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NRT2.4 and NRT2.5 Are Two Half-Size Transporters from the Chlamydomonas NRT2 Family

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Abstract: The NRT2 transporters mediate High Affinity Nitrate/NitriteTransport (HAN/NiT), which are essential for nitrogen acquisition from these inorganic forms. The NRT2 proteins are encoded by a multigene family in plants, and contain 12 transmembrane-spanning domains. *Chlamydomonas reinhardtii* has six *NRT2*, two of which (*NRT2*.5 and *NRT2*.4) are located in Chromosome III, in tandem head to tail. cDNAs for these genes were isolated and their sequence revealed that they correspond to half-size NRT2 transporters each containing six transmembrane domains. NRT2.5 has long *N*- and C- termini sequences without known homology. NRT2.4 also contains long termini sequences but smaller than NRT2.5. Expression of both studied genes occurred at a very low level, slightly in darkness, and was not modified by the N or C source. Silencing of *NRT2*.4 by specific artificial miRNA resulted in the inhibition of nitrite transport in the absence of other HANNiT (*NRT2.1/NAR2*) in the cell genetic background. Nitrite transport activity in the *Hansenula polymorpha* Δ*ynt*::*URA3 Leu2* mutant was restored by expressing *CrNRT2.4*. These results indicate that half-size NRT2 transporters are present in photosynthetic organisms and that NRT2.4 is a HANiT.

Keywords: half-size transporter; nitrite transport; gene expression; *Hansenula* complementation

1. Introduction

Nitrate is an essential mineral nutrient for plant growth and development. In fact, nitrate is reduced to ammonium and used to build biological molecules such as amino acids, proteins or nucleic acids. It is also a signal molecule that regulates the expression of genes for nitrate assimilation, root architecture, and other metabolic processes in the plant [1–3]. The nitrate concentrations in plant soils might change extremely from 10 μM in natural soils to 100 mM in fertilized soils [1]. Plants have displayed a sophisticated network of membrane transporters for the sensing, absorption, storage, and distribution of nitrate inside the organism. These nitrate transporters belong to four families: NRT1/PTR/NPF (nitrate transporter 1/peptide transporter/nitrate peptide transporter family), NRT2, chloride channel (CLC), and slow anion channel-associated 1 homolog 3 (SLAC1/SLAH), which have been reviewed [4,5].

Nitrate is also a nutrient for algae, which share many aspects for nitrate assimilation and regulation with land plants [6–8]. Water is the natural habitat for algae, where nitrate concentration is usually less than 1 μM and could increase several orders of magnitude due to contamination from plant fertilizers. The unicellular and model alga *Chlamydomonas reinhardtii* also has a high number of transporters to control cellular nitrate/nitrite and for adapting it to the changing environmental conditions, but this is still not fully understood. Thus, *Chlamydomonas* is a powerful green model that can provide more detailed analysis of the molecular function of genes for nitrate and nitrite transport studies [6–8]. This knowledge is key for understanding how the plants use nitrate fertilizers, one important topic in agronomy. In *Chlamydomonas,* the nitrate/nitrite transporters belong to the NPF and NRT2 as in plants, but this alga also has NAR1 transporters that are absent in plant genomes. NAR1 belongs to the formiate/nitrite transporter family (FNT) present in bacteria (FocA and NirC, in *E. coli*), fungi (NitA in *Aspergillus nidulans*), and yeast (NAR1 in *Hansenula polymorpha*). The structure of FNT is different from NPF and NRT2 transporters. FNTs are six transmembrane helixes proteins that can form a pentameric structure similar to aquaporin and behave more like a channel than a transporter [9]. The functions for the different NAR1/FNT can be summarized as: (1) in *E. coli*, NirC is involved in both nitrite uptake and export [10]; (2) in *Aspergillus nidulans,* NitA mediates specific high-affinity transport of nitrite and also has some role in nitrite efflux [11]; (3) in *Hansenula*, NAR1 mediates nitrate and nitrite efflux [12]; (4) in *Chlamydomonas reinhardtii* there exist six NAR1 and their functions have only been studied for NAR1.1 and NAR1.2. NAR1.1 is a plastidic nitrite transporter and, together with other key proteins for nitrate assimilation, is nitrate regulated and under the control of the major regulatory gene *NIT2* [13]. NAR1.2, also named LCIA, is a plastidic transporter with specificity for both nitrite and bicarbonate, and overexpressed under low CO_2 conditions [14]. Recently, it has been shown that NAR1.2 (LCIA) is a chloroplast envelope HCO_3^- transporter that forms part of a CO_2 concentrating mechanism (CCM) operating at very low CO_2 [15,16]. The CCM is essential in aquatic microalgae to accumulate CO_2 close to RUBISCO and to make efficient photosynthesis. More details for nitrate assimilation in microalgae have been recently reviewed [8].

The high-affinity nitrate transporters NRT2 are present in plants, algae, fungi, yeast, and bacteria [17–23]. This transporters family is also named NNP (Nitrate Nitrite Porter) (TC 2.A.1.8) and belongs to the Major Facilitator Superfamily (MFS) (TC 2.A.1) [24,25]. The structure of a typical NRT2 protein consists of 12 transmembrane helixes in two sets of six that form an *N*-terminal domain and a C-terminal domain linked by a hydrophilic central loop between helixes 6 and 7 [26]. This central loop is long, more than 90 residues, in fungal and yeast NRT2. The sequence identity between NRT2 proteins goes from 30% to 90%, but the highest conservation is in the middle of the protein where the MFS and NNP motifs are identified [18,24,26]. Meanwhile, the *Aspergillus* NRTA and NRTB, the *Hansenula* YNT, and the *E. coli* NARK and NARU do not require a second component for functionality; most of the NRT2 from plants and some from *Chlamydomonas* requires a second protein NAR2 [27–32]. In *Arabidopsis,* the two-components system NRT2/NAR2 is proposed to function as a tetrameric complex formed by two subunits from each of the two polypeptides [32,33]. All NRT2 studied are high-affinity transporters; in plants they are shown to work as high affinity nitrate transporters [32], whereas in fungi and yeast (AnNRTA, AnNRTB, HpYNT), as well as in *Chlamydomonas* (NRT2.1/NAR2), they are high affinity nitrate/nitrite transporters [11,22,34,35].

Chlamydomonas genome has six *NRT2* genes [8]. Two of them (*NRT2.4* and *NRT2.5*) are predicted to encode atypical NRT2 proteins in terms that they are half-size molecules. These genes have been analyzed in the present work and it is shown that NRT2.4 is a high affinity nitrite transporter.

2. Results

2.1. Half-Size Transporters Encoded by NRT2.4 and NRT2.5

Chlamydomonas genome predicts two *NRT2* genes in chromosome III that encode putative NRT2 transporters with six transmembrane helixes. Those transporter genes, named *NRT2.5* and *NRT2.4*, are in orientation from tail to head. The putative proteins contain elements from the first and second half of the typical twelve-transmembrane NRT2/NNP proteins. The isolation of the corresponding cDNAs was achieved according to the predictions from *Chlamydomonas* jgi v2 (Figure 1). Two amplification rounds with nested primers were performed, first with 5′UTR2.5-Qo and second with ATG2.5-Qi, and resulted in a 3-kb band, which was cloned and sequenced (Figure 1AB). The nucleotide sequence showed a polyadenylated transcript corresponding to *NRT2.5* where a canonical TGTAA polyadenylation signal was present 15 nt before the polyA tail, and the 3′UTR sequence, 171 nt-length, coincided with this region in the genome. The existence of the polyadenylated *NRT2.5* was confirmed in three *Chlamydomonas* strains: two wild types for nitrate assimilation 704 and 6145c, and the mutant D2 (Figure 1C). The sequencing data confirmed the existence of a *NRT2.5* transcript from different strains and support the presence of an atypical half-size NRT2 transporter in *Chlamydomonas*. To check whether a second half-size NRT2.4 transporter is also expressed, an RT-PCR amplification of the corresponding ORF was achieved (Figure 1D). Also, nested primers were used, the first round with 5′UTR_Nrt2.4-LoSpeI_Nrt2.4 and second round with ATG_Nrt2.4-LoSpeI_Nrt2.4. The amplified 1.5 Kb band (Figure 1D) was cloned, and its sequence corresponded to a cDNA for *NRT2.4*. Thus, the sequence analysis shows the existence of two independent cDNAs corresponding to *NRT2.5* and *NRT2.4* for which their exon/intron organization is shown in Figure 1, and the encoded proteins correspond to half-size NRT2 transporters (Figure 2).

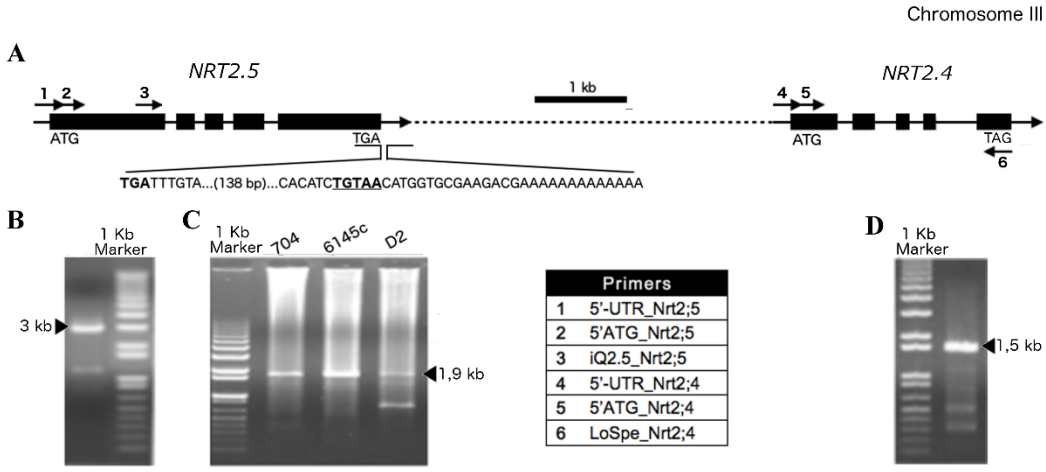

Figure 1. Amplification of cDNA corresponding to *NRT2.5* and *NRT2.4*. (**A**) Scheme of the genomic region containing *NRT2.4* and *NRT2.5,* where the exon/intron organization and the localization of primers used to amplify the corresponding cDNAs are indicated (**B**) Amplification of *NRT2*.5 cDNA with primers 1 and Q_0 in a first lap, and primers 2 and Q_i in a following lap. The 3-kb band, corresponding to full cDNA of *NRT2.5* was cloned and sequenced; (**C**) Probing the existence of polyadeylated *NRT2.5* in three *Chlamydomonas* strains, the WT strains 704 and 6145c, and in D2 strain (deleted at *NRT2.1, NRT2.2, NAR2*). RT-PCR was performed with primers 3 and Q_0 in a first lap, and primers 3 and Q_i in a following lap. The amplified 1.9 kb band was sequenced; (**D**) Amplification of the ORF corresponding to *NRT2.4*. The ORF of *NRT2.4*, a 1.5 kb band, was amplified with primers 4 and 6 in a first lap, and 5 and 6 in a second lap. In **B** and **D**, cDNA from the strain 704 was used.

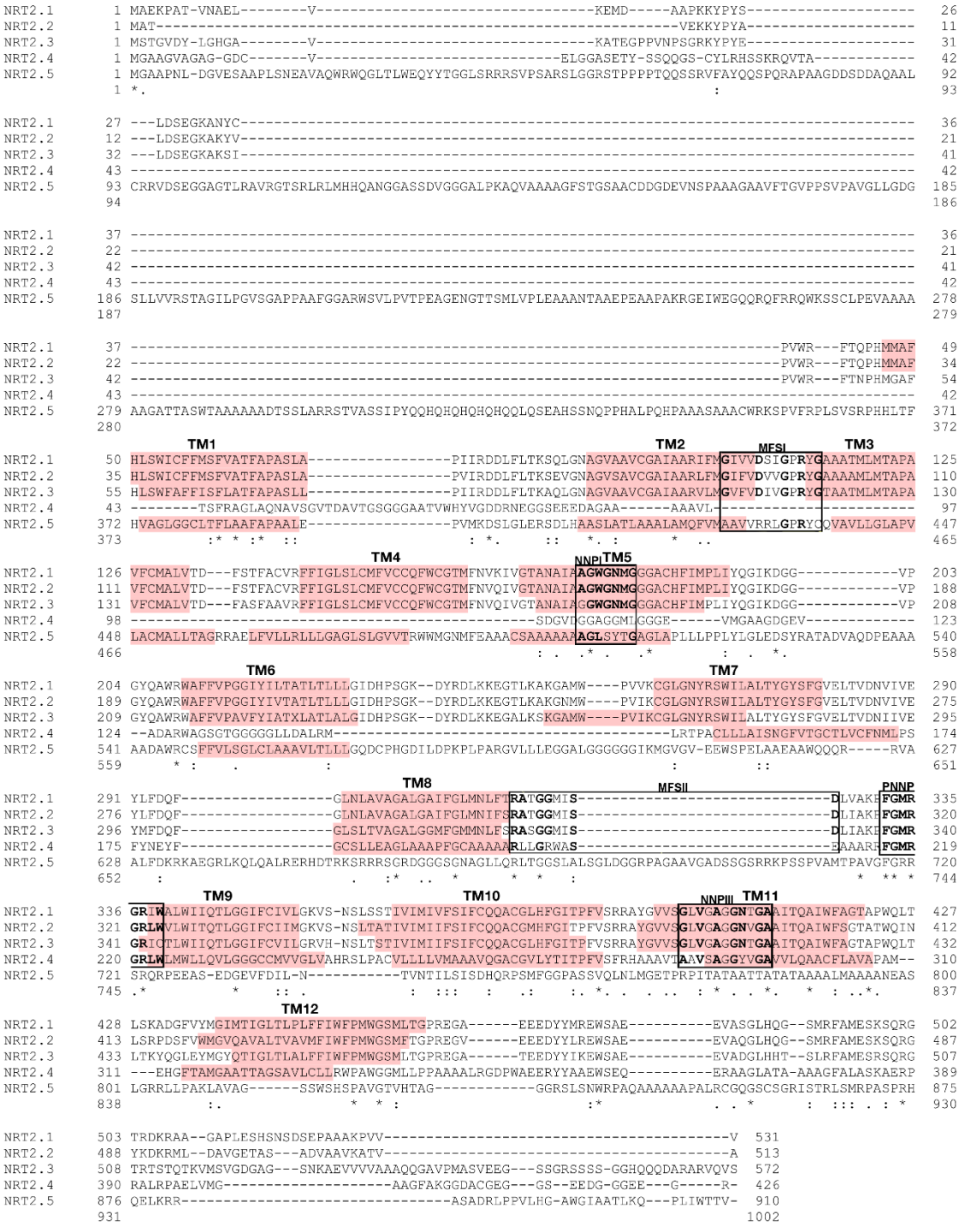

Figure 2. Alignment of NRT2 protein sequences from *C. reinhardtii.* This alignment was performed using TM-Coffee. Dashes refer to expansion of the sequence to allow maximal alignment. Sequences of transmembrane regions are shadowed and named TM1-12. Boxes indicate conserved signatures, MFSI, NNPI, MFSII, NNPII, and the photosynthetic NNP (PNNP) signature FGMRGR. Protein identification corresponds to: NRT2.1 (Cre09.g410850), NRT2.2 (Cre09.g410800), from Phytozome 10.3; NRT2.3 (AY669386.1) accession number in NCBI, NRT2.4 (KT971135), and NRT2.5 (KT971134). The prediction of transmembrane helices of each protein was obtained using the HMMTOP program [36].

These NRT2 proteins, 910 amino acids for NRT2.5 and 426 amino acids for NRT2.4, show homology with NRT2 transporters (Figure 2). The comparison of the *Chlamydomonas* NRT2.1-5 (Supplementary Table S1A) was performed by comparing one on one each of the two halves of NRT2.1-3 (from transmembrane domains 1 to 6, and 7 to 12) and NRT2.4 and 5. As known, there is a high identity between NRT2.1 and 2 (86%–94%) and between NRT2.2 and 2.3 about 86%. The identity degree between NRT2.4 and NRT2.1-3 is around 35%–37% and is similar between NRT2.5 and NRT2.1-3. The least identity is shared between NRT2.4 and NRT2.5. Comparisons of the *Chlamydomonas* full NRT2.1-5 proteins (Supplementary Table S1B) gave similar results, though with a smaller degree of conservation than above.

In addition, particular features were observed when analyzing NRT2.5 and NRT2.4 together with NRT2.1-3 transporters (Figures 2 and 3). The NRT2.5 transporters (910 residues) adjust to a six transmembrane protein with conservation with the first part of a typical NRT2 protein. NRT2.5 contains a MSF-I motif, between the second a third transmembrane domains, and a NNP-I motif into the fifth transmembrane helix. The expanding N-terminus (370 residues) and C-terminus (342 residues) have no homology with NRT2 proteins. This is usual within the NRT2/NNP family in which the middle region of the proteins shares a high degree of similarity, whereas the N- and C-termini share low homology [18].

The NRT2.4 protein (426 residues) has also six transmembrane helixes, which fits to the second part of a typical NRT2 protein (Figure 3). Thus, NRT2.4 contains a MSF-II motif between its second and third helix, which corresponds to the region between the eight and nine helixes in a typical NRT2. NRT2.4 also contains the NNP-II motif in its fifth transmembrane helix, which corresponds to the region in transmembrane 11 in a typical NRT2 protein. In addition, NRT2.4 contains a conserved motif FGMRGRLW considered as characteristic of eukaryotic photosynthetic NRT2 proteins [18]. The *N*-terminus (149 residues) and C-terminus (89 residues) sequences of NRT2.4, smaller than in NRT2.5, have no homology with NRT2 proteins.

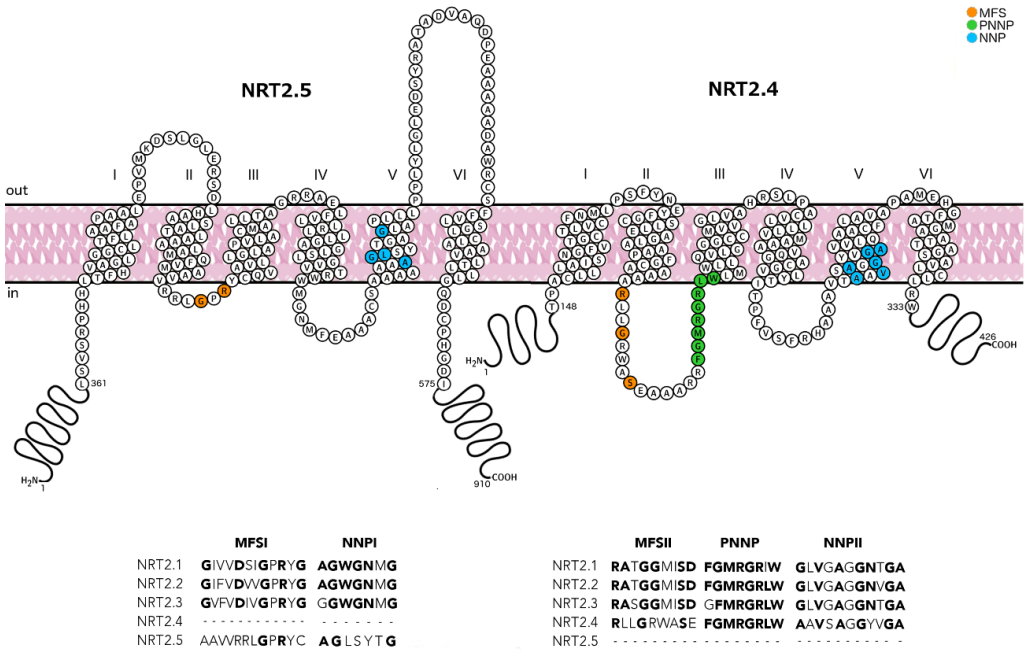

Figure 3. Predicted membrane topology for NRT2.4 and NRT2.5 from *Chlamydomonas*. The model was obtained by using the PROTTER program [37]. Strictly conserved amino acids corresponding to each motif signature are colored in orange for the Major Facilitator Superfamily motif (MFSI and II), blue for Nitrate Nitrite Porter motif (NNPI and II), and green for the Photosynthetic Nitrate Nitrite Porter motif (PNNP).

2.2. Expression of NRT2.4 and NRT2.5 is Not Regulated by Nitrogen

The main characteristics of the *Chlamydomonas NRT2.1*, *NRT2.2*, and *NRT2.3* expression are its up-regulation by nitrate, and that they are under the control of *NIT2*, the master gene for nitrate assimilation in the alga [7,38]. The expression of *NRT2.4* and NRT2.5 genes has been analyzed under different conditions: high *versus* low CO_2, ammonium *versus* nitrate, and light *versus* darkness (Figure 4). The results show that both genes have very low expression levels and that they are not regulated by nitrogen or carbon, but in the dark their expression seems to be slightly increased.

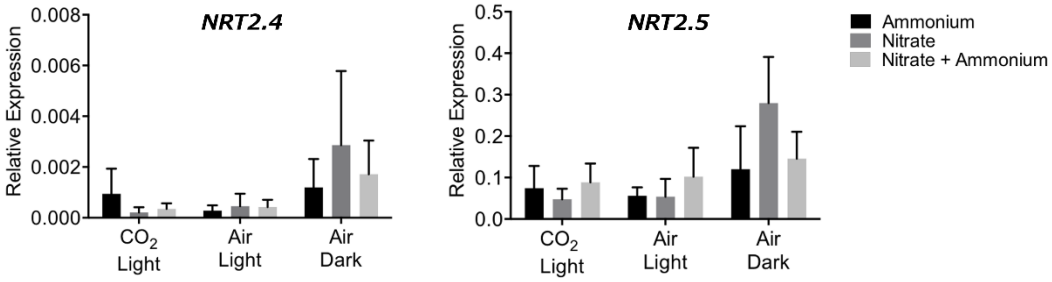

Figure 4. Expression of *NRT2.4* and *NRT2.5.* Ammonium-grown cells were transferred to medium containing 4 mM of the indicated nitrogen sources, in the presence of light or in the dark, and with or without CO_2 (5%). After three hours, samples were processed for real-time quantification by using specific primers indicated in Supplementary Table S2. Gene transcript levels are given as relative abundance with respect to the housekeeping gene ubiquitin ligase. The *Chlamydomonas* strain used is the WT strain 21gr.

2.3. NRT2.4 is a Nitrite Transporter

Although NRT2.5 and NRT2.4 are half-size of a typical NRT2, they could work as an NNP transporter: (1) by interaction of NRT2.5 (first part) and NRT2.4 (second part) to give a complete NRT2 protein; or (2) just as a half-sized NRT2.

An artificial microRNA technique has been used to silence *NRT2.4* [39]. The construction was cloned in a vector pChlamiRNA3 that confers paromomycin resistance, and was used for selection of the *Chlamydomonas* transformants (see Experimental Section). Three *Chlamydomonas* strains with different genetic backgrounds for *NRT2* transporters were transformed with the amiRNA construction. Strain 704 is a wild type, JA4 (contains *NRT2.1/NAR2* and *2.3* and lacks *NRT2.2*), and finally strain D2R4 (which has *NRT2.3* and lacks *NRT2.1*, *NRT2.2* and *NAR2*). Due to the low expression level of *NRT2.4*, the determination of the magnitude of silencing for this gene expression in the isolated transformants was subjected to a very high error. Different isolated paromomycin-resistant strains were used for the analysis of nitrite transport activity (Figure 5). The nitrite transport activity from the above strains and transformants under two conditions is shown. These cells were grown in an ammonium medium and nitrite transport activity was induced either directly (Figure 5A) or after induction of the cells in 4 mM nitrate for three hours (Figure 5B). No significant difference on this transport activity was observed between those strains. In Figure 5C,D, the nitrite transport activity from the strain JA4 and a transformant was compared under the same conditions as above and no significant differences were obtained between them as well. When *NRT2.4* was silenced under a D2R4 genetic background (five strains randomly isolated), after nitrate induction, transformants showed a lower nitrite transport activity than their parental strain (Figure 5E). The activity of the nitrite transport was from 100% in D2R4 to 14%–72% in D2AS strains. It is worth noting that to detect nitrite transport activity from the D2 strains and under the assayed conditions, high CO_2, nitrate pre-incubation is required to induce nitrite transport [38].

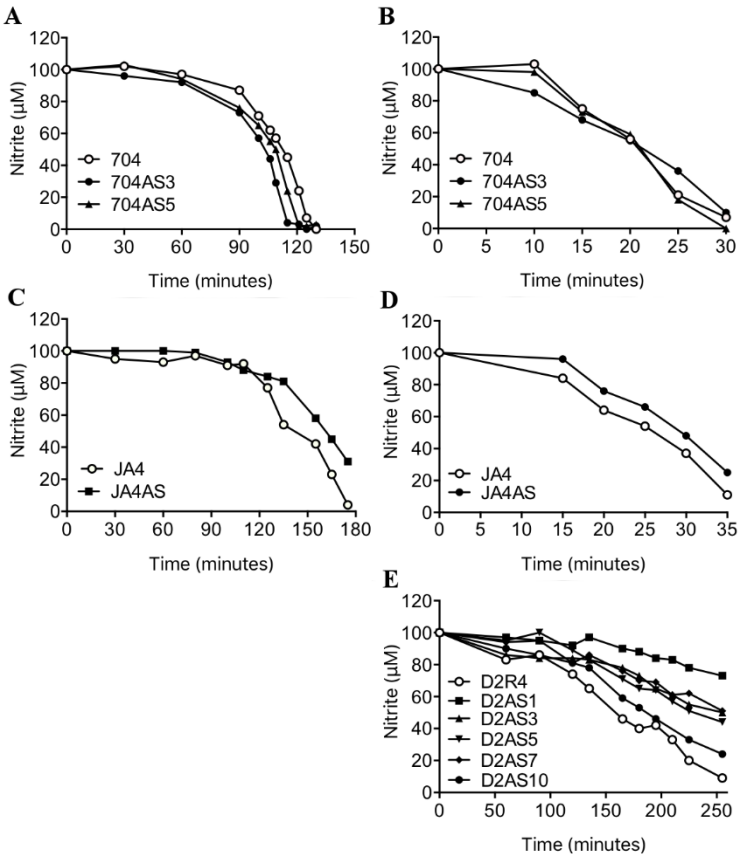

Figure 5. Nitrite uptake activity by *Chlamydomonas* strains when *NRT2.4* is silenced. *NRT2.4* silencing was performed by the artificial miRNA technique (see Experimental Section). Strains correspond to a wild type 704 and its corresponding silencing transformants (ami, 704AS3 and 704 AS5), JA-4 strain that contains *NRT2.1* and *NAR2* but lacks *NRT2.2*, as well as its corresponding ami (JA4S), and D2R4 that lacks of *NRT2.1, NRT2.2* and *NAR2*, as well as its corresponding ami (D2R4AS1, AS3, AS5, AS7 and AS10). (**A**) and (**C**), ammonium-grown cells were washed and transferred to 100 μM of nitrite. (**B**), (**D**) and (**E**) cells were previously induced in 4 mM nitrate medium during three hours and then transferred to 100 μM nitrite. The nitrite concentration in the medium was quantified along with time. Cells were always bubbled with 5% CO_2 enriched air. Cell density of the cultures was adjusted to 10 mg Chl/L. Data correspond to the average of a single replicate.

In *Chlamydomonas*, NRT2.1/NAR2 is the most efficient high affinity transporter and bispecific for nitrate and nitrite [6,7]. Thus, data from Figure 5 suggest that NRT2.4 participates in nitrite transport, but its activity is masked by NRT2.1/NAR2.

To determine whether this NRT2.4, half-size NRT2, is sufficient to mediate nitrite transport activity, its expression in yeast was assayed. The ORF for NRT2.4 was cloned into the yeast pYNR vector and used to complement the *Hansenula polymorpha* Δ*ynt*::*URA3 Leu2* mutant. This mutant has no YNT1, the only NRT2 transporter described in this yeast, and is affected in nitrate and nitrite transport [40]. Three yeast transformants expressing the *Chlamydomonas NRT2.4* (Δ*yntcrNRT2.4* L1-3) were assayed for nitrite transport activity under the conditions previously described [40]. Yeast cells were induced for 90 min in YG medium without nitrogen, and then nitrite transport activity was measured at pH 6.5 and using 100 μM nitrite. As shown in Figure 6, the Δynt mutant does not show nitrite transport activity, but the complementation with *CrNRT2.4* restores this activity, though it was less efficient than in the *Hansenula* wild type.

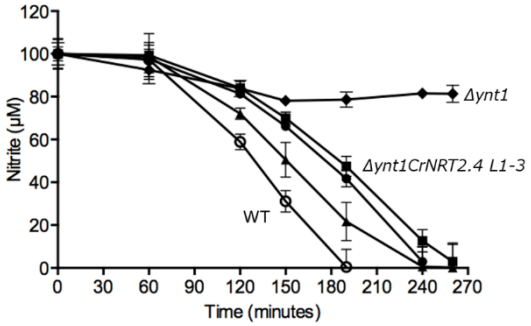

Figure 6. Nitrite uptake activity in *Hansenula polymorpha* expressing Cr*NRT2.4.* The *Hansenula* strains correspond to WT strain (open circles), Δynt1 mutant (closed diamonds) and three Cr*NRT2.4*::Δynt1 transformants (L1-3) (closed circles, squares, and triangles, respectively). Nitrite uptake was performed at pH 6.5 from cells induced for 90 min in minimal medium without nitrogen [40]. Cell density was adjusted at 660 nm and corresponded to 10 mg/mL (fresh weight).

3. Discussion

The genome of *Chlamydomonas reinhardtii* predicts six *NRT2* genes [8,41,42]. Three of them, NRT2.1 to NRT2.3, have been previously characterized [21,34,38,43] and correspond to the typical NRT2/NNP transporters with 12 transmembrane helixes. NRT2.1 together with NAR2 form a two-component high affinity system I, which is bispecific for nitrate and nitrite. NRT2.2 together with NAR2 form a high affinity system II, which is more specific for nitrate. The lack of *NRT2.1, NRT2.2,* and *NAR2* genes results in the absence of high affinity nitrate transport activity under high CO_2 in *Chlamydomonas* cells—which still have high affinity nitrite uptake activity, called system III—so it is NAR2 independent and was attributed to NRT2.3 [38]. A chlorate-resistant mutant isolated in the Δ[*NRT2.1, NRT2.2, NAR2*] background (strain DC2-III) resulted in the non- expression of this system III transport activity and the gene affected was found to be closely linked to *NIT2* [38]. Later on, in DC2-III, a mobile element was identified within *NIT2* at the end of *NIT2* causing its inactivation [44]. The activity of another transport system named IV was also proposed in *Chlamydomonas*, which has the following characteristics: (1) it is detected under low CO_2 conditions; (2) it has high-affinity for both nitrate and nitrite; and (3) it is inhibited by chloride and CO_2 but not by ammonium [38,45]. In summary, *Chlamydomonas* seems to take up nitrite efficiently from the medium by systems that are independent of NRT2.1, NRT2.2, and NAR2.

The typical NRT2 protein has 12 transmembrane helixes that form two domains, one N-terminal domain (TM1-TM6) and one C-terminal domain (TM7-TM12), where MFS and NNP motifs are identified [18,24,26]. In transmembrane five a conserved signature motif NNP I (A-G-W/L-G-N-M-G) is present. A second NNP signature is found in transmembrane 11 and is related to the sequence F/Y/K-x3-I/L/Q/R/K-x-G/A-x-V/A/S/K-x-G/A/S/N-L/I/V/F/Q-$x_{1,2}$-G-x-G-N/I/M-x-G-G/V/T/A. Another conserved sequence, R-[PA]-x-G-G-x-x-[SA]-D, is identified between transmembranes 8 and 9 and considered MFS II motif. Half of this sequence (G-x-x-[SA]-D) is closely related to a part of the MFS specific sequence motif, G-[RKPATY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM], which is located between transmembranes 2 and 3. However, the longest conserved sequence, F-G-M-R-G-R-L-W, is at the beginning of transmembrane 9 and found in photosynthetic organisms [18].

In this work, it is shown that *NRT2.4* and *NRT2.5* encode atypical NRT2 proteins that are half-size of a 12 transmembrane NRT2. These genes are in chromosome III in orientation *NRT2.5→NRT2.4* and correspond to the N- and C-termini domains of a full NRT2. The identity degree between whole proteins goes from 28% (NRT2.5 *versus* NRT2.1) up to 31% (NRT2.4 *versus* NRT2.1). However, in considering half molecules the identity degree increases up to 37% (NRT2.5 *versus N*-terminal of NRT2.1 or NRT2.4 *versus* C-terminal of NRT2.1).

Up to now, this is the first report of half-size NNP transporters. The 12 transmembrane helixes are the most frequent topology of MFS transporters and believed to be the result of an ancient gene duplication of a gene encoding a single domain with six transmembrane helixes. The maintenance of both domains in a single polypeptide chain could perhaps have been favored during evolution in order to facilitate substrate recognition and conformational changes during the transport [25]. It has been reported that halves of sucrose transporters StSUT1 from *Solanum tuberosum* show intermolecular interactions and reconstitute sucrose transport activity similar to the intact protein [46]. Also, heteromeric complementation is obtained with *N*-terminal halves from *Lycopersicon esculentum* SUT2 and C-terminal halves of StSUT1. However, single halves do not function independently. Our data suggest that NRT2.4, considered a second half of NRT2, complements nitrite uptake activity in *Hansenula polymorpha*. We do not know whether NRT2.4 plus NRT2.5, or single NRT2.5 are able to reconstitute transport activities with differential characteristics, and so further experiments will be required to answer this point.

The silencing of *NRT2.4* in *Chlamydomonas* strains with artificial micro RNA suggests that NRT2.4 participates in nitrite transport, but its activity is masked when the other efficient nitrite transporter NRT2.1/NAR2 is present. In *Chlamydomonas,* nitrite transport activity could result from oligomerization of NRT2.5 plus NRT2.4 or, like in *Hansenula,* only NRT2.4. As indicated above, a nitrite transport system III was proposed in *Chlamydomonas* [38]. However, the present data suggests NRT2.3, NRT2.4, and perhaps NRT2.5 could participate in this activity.

The expression pattern of *NRT2.4* and *NRT2.5* shows that these genes express very little and are not nitrogen regulated as *NRT2.1*, *NRT2.2,* and *NRT2.3* [21,38,43]. However, *NRT2.4* and *NRT2.5* show a co-regulation under our studied conditions and also in a minus nitrogen medium [47]. This co-regulation could support the idea that NRT2.5 (first half) plus NRT2.4 (second half) might constitute a NRT2 transporter.

Finally, although half-size NRT2 transporters have not been reported, recently a half-size NPF has been identified in rice [48]. It is the *OsNRT1.1b*, a splicing mRNA product of *OsNRT1.1*, which encodes a six-transmembrane nitrate transporter corresponding to the first half of OsNRT1.1a. Oocytes expressing *OsNRT1.1b* accumulate more nitrate, at 0.25 mM nitrate, than when the full size *OsNRT1.1a* is expressed.

To conclude, this work suggests that *Chlamydomonas reinhardtii* has half-size NRT2 transporters and that one of them (NRT2.4) is sufficient to work as a nitrite transporter. A complex picture seems to emerge for the nitrate/nitrite transporters, a consequence not only from the different families of transporters, but also of the numbers from each family, and the possibility of generating different protein complexes, *i.e.*, NRT2/NAR2, NRT2 alone, half-size NRT2s.

4. Experimental Section

4.1. Strains and Growth Conditions

The *Chlamydomonas reinhardtii* strains used in this work have seen studied previously and correspond to the following types: (1) wild type for nitrate assimilation: 6145c (cc-1691), 21gr (cc-1690), 704 is a WT having a reporter gene *pNIA1-ARS* [49]; (2) strain JA4 lacks *NRT2.2* and *NAR1.1,* and contains *NRT2.1/NAR2* [13,50]; and (3) strain D2R4, which lacks *NRT2.1, NRT2.2, NAR2, NIA1, NAR1,* and contains the reporter gene *pNIA1-ARS* [51].

Cells were grown in a minimum medium bubbled with 5% (*v*/*v*) CO_2-enriched air, and under continuous light [52]. The nitrogen source used for growth was ammonium chloride (8 mM). After growth, cells were washed out of ammonium and transferred to the medium indicated in the Figures. Nitrite transport was inferred from depletion of nitrite (100 μM) from the medium as described [38]. Experiments were done at least by duplicate. The *Hansenula polymorpha* strains WT and Δynt1 mutant were previously described, as the conditions for growth and assay for nitrite transport activity [40].

4.2. Silencing of NRT2.4

Artificial miRNA construct pChlamyRNA3-NRT2.4 was prepared following the protocol described [39]. The 90-nt oligonucleotides *NRT2.4-amiRNA-Fw* and *NRT2.4-amiRNA-Rev* (Supplementary Table S2) were designed by using the program WMD3 Web MicroRNA Designer [53] and contains a 21 nt amiRNA "TAATATGGTAATGTTAGGCTT" for which its target sequence is at the 3′UTR of *NRT2.4*. The double-stranded DNA oligonucleotide was cloned in *Spe*I into the vector pChlamyRNA3, under the control of *PSAD* promoter. The correct orientation of the construct was checked by sequencing, and then used to transform *Chlamydomonas* strains 704, JA4 and D2R4. Transformation was performed with 500 ng of plasmid DNA by using the glass-beads method [54], and the transformants isolated by paromomycin resistance [55].

4.3. Expression of CrNRT2.4 in Hansenula Polymorpha

The ORF corresponding to *NRT2.4* cDNA was cloned as a *Sal*I-*NRT2.4-Spe*I fragment into the yeast expression vector pYNR-EX and under the control of the yeast nitrate reductase [56]. The construct p*YNR-EX-CrNRT2.4* was confirmed by DNA sequencing and used to transform the high affinity nitrate/nitrite transport mutant Δynt1 (Δynt1::URA3 leu2). The transformants were obtained in a selection medium without leucine, and then checked for the presence of the construction by colony PCR [56].

4.4. cDNA Synthesis, Isolation of cDNA for NRT2.4 and NRT2.5 and Quantification of Gene Expression

Total RNA was isolated according to previously described methods [57]. The first strand cDNA was synthesized from total RNA by using QT primer and the iScript Selected cDNA Synthesis Kit [58] and by following manufacturer instructions.

The isolation of *NRT2.4* and *NRT2.5* cDNAs was designed considering the translation initiation codon from JGI v2 for primers design. Further versions were also considered [41,42]. See Supplementary Table S2 for the primers used, and Figure 1A for a scheme.

Quantification of *NRT2.4* and *NRT2.5* transcripts was achieved by real-time PCR on an iCycler MyiQ2 real-time PCR detection system (Bio-Rad) using SsoFastTM EvaGreen Supermix (Bio-Rad). For each gene, the data RNA levels were normalized using the ubiquitin ligase gene as an internal standard and expressed as relative values with respect to this housekeeping gene [59]. See Supplementary Table S2 for the primers used.

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