Short communication

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

Janet A. Minton a, Micah Rappa a, Amanda J. Stoffera a, Neil P. Schultes b, George S. Mourad a, * 

a Department of Biology, Indiana University-Purdue University Fort Wayne, 2101 East Coliseum Blvd., Fort Wayne, IN 46805, USA 
b Department of Plant Pathology and Ecology, The Connecticut Agricultural Experiment Station, 123 Huntington St, New Haven, CT 06511, USA

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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; Collie 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 12 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), Chlamydomonas reinhardtii (CrNCS1), Zea mays (ZmNCS1) and Setaria viridis (SvNCS1) include adenine, guanine, uracil, cytosine, hypoxanthine, allantoin, xanthine, uric acid, 8-azaguanine, 8-azaadenine and 5-fluorocytosine (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...
transporter, respectively (Pantazopoulou and Dialinas, 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 MHPI 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 reverted 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 mutagenesis and functional analysis in 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 overlay 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 nucleobase and 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-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 [3H]-adenine, [3H]-guanine, [3H]-hypoxanthine, [3H]-xanthine and [3H]-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 [3H]-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 Km of 10.06 μM ± 0.25 and 5.15 μM ± 0.9, respectively (Fig. 3A, B). In the presence of the proton ionophore CCCP, [3H]-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...
expressing pRH561 (Schein et al., 2013). Second, Viridiplantae NCS1 have a very broad solute specificity. First, plant NCS1 share substantial amino acid sequence similarities among plant NCS1 do not follow evolutionary sequence among plant NCS1. For example, the ability to transport xanthine is not associated solely with nonvascular 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).

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.

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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'-CCGGGTACCTGAGATGGTATCCATTTTTGGGCAGTTG) and PpNCS1A2 (5'-CCGGGTACCTGAGATGGAAGGCGTGCTGCGCAGTGTG) and the predicted coding region derived from locus Pp1s171_59V6.1 was amplified using oligonucleotides PpNCS1B1 (5'-CCGGGTACCTGAGATGGAAGGCGTGCTGCGCAGTGTG) and PpNCS1B2 (5'-ACGGGTACCTGAGATGGAAGGCGTGCTGCGCAGTGTG) 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 XhoI and NotI 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 [MATa, fyc2Δ::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0] (Winzeler et al., 1999) and ATCC® 400315™ [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.
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 OD600 ≈ 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 Metroclean membrane filter (Gelman Sciences, Ann Arbor, MI). Filters were washed with 8 mL of water and radioactivity was measured using Beckman LS6500 scintillation counter. FCY2A RG191 strain expressing either PpNCS1A or PpNCS1B was used to assay uptake of [8-3H]-guanine at 1 µM, [2,8-3H]-adenine at 0.25 µM, [8-3H]-hypoxanthine at 0.5 µM and [8-3H]-xanthine at 1 µM. FUR4J ATTC#4003158 strain expressing either PpNCS1A or PpNCS1B was used to assay uptake of [5,6-3H]-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 [2,8-3H]-adenine by RG191 expressing PpNCS1A or PpNCS1B and for uptake of 0.5 µM [5,6-3H]-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 OD600 ≈ 4. Cells were then added to reaction tubes containing radioabeled 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 [2,8-3H]-adenine at 0.5 µM and varying concentrations of unlabeled adenine (0, 0.5, 2, 4, 10, 20, 50, 100, 150, 500, 1500 µM). Homologous competition was also carried out with [5,6-3H]-uracil at 1.0 µM and unlabeled uracil at a range of concentrations similar to unlabeled adenine given above. Km were calculated by fitting a nonlinear regression to the data points and analyzing using the one-site-homologous model in the Prism 6 software application. Radiolabeled nucleobase uptake of [2,8-3H]-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-hydroxy-3-methylglutaric acid (3-CM) 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.

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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.

References


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