The nucleobase cation symporter 1 of *Chlamydomonas reinhardtii* and that of the evolutionarily distant *Arabidopsis thaliana* display parallel function and establish a plant-specific solute transport profile

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The single cell alga *Chlamydomonas reinhardtii* is capable of importing purines as nitrogen sources. An analysis of the annotated *C. reinhardtii* genome reveals at least three distinct gene families encoding for known nucleobase transporters. In this study the solute transport and binding properties for the lone *C. reinhardtii* nucleobase cation symporter 1 (CrNCS1) are determined through heterologous expression in *Saccharomyces cerevisiae*. CrNCS1 acts as a transporter of adenine, guanine, uracil and allantoin, sharing similar — but not identical — solute recognition specificity with the evolutionary distant NCS1 from *Arabidopsis thaliana*. The results suggest that the solute specificity for plant NCS1 occurred early in plant evolution and are distinct from solute transport specificities of single cell fungal NCS1 proteins.

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1. Introduction

Nucleobase biochemistry serves diverse needs during a plant’s life cycle. Purines and pyrimidines are central to nucleic acid biochemistry, ATP synthesis, carbohydrate, glycoprotein and phospholipid metabolism, as well as the biosynthesis of many secondary metabolites such as cytokines and caffeine [1,2]. Nucleobase biochemistry is comprised of an intricate balance of *de novo* synthesis, catabolism and salvage pathways. A shared aspect is the highly compartmented nature of these pathways. In turn, a myriad of transport systems has evolved to serve the movement of nucleobase compounds — both within and between cells. Purine and pyrimidine biochemistries involve plastidic and cytoplasmic exchange [2,3]. Nucleobases are also moved between adjacent cells as seen in the generation of ureides (purine derivatives) in soybean nodule bacteroid-containing and nonbacteroid-containing cells [4]. Longer distance nucleobase transport involving different tissues is key to nitrogen source—sink relationships as seen in endosperm to cotyledons nucleobase movement in germinating castor bean seedlings [5].

In plants a surprising number of nucleobase transporters fill the needs of nucleobase movement — no less than five distinct gene families encoding nucleobase transporters are present in the *Arabidopsis* genome. Two transporter families are unique to plants. *Arabidopsis* contains 21 purine permeases (PUP) some of which transport adenine, cytosine and cytokinins [6–8]. Of the eight ureide permeases (UPS) in *Arabidopsis*, three are known to transport allantoin, uracil, uric acid and xanthine [9–11]. The remaining three nucleobase transporter families have orthologs in both prokaryotic and eukaryotic taxa. The nucleobase-ascorbate transporters (NATs) are ubiquitous and cooperatively transport a large array of solutes including the oxidized purines xanthine and uric acid, uracil and ascorbate [12,13]. None of the twelve *Arabidopsis* NAT loci have been functionally characterized, however, the maize leaf permease 1 is known to transport xanthine and uric acid [14,15]. The AzaA-like transporter family in *Arabidopsis* transports adenine, guanine, and uracil [16]. The lone nucleobase cation symporter 1 in *Arabidopsis* (AtNCS1) facilitates the movement of adenine, guanine, and uracil [17]. Genes that encode for NCS1 proteins are found throughout the plant kingdom [17,18].

Most fungi contain multiple NCS1 as exemplified by the canonical *Saccharomyces cerevisiae* FCY2-subfamily — transporting adenine, guanine, cytosine and hypoxanthine — and the FUR4-like subfamily — transporting uracil, uridine, allantoin and other metabolites [13]. In single cell microbes, robust nucleobase transport systems are mainly dedicated to scavenging and importing...
nucleobases from the environment to serve as nitrogen sources [13,19]. AtNCS1 has an interesting solute transport profile — distinct from the canonical FCY2 and FUR4 profiles. The question arises as to whether the AtNCS1 profile is unique or representative of plant NCS1 properties. To address this question, the function of another plant NCS1 is studied. As with evolutionary distant microbes, *Chlamydomonas reinhardtii* is a free-living single cell organism capable of importing and utilizing exogenous purines [20,21]. At the same time, it is firmly rooted in the plant kingdom performing photosynthesis as its distant multi-cellular higher plant relatives. In this study we determine the solute transport and binding profiles of the NCS1 from this evolutionarily and pivotally placed single cell alga.

2. Results

2.1. *C. reinhardtii* genome encodes a NCS1 protein

A homology search of the *C. reinhardtii* protein database with the AtNCS1 protein sequence identified one predicted 520 amino acid protein encoded by locus XM_001694880 (CHLRE-DRAFT_191336) [22] EST clone 1031058E09.1 corresponds to a full-length cDNA containing the open reading frame for protein XP_001694932.1 now referred to as CrNCS1 with the start methionine designated by the solid square in Fig. 1. A hand analysis of the genomic sequence directly upstream of this locus reveals an additional sixteen amino acids in frame containing a predicted chloroplast cleavage sequence (Fig. 1). — this polypeptide extension is not supported by available cDNA clones. Despite the large evolutionary distance separating *Arabidopsis thaliana* and *C. reinhardtii* their NCS1 proteins share substantial amino acid similarity (43/74% identity/similarity). Extensive similarities are also evident between CrNCS1 and select bacteria and fungi NCS1s with determined functions. NCS1 proteins are integral membrane proteins commonly with twelve transmembrane-spanning domains [18].

Fig. 1 reveals that transmembrane-spanning domains for CrNCS1 overlap with those predicted in Arabidopsis NCS1, fungal FCY2 and FUR4-type members and the bacterial NCS1 benzyl-hydantoin transporter from *Microbacterium liquefaciens*, MHP1 (derived from X-ray crystal structure studies). In addition, many of the amino acids identified as highly conserved among NCS1, those involved in cation binding, solute binding and those important in solute transport are present in CrNCS1 (Fig. 1). A phylogram shows that both CrNCS1 and AtNCS1 are more closely related to fungal FUR4 rather than the fungal FCY2 subgroups of NCS1 proteins (Fig. 2). Of the eighteen highly conserved amino acids presented in Fig. 1, sixteen are shared with CrNCS1 and the FUR-like NCS1, while at only two of these positions do both plant and FCY2-like sequences differ from FUR4-like and MHP1 residues.

2.2. Expression of CrNCS1 in *S. cerevisiae* confers specific growth abilities on nucleobases or nucleobase analogs

Heterologous complementation studies in *S. cerevisiae* were performed to identify the substrate specificity of CrNCS1. This series of experiments test the ability of yeast harboring the CrNCS1 coding region to grow on media supplemented with toxic nucleobase compounds or on medium containing certain nucleobases as the sole nitrogen source. Yeast expression plasmids containing CrNCS1 (pRH559 and pNS481) were transformed into yeast strains deficient in fcy2 (adenine—cytosine—guanine transport) or fur4 (uracil transport), full1 (uridine transport), and dal4 (allantoin transport). Yeast strains deficient in fcy2 and carrying pRH559 were grown on media containing 8-azaadenine (8-AZA), 8-azaguanine (8-AZG), 5-fluorocytosine (5-FC), while strains deficient in full1 and carrying pNS481 were grown on media supplemented with 5-bromo-2'-deoxyuridine (5-BrdU). Yeast strains deficient in fur4 and carrying pRH559 and strains deficient in dal4 and carrying pNS481 were grown on uracil or allantoin, respectively, as the sole nitrogen source. Results show that yeast strains containing CrNCS1 expression plasmids displayed sensitivity to growth on 8-AZA and 8-AZG but no sensitivity to 5-FC or 5-BrdU was observed (Fig. 3A and B). Yeast strains harboring CrNCS1 are able grow on allantoin or uracil as sole nitrogen sources (Fig. 3C and D).

2.3. Radiolabeled nucleobase transport studies further define the solute transport specificity of CrNCS1

Growth studies suggest that adenine, guanine, and uracil are transported by CrNCS1. This was directly tested using radiolabeled nucleobase compounds. Yeast strains deficient in fcy2 with pH559 showed significant increase in the uptake of the purines [3H]-adenine, [3H]-guanine, and strains deficient in fur4 containing pH559 showed significant increase in the uptake of the pyrimidine [3H]-uracil when compared to controls (Fig. 4A—C). Only 25% as much adenine compared to guanine is taken up during a period of linear uptake rate (Fig. 4A and B).

2.4. Biochemical properties of CrNCS1 solute recognition

Time course experiments monitoring the uptake of [3H]-adenine or [3H]-uracil in CrNCS1 containing yeast lines carried out over a range of 50 and 75 min, respectively, remained linear — neither reaching saturation (Fig. 5A and B). The slow velocity and lack of saturation may reflect a low level of functional CrNCS1 in yeast plasma membranes and is not uncommon with heterologous expression systems. Yeast lines containing CrNCS1 showed a linear uptake rate (Fig. 4A and B).

![Fig. 1](image1.png)

**Fig. 1.** Amino acid sequence similarity and phylogenetic relationships of CrNCS1 and NCS1 proteins from select organisms. Phylogenetic relationships of CrNCS1, AtNCS1, MHP1, ScFUR4, ScDAL4, ScFUI1, ScFCY2, AnFURA, AnFURD and AnFCYB generated through Phylogeny.fr [40] using MUSCLE alignment parameter and tree construction using maximum likelihood [41–43].
expression (i.e. similar rates and lack of saturation with trans-zeatin uptake in yeast expressing the A. thaliana PUP1 gene [6]). The rate of adenine or uracil accumulation within this time interval is $1.30 \times 10^{-2}$ nmol/min 10^9 cells and $4.54 \times 10^{-3}$ nmol/min 10^9 cells, respectively. A series of homologous competitive uptake assays using [3H]-adenine/adenine or [3H]-uracil/uracil were performed to determine the affinity for the respective purine and pyrimidine (Fig. 5C and D). The results show that CrNCS1 acts as a high affinity transporter of adenine ($K_m = 2.46$ μM) and uracil ($K_m = 5.9$ μM).

The solute binding capacity of CrNCS1 was further refined through a series of heterologous competition assays with yeast harboring CrNCS1. The data show that [3H]-adenine uptake is efficiently competed by unlabeled adenine, guanine, xanthine, cytosine or 5-FC but only marginally competed by hypoxanthine.
Similarly, $[^3H]^{-}$uracil uptake is efficiently competed by unlabeled uracil, xanthine and uric acid (Fig. 6B). Direct uptake experiments reveal that neither $[^3H]^{-}$xanthine nor $[^3H]^{-}$hypoxanthine is transported into yeast harboring CrNCS1 compared to control strains (Fig. 7A and B).

In the presence of protonophore CCCP, $[^3H]^{-}$adenine uptake by the yeast strain containing pH559 is inhibited, but the presence of ouabain has no effect on $[^3H]^{-}$adenine uptake compared to control (Fig. 8). This result is consistent with the functioning of CrNCS1 as a proton — but not Na$^+$ — driven nucleobase cation symporter.

3. Discussion

3.1. CrNCS1 is a transporter of adenine, guanine, uracil and allantoin

The free-living, single cell alga C. reinhardtii utilizes purines from the environment as nitrogen sources, as do other numerous microbes, yet like its higher plant relatives is photosynthetic. This report investigates whether CrNCS1 functions similarly to microbial or the Arabidopsis NCS1 proteins. To address this question, the biochemical function of the NCS1 transporter from C. reinhardtii was determined through heterologous complementation studies using nucleobase-transport deficient S. cerevisiae strains. CrNCS1 is an adenine, guanine, uracil and allantoin transporter with high affinities for adenine and uracil and has a solute transport profile similar to that of Arabidopsis NCS1. Transport was assayed by two independent methods. First, growth studies were used to investigate the ability of CrNCS1 to allow nucleobase transport deficient yeast strains to use uracil or allantoin as the sole nitrogen source (Fig. 3C and D), or for the ability to grow in the presence of toxic nucleobase analogs 8-AZA, 8-AZG, 5-FC or 5-BrdU (Fig. 3A and B). The results support the premise that CrNCS1 facilitates the transport of adenine, guanine, uracil and allantoin compounds but not cytosine or uridine (Fig. 3). A second set of experiments utilizes radiolabeled nucleobases to monitor the transport across biological membranes in yeast harboring CrNCS1. Here results show that CrNCS1 transports the purines adenine and guanine, and the pyrimidine uracil (Fig. 4A-C). Such results are in accordance with the growth experiments. Kinetic parameters for adenine and uracil were determined in a series of homologous competition experiments and show that CrNCS1 has a high affinity for adenine with a $K_m$ of 2.46 $\mu$M and for uracil with a $K_m$ of 5.9 $\mu$M (Fig. 5C and D). The solute specificity of CrNCS1 was further characterized through heterologous competition assays. Here it was determined that adenine, guanine, uracil, xanthine and uric acid are efficient competitive inhibitors while cytosine, 5-FC and hypoxanthine are marginal inhibitors (Fig. 6A and B). Subsequent experiments show that CrNCS1 neither transports xanthine nor hypoxanthine (Fig. 7A and B). Data reveal that solute transport is inhibited by the protonophore inhibitor CCCP but not the Na$^+$ gradient destabilizing compound ouabain which suggests that CrNCS1 is a proton-driven symporter — a hallmark of most NCS1 family members (Fig. 8).
3.2. CrNCS1 solute specificity correlates with in alga nucleobase uptake data

How does the solute transport specificity of CrNCS1, determined through heterologous studies, match with data from in vivo studies of purine utilization in C. reinhardtii? Previous work detailing purine utilization showed that C. reinhardtii: a) has at least two distinct transport systems one for adenine, guanine and possibly hypoxanthine and one for xanthine and uric acid [20,21]; b) can also take up and use allantoin [23]; c) adenine uptake is competitively inhibited by guanine, hypoxanthine, xanthine and uric acid and d) $K_m$ values are similar for adenine and guanine, but adenine is taken up at 35% of the rate of guanine [21]. Our data matches well in that we find that CrNCS1: a) transports adenine, guanine, and allantoin; b) adenine or uracil transport is competitively inhibited by guanine, hypoxanthine,
cytosine and xanthine; c) does not transport xanthine and that d) adenine uptake is slower than that of guanine. Conversely, CrNCS1 does not transport hypoxanthine. Now that the complete genome of *C. reinhardtii* is known this difference in transport can be accounted for. The *C. reinhardtii* genome appears to contain a single gene encoding for one NCS1, two for AZG transporters and five genes encoding for NAT transporters (Phytozome v9.0 http://www.phytozome.com/). In *Aspergillus* species, AZG transporters move hypoxanthine, adenine and guanine and several members of the NAT family have a low affinity for hypoxanthine suggesting that hypoxanthine may be moved by members of these families in *C. reinhardtii* [13]. Interestingly, allantoin transport in *C. reinhardtii* may be controlled by CrNCS1 as no genes encoding for PUP or UPS transporters are evident. In contrast, UPS1 & 2 proteins in Arabidopsis and soybean are the major transporters of allantoin [9,11,24], suggesting that these plant-specific transporters arose among land plants.

### 3.3. A canonical plant NCS1 solute specificity profile is defined

What does this study say about the evolution of NCS1 function in the plant world? Among plants it appears that NCS1 proteins are more closely related to the FUR4 rather than the FCY2 subfamily. The phylogenetic tree of selected FUR4 and FCY2 proteins portrayed in Fig. 2 clearly details this and mirrors the same result seen in recent and more extensive phylogenetic analysis using multiple plant, fungal and bacterial NCS1 proteins [17,18]. However, a plant NCS1 origin is most likely not the result of horizontal gene transfer from fungi as such transfers are rare and the few instances rigorously supported by bioinformatic and phylogenetic analysis do not include NCS1s [25]. Despite the approximately one billion years of evolutionary time marking the divergence of single cell *C. reinhardtii* from the higher dicotyledonous plant *A. thaliana* [22], a canonical solute specificity profile for plant NCS1 emerges – defined by the transport of adenine, guanine and uracil but not cytosine derivatives. CrNCS1 solute transport does not mimic FCY-like transport patterns since it does not transport 5FC and hypoxanthine. Although CrNCS1 transports solutes similar to those associated with FUR4 transporters (uracil and allantoin), CrNCS1 differs from individual FUR4 members in that it has a wider range of solute transport. In essence, the evolutionary linkage of plants and the role NCS1 plays in plant metabolism, trumps consideration of single cell lifestyle at least in terms of NCS1 solute specificity. This is not surprising given the substantial overall amino acid sequence identity/similarity (43/74%), conserved key amino acids identified in NCS1 function and remarkable secondary structure similarity observed among plant NCS1 proteins [17]. Indeed the plastid local of AtNCS1 and likely CrNCS1 point to a role in plant-specific nucleobase salvage distinct from that of microorganisms [3]. CrNCS1 is clearly capable of allantoin transport, however, the loss of AtNCS1 in Arabidopsis does not diminish that ability to grow on allantoin [17].

The solute transport and binding profiles of the two plant NCS1s, CrNCS1 and AtNCS1, have a broad solute transport profile and are a curious mix of those profiles observed in the fungal FCY2 and FUR4 subfamilies of the NCS1 proteins. Among the fungal FCY2-like transporters – *S. cerevisiae* (FCY2, 21 & 22), *Candida albicans* (FCY21) [26] and *Aspergillus nidulans* (FCYB) [27] – the solute transport specificities are conserved in moving cytosine, adenine, guanine and hypoxanthine. Although amino acid sequence similarity unites the FUR4-like fungal members, there is a little direct link between specific sequences and transport of uracil,
allantoin, uridine and other metabolites, suggesting a wider degree of convergent evolution in this subfamily [13,28]. As with the fungal FCY2 members, CrNCS1 transports adenine, guanine but unlike FCY2 it does not transport hypoxanthine or 5FC. CrNCS1 shares solute transport profiles with the S. cerevisiae FUR4 subgroup in moving uracil and allantoin but not uridine. In A. nidulans, FURD transports uracil and is competitively inhibited by xanthine and uric acid as is CrNCS1, however, unlike FURD, CrNCS1 does not transport 5FC or xanthine [29].

An ever-sophisticated picture is emerging detailing the molecular function of NCS1 proteins. Recent investigations have developed three-dimensional structures of MHP1 in outward-facing, occluded and inward-facing states [30,31]. The MHP1 data and a molecular modeling assisted site-directed mutagenesis of A. nidulans FCYB [32], have identified key amino acids involved in cation and substrate binding and/or transport. Although many of these amino acids are common to CrNCS1 (Fig. 1), it is as yet not possible to directly link amino acid sequence to solute specificity. For example, several amino acids in FCYB identified as essential for function, when altered to FUR4-like and plant residues, fail to alter solute recognition beyond FCY2-like profiles (Fig. 1 † residues) [32]. A more clear understanding of sequence-to-function predictions in NCS1 will require more data. To this end, current studies in our lab are in progress to determine if other plant NCS1 proteins from evolutionarily representative species share the same biochemical characteristics as AtNCS1 and CrNCS1.

The data presented here support the contention that the solute specificity of plant NCS1 arose early in plant evolution and define a plant solute transport profile. It is as yet unclear how solid this emerging consensus is. Are the solute transport and binding profiles among plant NCS1 proteins conserved with little variability across evolution? Or, despite sequence similarities, is a wide range of solute transport specificities reflecting convergent evolution evident?

4. Methods

4.1. Construction of yeast expression plasmids

Yeast expression vectors pRG399 and pRG402 carry constitutive PMA1 promoter and CYC1 termination cassette, a 2 υ origin of replication and a LEU2 or TRP1 gene, respectively [16]. Oligonucleotide primers CrNCS1A (5′-ccgccgcatatagggatgtrctcggttcatcata3′) and CrNCS1B (5′-ataagatgccgcatagtctgctcgccgagccgaggc3′) were used to amplify a 1588 bp DNA fragment from EST clone 1031058E09.y1 (a kind gift of Dr. Mark L. Heinmickel, Stanford University, CA). The DNA fragment was cleaved with Xho I and Not I and cloned into the same restriction sites within the multiple cloning site of yeast expression vectors pRG399 and pRG402 to generate plasmids pRH559 and pNS481, respectively.

4.2. S. cerevisiae strains and transformations

S. cerevisiae strains RG191 [MAT α, fyc2Δ::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0], ATCC#4003158 [MAT α, his3Δ, leu2Δ, met15Δ, ura3Δ, fur4Δ] [33], RW105Δdal4A [α, his3Δ, trp1Δ, fur4Δ, dal4Δ::HIS3], RW128 [α, fur4Δ his3Δ trp1Δ ful1Δ::HIS3] [34] and NC122-sp6 [MAT α leu2 fur4Δ] [35] were grown in Synthetic Complete medium (SC) at 30 °C. Yeast transformations were performed using the lithium acetate method [36]. Sensitivity to toxic nucleobase analogs as well as ability to utilize a solute as a sole nitrogen source was analyzed by adding filter-sterilized stock solutions to the autoclaved SC media.

4.3. Radiolabel uptake by yeast expressing CrNCS1

Yeast grown for 24 h in 5 mL liquid culture at 30 °C were pelleted, rinsed and re-suspended in citrate buffer to an OD600 ≈ 4. Cells were then incubated for 0 and 2.5 or 5 min in a 100 mM citrate buffer solution (pH = 3.5) with 1% glucose containing radiolabeled nucleobases. 25 μL aliquots were added to 4 mL ice-cold water and filtered through a 0.45 μM Memcel membrane filter (Gelman Sciences, Ann Arbor, MI). Filters were then washed with 8 mL of water and radioactivity was measured by scintillation counter. Statistical analysis used an independent paired t-test. Significance was measured at P = 0.05 (*). [8-3H]-guanine, [2,8-3H]-adenine, or [3H]-uracil assays were performed at 0.25 μM of the appropriate radiolabeled nucleobase and samples were taken at 0 and 2.5 min [8-3H]-hypoxanthine or [8-3H]-xanthine assays were performed at 0.5 μM radiolabel nucleobase and samples were taken at 0 and 5 min (Moravek, Brea, CA).

4.4. Transport kinetics of CrNCS1 expressed in yeast

Time courses for the uptake of 0.5 μM [2,8-3H]-adenine or [3H]-uracil were performed using yeast cells (OD600 ≈ 4) expressing CrNCS1. For kinetic analysis, homologous competition was performed using yeast cells (OD600 ≈ 4) expressing CrNCS1 at varying concentrations (0, 0.5, 2, 4, 10, 20, 50, 100, 150, 500, 1500 μM) of unlabeled homologous competitor (adenine or uracil) in the presence of 1 μM of its respective radiolabeled nucleobase. Data were fitted using nonlinear regression and analyzed using the one site-homologous model (Prism 6) assuming hot and cold ligands have identical affinities for the receptor. Reactions were stopped at 5 min and radiolabeled uptake was assayed as described above.

4.5. Radiolabeled substrate competition study of CrNCS1 expressed in S. cerevisiae

Radiolabeled uptake was performed as described above for [2,8-3H]-adenine, or [3H]-uracil in the presence of various cold competitors. Yeast cells (OD600 ≈ 4) expressing CrNCS1 were incubated for 5 min in the presence of 1 μM [2,8-3H]-adenine and 1 mM unlabeled adenine, guanine, cytosine, 5-fluorocytosine, xanthine, or hypoxanthine. Alternatively, yeast cells expressing CrNCS1 (OD600 ≈ 4) were incubated for 5 min in the presence of 1 μM [3H]-uracil and 1.5 mM uracil, uric acid or xanthine.

4.6. Radiolabeling and uncoupler uptake by yeast expressing CrNCS1 in the presence of a protonophore or a Na+ pump uncoupler

Radiolabeled uptake was performed as previously described at 1 μM [2,8-3H]-adenine. Yeast cells expressing CrNCS1 were exposed to 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) or 1 mM ouabain octahydrate. The reaction was incubated at 30 °C and aliquots were withdrawn at 5 min.

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