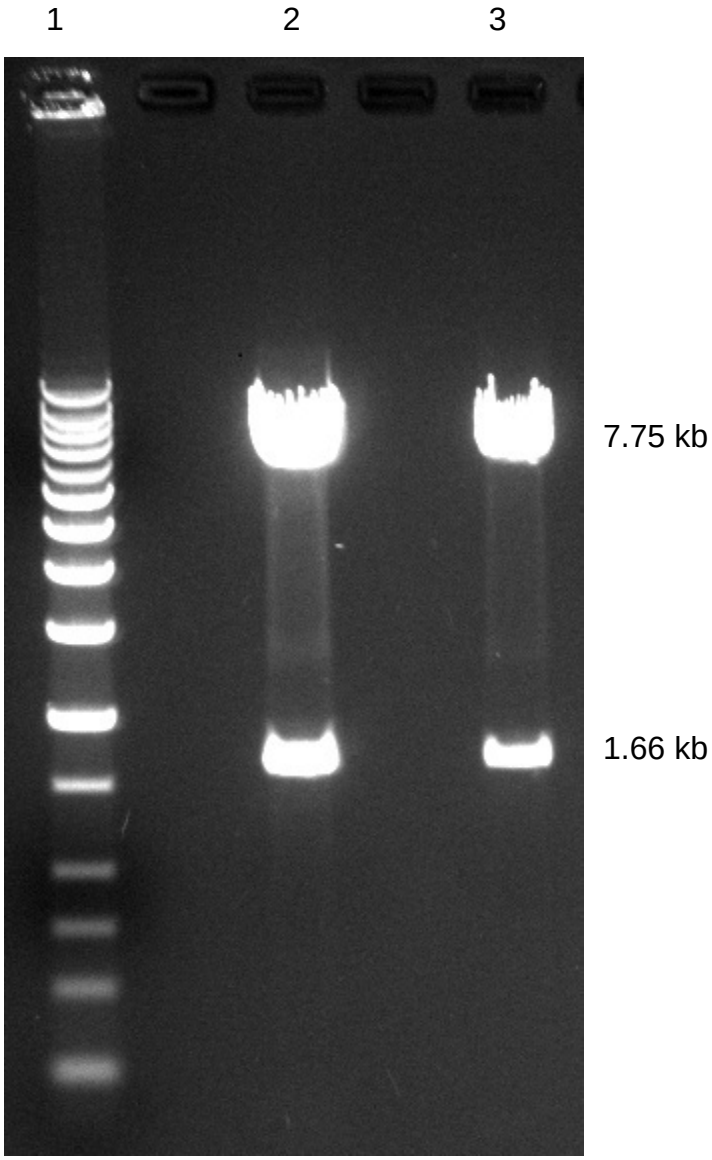

Figure 13. Gel electrophoresis image of a restriction digest for plasmid pRH163 (*AtNAT2*) with *Xho* I and *Not* I. *AtNAT2* band is seen at approximately 1.66 kb and the remaining 7.75 kb is pRG399 (empty expression vector).

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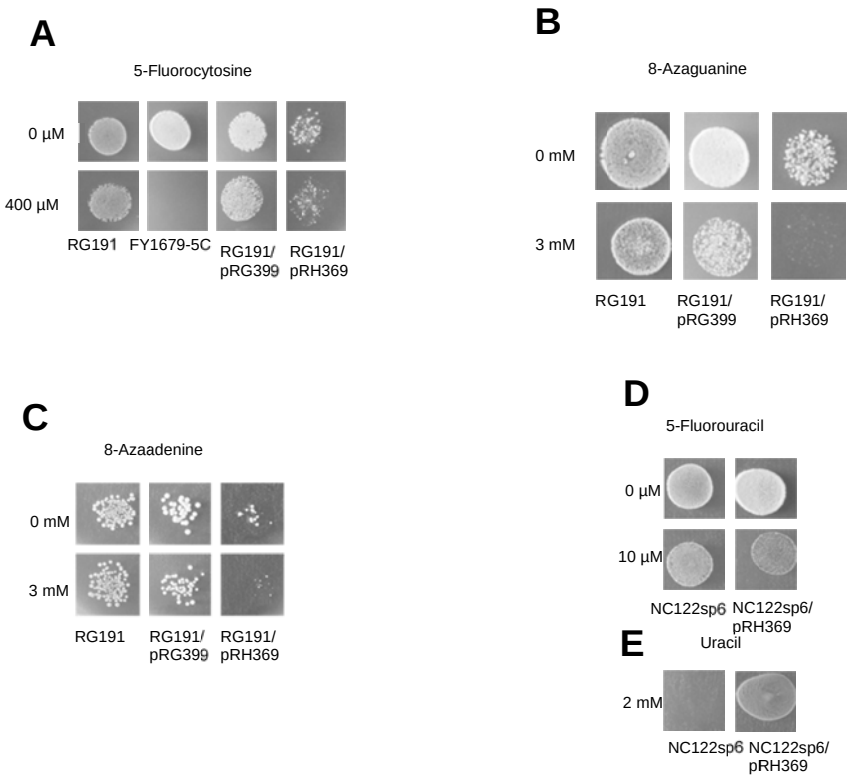
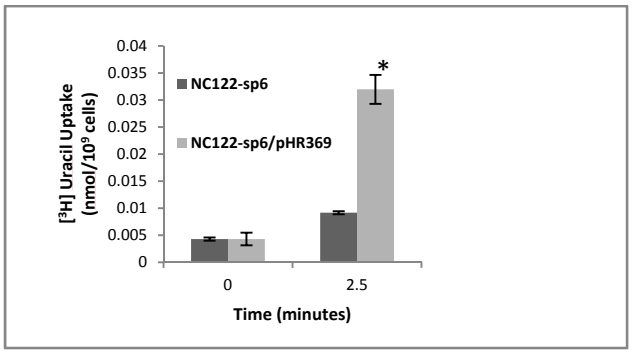
APPENDIX

APPENDIX



A.1. Growth of *Saccharomyces cerevisiae* expressing *AtNCS1* on toxic purine and pyrimidine analogues. Growth pattern of yeast strains: deficient for the adenine-guanine-cytosine *fcy2* (RG191); deficient for the uracil transporter *fur4* (NC122-sp6); wild type for FCY2 and FUR4 (FY1679-5C); with an empty expression vector (pRG399); or with the AtNCS1 coding region placed under constitutive transcriptional control (pRH369) were grown on nutrient media with increasing amounts of 5-Fluorocytosine (A), 8-Azaguanine (B), 8-Azaadenine (C), or 5-Fluorouracil (D). The growth pattern of NC122-sp6 alone or containing pRH369 on nutrient media with uracil as the sole nitrogen source is presented in (E) (Mourad et al., 2012). This work was performed by Kevin Ann Hunt a student in the lab of Dr. George Mourad.

(A)



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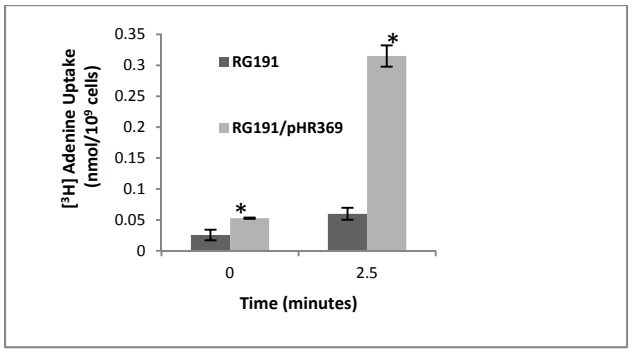
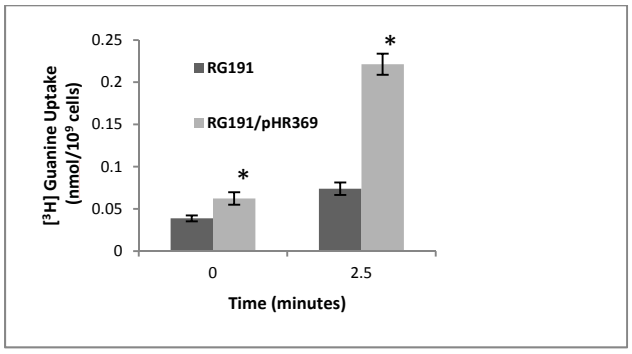


(C)



A.2. RG191 and RG191+pRH369 were incubated (A) [3H]-adenine, (B) -guanine, or (C) -uracil in citrate buffer (pH 3.5) and aliquots were taken at 0 and 2.5 min. Values shown are the mean of at least three independent experiments. Error bars indicate the standard error of the mean. Statistical analysis used an independent paired t-test. Significance was measured at *P = 0.05 (Mourad et al., 2012). This work was performed by Julie Crosby a student in the lab of Dr. George Mourad.