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12-29-2015

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Short communication

Heterologous complementation studies reveal the solute transport profiles of a two-member nucleobase cation symporter 1 (NCS1) family in *Physcomitrella patens*

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ABSTRACT

As part of an evolution-function analysis, two nucleobase cation symporter 1 (NCS1) from the moss *Physcomitrella patens* (PpNCS1A and PpNCS1B) are examined — the first such analysis of nucleobase transporters from early land plants. The solute specificity profiles for the moss NCS1 were determined through heterologous expression, growth and radiolabeled uptake experiments in *NCS1*-deficient *Saccharomyces cerevisiae*. Both PpNCS1A and 1B, share the same profiles as high affinity transporters of adenine and transport uracil, guanine, 8-azaguanine, 8-azaadenine, cytosine, 5-fluorocytosine, hypoxanthine, and xanthine. Despite sharing the same solute specificity profile, PpNCS1A and PpNCS1B move nucleobase compounds with different efficiencies. The broad nucleobase transport profile of PpNCS1A and 1B differs from the recently-characterized Viridiplantae NCS1 in breadth, revealing a flexibility in solute interactions with NCS1 across plant evolution.

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1. Introduction

Nucleobase biochemistry in plants is marked by extensive *de novo* synthesis, salvage and catabolism pathways (Zrenner et al., 2006). This metabolic complexity reflects the extensive requirements for nucleobases throughout the plant life cycle including; continual DNA and RNA synthesis and salvage, nitrogen storage in endosperm and release upon germination, nitrogen acquisition and long-distance transport exemplified by the ureide cycle and production of cytokinins and purine alkaloids (Stasolla et al., 2003; Ashihara et al., 2008; Frébort et al., 2011; Collier and Tegeder, 2012). Nucleobase biochemistry utilizes multiple subcellular compartments within cells, between adjacent cells or between tissues - necessitating a robust membrane transporter system to move intermediates.

Five nucleobase transporter families are present in higher plant genomes. *Arabidopsis thaliana* genome contains 21 loci that encode for Purine Permease (PUP), known to move purines, cytokinins and pyridoxine (reviewed in Jelesko, 2012; Szydlowski et al., 2013).

Three of the five Ureide Permease (UPS) in Arabidopsis move allantoin, uracil, uric acid and xanthine (Desimone et al., 2002; Schmidt et al., 2004, 2006). Arabidopsis genome contain 12 Nucleobase Ascorbate Transporter (NAT) loci (Maurino et al., 2006). NAT3 and 12 transport adenine, guanine and uracil, while the maize NAT Leaf Permease 1 (LPE1) transports xanthine and uric acid (Argyrou et al., 2001; Niopek-Witz et al., 2014). The two azaguanine-like transporters (AZG) in Arabidopsis transport adenine, guanine, and uracil (Mansfield et al., 2009). Arabidopsis contains one nucleobase cation symporter 1 (NCS1) (Mourad et al., 2012; Witz et al., 2012). The solute specificity (both solute transport and competitive binding) for NCS1 from *A. thaliana* (AtNCS1), *Chalmydomonas reinharditii* (CrNCS1), *Zea mays* (ZmNCS1) and *Setaria viridis* (SvNCS1) include adenine, guanine, uracil, cytosine, hypoxanthine, allantoin, xanthine, uric acid, 8-azaguanine, 8-azaadenine and 5-flurocytosine (Mourad et al., 2012; Witz et al., 2012; Schein et al., 2013; Witz et al., 2014; Rapp et al., 2015).

In contrast to plants, microbial genomes contain numerous NCS1 loci (Ma et al., 2013). NCS1 proteins sort into two different sub-families based upon amino acid sequence similarity: the FUR4-like and the FCY2-like NCS1, named after the *Saccharomyces cerevisiae* FUR4 uracil transporter and FCY2 adenine, guanine, cytosine

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transporter, respectively (Pantazopoulou and Diallinas, 2007). Upon amino acid sequence similarity and phylogenetic analysis Viridiplantae NCS1 always clade with FUR4-like subfamily (Mourad et al., 2012; Schein et al., 2013; Ma et al., 2013), although horizontal transfer of NCS1 into plants seems to stem from bacterial rather than fungal origins (Yue et al., 2012).

Recently, three dimensional structures for the outward-facing, occluded and inward facing states for the NCS1 from *Microbacterium liquefaciens* the sodium-hydantion transporter MHP1 have been determined, revealing amino acids important for solute and cation interactions (Weyand et al., 2008; Shimamura et al., 2010). NCS1 proteins belong to the LeuT superfamily that is characterized by two five helix inverted repeats that are believed to function as a "rocking-bundle" model (Shimamura et al., 2010). Key amino acids in the presumptive solute and cation binding pocket (including TMI, III, VI & VIII) have been identified - some residues are conserved among NCS1 while others vary (Suppl. Fig. 1) (Weyand et al., 2008; Krypotou et al., 2012; Mourad et al., 2012; Schein et al., 2013; Witz et al., 2014). Molecular model directed mutagenesis and functional analysis of NCS1 including *Aspergillus nidulans* FCYB (Krypotou et al., 2012) and recently AtNCS1 (also called PLUTO) (Witz et al., 2014) corroborate such interactions. Here we extend our functional analysis of NCS1 by leveraging evolutionary divergence among the Viridiplantae. In this report we determine the solute transport profile of two NCS1 from the moss *Physcomitrella patens*.

2. Results

2.1. Physcomitrella patens genome contains two loci encoding for NCS1 proteins

The *P. patens* genome contains two loci which encode for distinct NCS1 proteins (Rensing et al., 2008). Loci Pp1s34_250V6 (Phypa_120934) and Pp1s171_59V6 (Phypa_191990) encode for a 540 amino acid polypeptide (here named PpNCS1A) and 612 amino acid polypeptide (here named PpNCS1B), respectively. PpNCS1A and 1B share extensive predicted amino acid similarity − 68.6% amino acid identity and 81.2% amino acid similarity (Suppl. Fig. 1). As expected the levels of amino acid identity/similarity are lower upon comparison with CrNCS1 and AtNCS1 for PpNCS1A 52.2/76.9% and 48.3/76.5% respectively, and for PpNCS1B 49.7/73.2% and 46.5/77.5%, respectively. Bacterial, fungal, and plant NCS1 contain twelve TM (transmembrane domains) that overlap upon amino acid alignment (Suppl. Fig. 1). Despite the relatively low level of amino acid sequence similarity among all NCS1 proteins, secondary structures and a number of key amino acid residues involved in solute and cation interactions are conserved across taxa (Suppl. Fig. 1) (Weyand et al., 2008; Mourad et al., 2012; Krypotou et al., 2012; Schein et al., 2013; Witz et al., 2014). A number of the identified amino acids have been the subject of site-directed mutagenesis and functional analysis in the *A. nidulans* FCYB and AtNCS1 (Suppl. Fig. 1) (Krypotou et al., 2012; Witz et al., 2014).

2.2. Growth of S. cerevisiae containing PpNCS1A and PpNCS1B on nucleobases or nucleobase analogs

Heterologous expression experiments test the ability of *S. cerevisiae* cells with *PpNCS1A* or *PpNCS1B* coding regions to grow on medium containing toxic nucleobase analogs. Plasmids containing *PpNCS1A* (pRH561) or *PpNCS1B* (pRH573) were transformed into *fcy2* (adenine-cytosine-guanine-hypoxanthine transport) deficient yeast strains. Yeast strains deficient in *fcy2* and carrying pRH561 or pRH573 were grown on medium supplemented with 8-azaadenine (8-AZA), 8-azaguanine (8-AZG), or 5-fluorocytosine (5-
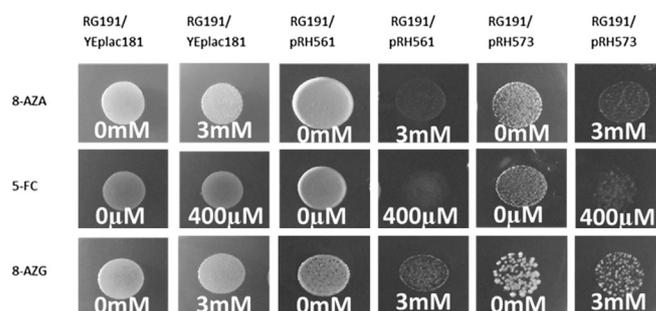

Fig. 1. Growth sensitivity of yeast expressing *PpNCS1A* or *PpNCS1B* to toxic nucleobase analogs. A *S. cerevisiae* strain deficient in *fcy2* (RG191) and containing the empty vector YEplac181 (control) or the vector with *PpNCS1A* (pRH561) or *PpNCS1B* (pRH573) was grown on medium containing toxic analogs 8-AZA, 8-AZG or 5-FC at the indicated concentrations at 30 °C for two days.

FC) (Fig. 1). These growth studies show that yeast cells containing either *PpNCS1A* or PpNCS1B were sensitive to 8-AZA, 5-FC and 8-AZG (Fig. 1).

2.3. Radiolabeled nucleobase uptake studies define the solute specificity of PpNCS1A and PpNCS1B

To further characterize the transport profile of PpNCS1A and PpNCS1B, radiolabeled nucleobase uptake was tested. Yeast deficient in *fcy2* and containing either pRH561 (*PpNCS1A*) or pRH573 (PpNCS1B) showed significant increase in the uptake of [^{3}H]-adenine, [^{3}H]-guanine, [^{3}H]-hypoxanthine, [^{3}H]-xanthine and [^{3}H]-cytosine when compared to controls (Fig. 2A−E). Similarly, yeast strains deficient in *fur4* and containing either pRH561 or pRH573 displayed a statistically different level of uptake for [^{3}H]-uracil when compared to control strains (Fig. 2F). Interestingly, yeast cells containing *PpNCS1A* showed high levels of guanine and hypoxanthine uptake relative to cytosine uptake (G/C = 0.89 and HX/C = 0.36) than did *PpNCS1B*-containing yeast (G/C = 0.21 and HX/C = 0.01). The reverse was shown for xanthine relative to cytosine uptake for *PpNCS1A*-containing (X/C = 0.022) vs. *PpNCS1B*-containing (X/C = 0.15) yeast (Fig. 2).

2.4. Biochemical properties of PpNCS1A and PpNCS1B

Homologous competition assays were performed to investigate the affinity of adenine for *PpNCS1A* and *PpNCS1B*. Yeast containing *PpNCS1A* and *PpNCS1B* have high affinities for adenine with K_m of 10.06 μM ± 0.25 and 5.15 μM ± 0.9, respectively (Fig. 3A, B). In the presence of the proton ionophore CCCP, [^{3}H]-adenine uptake by yeast cells containing *PpNCS1A* or *PpNCS1B* was significantly inhibited, however no effect was observed in the presence of Ouabain (Fig. 3C). This is consistent with the functioning of *PpNCS1A* and *PpNCS1B* as proton-driven nucleobase cation symporters.

3. Discussion

This report presents data and analysis on nucleobase transporters from early land plants. Both PpNCS1A and PpNCS1B share the same broad solute specificity profile, transporting adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and the toxic analogs 8-AZA, 8-AZG, and 5-FC (Figs. 2 and 3). Both transporters can competitively bind uric acid (data not shown). This is the first instance of a eukaryotic NCS1 moving xanthine. Several other plant nucleobase transporters move xanthine including the maize NAT leaf permease 1, Arabidopsis AtUPS1, 2 & 5 and the Arabidopsis
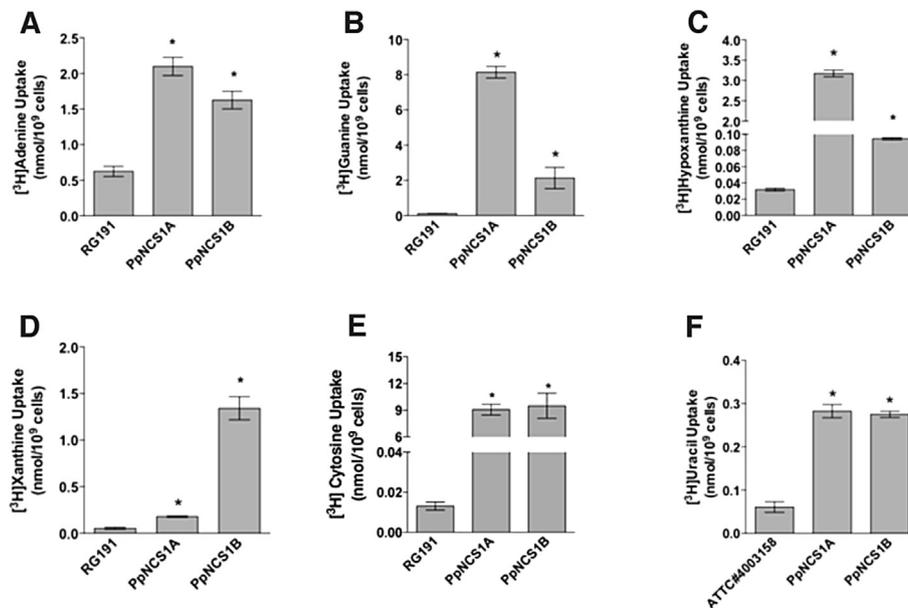
Fig. 2. Uptake of ^{3}H-adenine (A), ^{3}H-guanine (B), ^{3}H-hypoxanthine (C), ^{3}H-xanthine (D), ^{3}H-cytosine (E), or ^{3}H-uracil (F) by yeast strains RG191 (A—E) or ATTC#4003158 (F) expressing pRH561 (*PpNCS1A*) or pRH573 (*PpNCS1B*). An independent paired *t*-test measured statistical significance at P < 0.05. All error bars represent SEM.

AtAZG1 and AtAZG2 (Argyrou et al., 2001; Schmidt et al., 2006; Schultes & Mourad pers comm).

Despite the fact that the solute transport profiles of PpNCS1A and 1B are the same, the transporters behave differently in regard to how well certain nucleobases are transported. Both transporters have a high affinity for adenine with a Km of 10.06 μM for PpNCS1A and 5.15 μM for PpNCS1B (Fig. 3) and lesser affinities for uracil, Km of 14.8 μM for PpNCS1A (data not shown). Indeed the ratio of G/C (0.89) and HX/C (0.36) transport (in yeast under identical conditions) for PpNCS1A is high while the ratio of X/C (0.022) is low. The opposite trend is seen for PpNCS1B where X/C ratio is high (0.15) relative to low ratio for HX/C (0.01). The differences in transport behavior are not surprising as PpNCS1A and PpNCS1B differ by 47 amino acids in their presumptive "mature forms".

While most other plants contain one NCS1 locus, *P. patens* contains two NCS1 loci. The expression patterns of *PpNCS1A* and *1B* were compared using GENEINVESTIGATOR (Hruz et al., 2008) on microarray gene expression data including developmental stages, culture forms and stress conditions (Hiss et al., 2014). *PpNCS1A* (*Phypa_120934*) displays consistent moderate to low levels of expression among all conditions, very similar to that observed for reference gene Phypa_173694 identified in Hiss et al., 2014. This expression pattern is consistent for generic "housekeeping genes". On the other hand, the gene expression pattern of *PpNCS1B* (Phypa_191990) is more dynamic. *PpNCS1B* is expressed at a higher level than *PpNCS1A* particularly under phyllid dedifferentiation conditions. PpNCS1B expression is downregulated under the following stress conditions: spore germination; spore germination shifted from pH5.8 to pH4.5; shift from long to short day conditions; in darkness, and during dehydration and rehydration.

Viridiplantae NCS1, of known function, share several common features. First, plant NCS1 share substantial amino acid sequence similarities and secondary structure (Suppl. Fig. 1; Mourad et al., 2012; Schein et al., 2013). Second, Viridiplantae NCS1 have a very broad solute specificity profile. A canonical plant solute specificity profile is now defined as transport of the purines adenine and guanine with variable abilities to transport or competitively bind other nucleobase and related compounds including, hypoxanthine, xanthine, 8AZA, 8AZG, cytosine, 5FC, uracil, 5FU, allantoin and uric acid. For NCS1, a broad rather than narrow solute specificity

appears constant throughout Viridiplantae evolution. Third, each plant NCS1 displays a unique solute transport and recognition pattern. Fourth, the solute specificity profiles among plant NCS1 do not follow evolutionary sequence among plant NCS1. For example, the ability to transport xanthine is not associated solely with non-vascular plants and thereafter lost among vascular plants-as CrNCS1 but not PpNCS1A & B lack xanthine transport. Similarly, the ability to transport uracil is present across Viridiplantae, except for among the grasses *Z. mays* and *Setaria viridis* NCS1 (Rapp et al., 2015). These features reflect the promiscuous and flexible nature of solute recognition among plant NCS1. Indeed, the solute transport/binding diversity observed is most likely not solely due to previously identified essential and conserved amino acids, but rather to other, as yet, unidentified residues that direct subtle solute discrimination on a species basis.

Site-directed mutagenesis studies focus on residues within the pocket region that appear to interact with the solute or cation in the *A. nidulans* FCYB and AtNCS1 (PLUTO) transporters (Krypotou et al., 2012, Witz et al., 2014). Although some residues are important for substrate binding or transport, particular amino acid residues and associated replacements do not conform to a "one size fits all" scenario. One amino acid in TMIII is illustrative — the glutamic acid (E227 for AtNCS1) and cognate asparagine (N163 for AnFCYB) (Suppl. Fig. 1) (Krypotou et al., 2012; Witz et al., 2014). For AtNCS1 the site directed change E227Q abolishes uracil transport (Witz et al., 2014), suggesting that this is important for uracil transport in NCS1. However, glutamic acid at that position does not correlate with uracil transport when viewed across evolutionary space (Fig. 4). Although E227 of AtNCS1 is conserved in a majority of vascular land plants, (Q) is conserved in non-vascular land plants and algae, as well as in fungal and bacterial uracil or allantoin transporters (Fig. 4). This data reveals that solute discrimination and binding are species-specific, multi-residue dependent and likely the result from the interaction of other, yet to be identified, residues. The evolution-function analysis offers other gateway into discerning NCS1 solute interactions in a number of different ways. For example, xanthine is uniquely transported by PpNCS1A & 1B but can only competitively bind to CrNCS1, ZmNCS1 and SvNCS1 (Fig. 2; Schein et al., 2013; Rapp et al., 2015). Given the close amino acid sequence similarities among plant NCS1, a systematic and
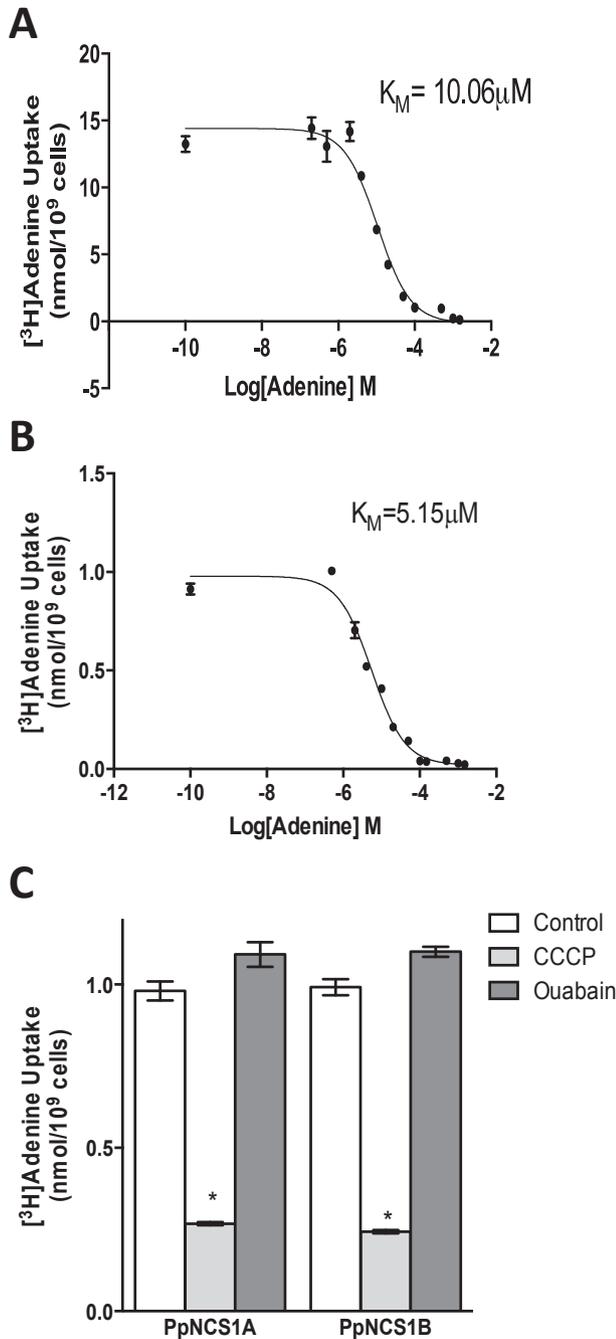
Fig. 3. Biochemical properties of PpNCS1A and PpNCS1B. Competition of [2, 8-³H]-adenine with varying concentrations of unlabeled adenine for uptake by *fcy2Δ* yeast (RG191) expressing *PpNCS1A* (A) or *PpNCS1B* (B). Kinetic parameters were determined using GraphPad Prism software. Uptake of [2,8-³H]-adenine by RG191 expressing *PpNCS1A* or *PpNCS1B* in the presence of the protonophore CCCP or the sodium pump uncoupler ouabain (C). Error bars represent SEM.

Fig. 4. Alignment of TMIII and uracil transport function of Viridiplantae NCS1. ClustalW alignment of NCS1 TMIII across evolutionary space represented by *A. nidulans* AnFCYB (ACA81792.1), *S. cerevisiae* ScFCY2 (P17064), *Bacillus subtilis* allantoin transporter BcALL (P94575), *E. coli* allantoin transporter EcALL (P75712), *S. cerevisiae* allantoin permease ScDAL4 (CAA78826.1), *S. cerevisiae* uracil permease ScFUR4 (CAA29986.1), *Ostreococcus tauri* OtNCS1 (XP_003080073), *Micromonas sp. RCC299* MsNCS1 (XP_002499820), *Volvox carteri* VcNCS1 (Vocar20015267m), *C. reinhardtii* CrNCS1 (XP_001694932.1), *Selaginella moellendorffii* SmNCS1 (XP_002970317.1), *Adiantum capillus-veneris* AcNCS1 (Schultes and Mourad, pers. comm.), *Picea glauca* PgNCS1 (BT114389.1), *Amborella trichopoda* ArNCS1 (XP_006826231.1), *Zea mays* ZmNCS1 (NP_001136535.1), *Setaria viridis* SvNCS1 (AHC53692.1), *Nicotiana sylvestris* NsNCS1 (Schultes and Mourad, pers. comm.), and *A. thaliana* AtNCS1 (NP_568122.2). Highly conserved tryptophan (W in bold) at position 223 (AtNCS1 numbering) and variable glutamine/glutamic acid (Q/E bold with gray shade/boxed) at position 227 are indicated. Transport indicates functional transport of uracil (URA) or not (no), allantoin (ALL), or not determined (n.d.).

GATGGTATCCATTTTTGGGCAGTTG 3′) and PpNCS1A2 (5′ CGGGA TCCGCGGCCGCTCACTGACTTAGCAGATGCCTTTTCG 3′) and the predicted coding region derived from locus Pp1s171_59V6.1 was amplified using oligonucleotides PpNCS1B1 (5′ CCGGGTACCTCGA-GATGGAAGGCGTGCTGCGCAGTGTG 3′) and PpNCS1B2 (5′ ACGC GTCGACTTATACATTAGCCACTCGCAAGCG 3′) from reverse transcribed *Physcomitrella patens* mRNA (a kind gift from Dr. Magdalena Bezanilla, University of Massachusetts, Amherst MA, USA). The resulting 1655 bp and 1863 bp DNA fragments were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA), sequenced, cleaved with restriction endonucleases *Xho* I and *Not* I and cloned into yeast vector pRG399 (Mansfield et al., 2009) to generate plasmids pRH561 (for PpNCS1A) and pRH573 (for PpNCS1B).

4.2. S. cerevisiae strains, transformation and growth conditions

S. cerevisiae strains RG191 [*MATα*, *fyc2Δ::kanMX4*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*] (Winzeler et al., 1999) and ATCC® 4003158™ [*MATa*, *fur4Δ::kanMX4*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*] (ATCC, Manassas, VA, USA) were grown in Synthetic Complete medium (SC) at 30 °C. Yeast transformations were performed by the lithium acetate method (Gietz and Woods, 2002). Sensitivity to toxic nucleobase was analyzed by adding filter-sterilized stock solutions to the SC media at the designated concentrations.

combined evolution-function and molecular modeling study may shed informative results for NCS1 solute discrimination.

4. Materials and methods

4.1. Construction of yeast expression plasmids

The predicted coding region from Pp1s34_250V6.1 was amplified using oligonucleotides PpNCS1A1 (5′ CCGGGTACCTCGA

4.3. Radiolabel uptake by yeast expressing PpNCS1A or PpNCS1B

Yeast grown liquid cultures at 30 °C were pelleted, rinsed, and re-suspended in 100 mM citrate buffer, pH 3.5, and 1% glucose to an $OD_{600} \approx 4$. Cells (15 μL) were added to 15 μL radioisotope/100 mM citrate buffer (pH 3.5) with 1% glucose, mixed and incubated at 30 °C for 5 min. 25 μL aliquots were added to 4 mL ice-cold water and filtered through a 0.45 μm Metricel membrane filter (Gelman Sciences, Ann Arbor, MI). Filters were washed with 8 mL of water and radioactivity was measured using Beckman LS6500 scintillation counter. FCY2Δ RG191 strain expressing either *PpNCS1A* or *PpNCS1B* was used to assay uptake of [8-^{3}H]-guanine at 1 μM, [2,8-^{3}H]-adenine at 0.25 μM, [8-^{3}H]-hypoxanthine at 0.5 μM and [8-^{3}H]-xanthine at 1 μM. *FUR4Δ* ATTC#4003158 strain expressing either *PpNCS1A* or *PpNCS1B* was used to assay uptake of [5,6-^{3}H]-uracil at 1 μM. An independent paired *t*-test was used to measure statistical significance. Significance was measured at P = 0.05 (*). All radioisotopes were from Moravek, Brea, CA, USA.

4.4. Biochemical properties of PpNCS1A and PpNCS1B

Kinetic analysis was performed by time course and homologous competition assays. Time courses were done for uptake of 0.25 μM [2,8-^{3}H]-adenine by RG191 expressing PpNCS1A or PpNCS1B and for uptake of 0.5 μM [5,6-^{3}H]-uracil by the ATCC® 4003158™ strain expressing PpNCS1A. Cells were grown overnight in SC liquid medium, pelleted, re-suspended in citrate buffer as above and concentrated to $OD_{600} \approx 4$. Cells were then added to reaction tubes containing radiolabeled nucleobase, incubated, sampled and filtered as described above. Samples were taken at 0, 5, 10, 40, 60 and 90 min. Homologous competition experiments were done with [2,8-^{3}H]-adenine at 0.5 μM and varying concentrations of unlabeled adenine (0, 0.5, 2, 4, 10, 20, 50, 100, 150, 500, 1000, 1500 μM). Homologous competition was also carried out with [5,6-^{3}H]-uracil at 1.0 μM and unlabeled uracil at a range of concentrations similar to unlabeled adenine given above. K_m were calculated by fitting a nonlinear regression to the data points and analyzed using the one site-homologous model in the Prism 6 software application.

Radiolabeled nucleobase uptake of [2,8-^{3}H]-adenine by RG191 expressing either *PpNCS1A* or *PpNCS1B* was carried out alone at a concentration of 1 μM, in the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at a concentration of 100 μM or ouabain octohydrate at a concentration of 1 mM. Reaction mixtures were incubated at 30 °C and sampled at 5 min.

Conflicts of interest

The authors of the manuscript have no conflict of interest to declare.

Authors contribution

Study conception and design: Schultes, Mourad.
Acquisition of data: Minton, Rapp, Stoffer.
Analysis and interpretation of data: Minton, Schultes, Mourad.
Drafting of manuscript: Minton, Schultes, Mourad.
Critical revision: Schultes, Mourad.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.12.014.

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