# A high affinity nitrate transport system from *Chlamydomonas* requires two gene products

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**Abstract A nitrate-regulated cluster of genes involved in nitrate transport and assimilation has been identified in *Chlamydomonas reinhardtii*. Mutant strains of the alga, which are defective in some aspect of transport and assimilation have been used to assign functions to these genes. This analysis has suggested that two gene products are necessary to obtain a functional high affinity nitrate system in *Chlamydomonas* [Quesada et al. (1994) Plant J. 5, 407–419]. In this paper we have tested this hypothesis by injecting *Xenopus* oocytes with mRNA prepared from these two cDNAs, *Nrt2;1* and *Nar2*, and then assaying the oocytes for nitrate transport activity. Oocytes injected with single types of mRNA did not show any nitrate transport activity. Furthermore, *Nar2* mRNA was toxic to oocytes, with nearly 60% of the oocytes dead 3 days after the injection. However, when oocytes were injected with a mixture of two mRNAs prepared from *Nrt2;1* and *Nar2*, a high affinity nitrate transport activity could be measured. However, the $K_m$ for nitrate of this transport system was 28 μM which is higher than the value of 1.6 μM which had been obtained by the analysis of mutant phenotypes. The pH-dependence of the nitrate-elicited currents was consistent with a proton-cotransport mechanism. These results prove that two gene products are required to produce a functional high affinity nitrate transport system and that this process does not involve transcriptional regulation.**



*Key words:* Coexpression; Nitrate transport; *Chlamydomonas*; Proton cotransport; *Xenopus* oocytes

## 1. Introduction

Nitrate uptake has been classified into high and low affinity systems and these systems have been further divided into constitutive and inducible [1]. The genes encoding many of these different classes of nitrate transporter have been identified and their corresponding cDNAs isolated [2–4]. One of the best ways to prove the function of a gene is to express the protein in a heterologous expression system, such as yeast or *Xenopus* oocytes. Although there are several reports on the characterisation of expression of the low affinity nitrate uptake systems (*Nrt1*) in oocytes [2,5], which has been recently found to show a high affinity nitrate transport activity [6,7], there are none for the high affinity nitrate transporters of the *Nrt2* family.

The nitrate uptake systems of the unicellular alga, *Chlamydomonas reinhardtii* have been characterised by phenotype analysis of various different mutant strains. A 32-kb region of the *Chlamydomonas* genome has been shown to contain three genes that appear to be involved in the uptake of nitrate and nitrite [8]. Database comparison of the sequence information for two of these genes, *Nrt2;1* and *Nrt2;2* shows them to be related to CRNA [9], a nitrate transporter from the fungus: *Emericella* (*Aspergillus*) *nidulans* [3]. A mutant lacking these genes and *Nar2* was unable to use nitrate as the only nitrogen source, but only when either one of the *Nrt2* genes and *Nar2* were present was nitrate uptake activity restored. Further phenotype analysis showed that the *Nrt2;1* and *Nar2* genes were required for a high affinity nitrite/nitrate transport system, while *Nrt2;2* and *Nar2* encoded a nitrate-specific system [10]. Two high affinity nitrite transport systems, possibly encoded by *Nrt2;3* and *Nrt2;4* genes and having a differential regulation of their activity, have also been characterised recently [11]. In this paper, to provide proof of function and to demonstrate that *Nar2* is not involved in the regulation of the transcription of the *Nrt2;1* gene we have co-injected mRNAs obtained from both genes into *Xenopus* oocytes and then assayed for nitrate transport activity.

## 2. Materials and methods

### 2.1. Synthesis cRNA from of a full-length Nrt2;1 and Nar2 cDNA

The full-length *Nrt2;1* and *Nar2* cDNA were cloned in pUC19 and pBluescript II KS vector in *Eco*RI–*Hin*dIII sites with the 5′ end in the *Eco*RI site [9], named pUC19Nrt2;1 and pBSNar2, respectively. *Nar2* cDNA was provided by P.L. Lefebvre (University of Minnesota, MN, USA). We chose *Nrt2;1* rather than *Nrt2;2* [10] for the oocyte expression experiments because a full-length cDNA was available for this gene. The *Nrt2;1* cDNA was excised from pUC19Nrt2;1 with *Eco*RI and *Hin*dIII restriction digestion. The *Nrt2;1* cDNA was then subcloned into the pBluescript II KS vector at the compatible sites, resulting in the construct pBSNrt2;1. For in vitro synthesis of cRNA both pBSNrt2;1 and pBSNar2 were linearised by digestion with *Xho*I, and the full-length mRNAs were synthesised using a T7 RNA transcription kit (Ambion). Purification of the mRNA and confirmation of its size and integrity by gel electrophoresis were performed according to protocols provided by the supplier of the in vitro transcription kit.

### 2.2. Oocyte preparation, injection and electrophysiology

Oocytes were prepared, stored and injected with mRNA as described in an earlier paper [5]. Healthy oocytes at stage V or VI were chosen for injection with 50 nl *Nrt2;1* or *Nar2* cRNA (1 ng/nl), or 50 nl DEPC treated water as a control. Some oocytes were injected with 50 nl of mRNA containing a mixture of *Nrt2;1* and *Nar2* mRNA (1:1) made by mixing the stock solutions (2 ng/nl). The same amount of each type of mRNA was injected into each oocyte. The % mortality of injected oocytes was recorded after 3 days and the electrophysiological measurements were performed 3–6 days after mRNA injection. An oocyte was defined as dead by

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having a resting membrane potential of 0 mV, which in many cases was accompanied by cytoplasm leaking from the cell.

Oocyte currents were measured using the two microelectrode voltage-clamp method [12]. Electrodes were filled with 3 M potassium chloride and had tip resistance ranging from 0.5 to 2 MΩ. All electrophysiological measurements were made in a saline containing (in mM) 116 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4. MES was added in place of HEPES for pH 5.5 saline; the pH was adjusted by the addition of 5 M NaOH solution. For steady-state current measurements, the oocyte membrane potential was clamped at −50 mV from which the membrane was pulsed to the test potentials for 120 ms from −10 mV to −150 mV with −20 mV increments, followed by a 1-s interval at the holding potential.

Nitrate-elicited current voltage difference (*I-V*) curves were obtained by subtracting the currents measured before nitrate addition from those obtained during nitrate addition [5]. At a particular membrane potential, the steady-state nitrate-dependent currents were measured as a function of external nitrate concentration, and the resulting dose-response curves were fitted to a Michaelis–Menten relationship by a non-linear least squares method using the SigmaPlot software (Jandel Scientific, Germany). These fits yielded the maximal currents $i_{max}$ and the half-maximal nitrate concentrations $K_m$ [13]. The oocytes were allowed to adjust to changes in external pH ($pH_o$) for at least 5 min before obtaining *I-V* curves.

## 3. Results and discussion

Fig. 1 shows the percentage mortality of oocytes 3 days after mRNA-injection. Oocytes injected with *Nrt2;1*-mRNA and water had similar survival rates with mortality at less than 5%. However, 56% of *Nar2*-mRNA and 20% of (*Nrt2;1*+*Nar2*)-mRNA injected oocytes were dead 3 days after injection. By 5 days, all the oocytes injected with *Nar2*-mRNA were dead and 70% of those injected with the mixture of mRNAs had died. These results show that either the protein encoded by the RNA or the actual mRNA was toxic to the oocytes and this toxicity was ameliorated in oocytes co-injected with *Nrt2;1* mRNA.

Oocytes which had been injected with each of the treatments shown in Fig. 1 were assayed for nitrate transport activity by measuring nitrate-elicited currents over a range of different membrane voltages (see Section 2). Only oocytes which had been injected with a mixture of mRNAs encoding both *Nar2* and *Nrt2;1* showed any nitrate-elicited currents. Fig. 2 shows the mean voltage- and $pH_o$-dependence of the

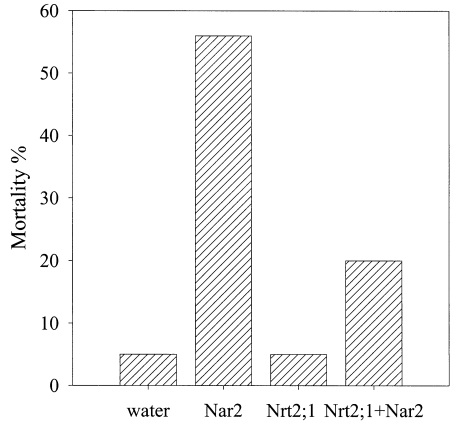


Fig. 1. Mortality of injected oocytes. The health of oocytes was assessed 3 days after injection with 50 nl of DEPC treated water, *Nar2* RNA (1 ng/nl), *Nrt2;1* RNA (1 ng/nl) or a 1:1 mixture of *Nar2* and *Nrt2;1* RNAs (2 ng/nl). The number of dead oocytes was recorded and % mortality of the total injected was calculated.

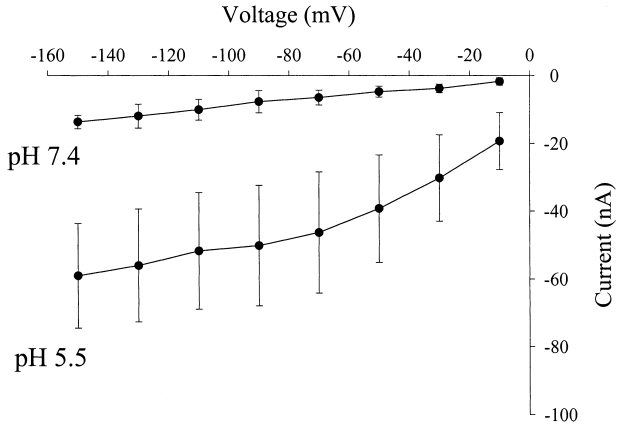


Fig. 2. Voltage- and pH-dependence of nitrate-elicited currents in oocytes injected with a mixture of *Nar2* and *Nrt2;1* mRNA. The *I-V* difference curves are shown as the means ± S.E.M. for five oocytes and nitrate was applied as the sodium salt (0.1 mM). For the details of voltage clamp conditions see Section 2.

nitrate-elicited currents for five oocytes, and there was a seven-fold increase in the magnitude of the current as the $pH_o$ was decreased from 7.4 to 5.5. This $pH_o$-dependence suggests that the transport mechanism may be by cotransport with protons, because at $pH_o$ 5.5 there is a larger proton gradient across the plasma membrane driving cotransport with nitrate. All other treatments failed to show any nitrate-elicited current at either $pH_o$ 7.4 or 5.5 (data not shown). The nitrate-elicited current increased as the membrane potential became more negative at both external pHs. At $pH_o$ of 5.5 the mean current increased from −39.2 ± 15.8 nA at −50 mV to −50.2 ± 17.8 nA at −90 mV. These nitrate-elicited currents were also measured as the external nitrate concentration was changed (Fig. 3). The $K_m$ for nitrate generated from fitting this data was voltage independent with a mean value of 28 ± 7 μM and the $i_{max}$ ($V_{max}$) was voltage-dependent and increasing from −63.7 ± 30.8 nA at −90 mV to −255.4 ± 27.5 nA at −150 mV. These experiments show that when *Xenopus* oocytes were injected with each of the single types of mRNA no nitrate transport activity could be measured. However, after coinjection of a mixture of mRNA prepared from the *Nar2* and *Nrt1;2* cDNAs gave a functional nitrate transport system. The calculated $K_m$ value is within the μM range, but is significantly higher than that obtained by in vivo assays in *Chlamydomonas* cells [10]. These different values might reflect the effects of differences in folding, glycosylation, or lipid environment on the individual proteins or their interactions when expressed in oocytes versus *Chlamydomonas*. Although in oocytes the $K_m$ for nitrate transport was independent of membrane voltage, the parameter may depend on $pH_o$. In the oocyte experiments the $K_m$ was measured at $pH_o$ 5.5, but at 7.6 in *Chlamydomonas* [10]. A plant hexose transporter expressed in oocytes showed a decrease in $K_m$ for sugar as $pH_o$ was increased [14].

There are already several examples of transport systems that require two genes for functional activity. When more than one protein or RNA species is required for activity this may be because the functional complex contains more than one protein subunit (e.g. receptors [15]); or because a second protein mediates the correct assembly of the transporter (e.g. pumps; [16]). There are also examples of two proteins interacting in the plasma membrane to modify the protein's prop-

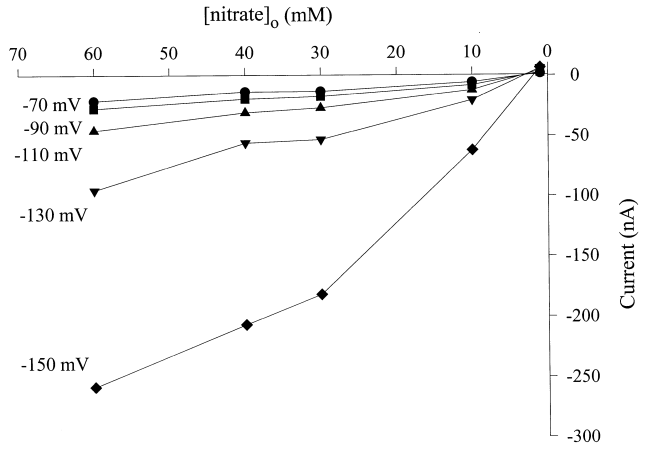


Fig. 3. Nitrate-elicited currents of *Nrt2;1* and *Nar2* expressed in *Xenopus* oocytes. The *Nrt2;1* and *Nar2* mRNA co-injected oocytes were treated with the indicated concentrations of sodium nitrate in pH 5.5 saline. The nitrate-elicited currents were used to determine *I-V* difference curves (see Section 2) at five different voltages, −70 (●), −90 (■), −110 (▲), −130 (▼) and −150 (◆) mV. The nitrate-induced current-voltage relationship was obtained by subtracting the *I-V* relations determined before nitrate addition from that obtained after nitrate addition. The voltage response curves were obtained by plotting nitrate-elicited currents against external nitrate concentrations, and fitted to the Michaelis–Menten equation at the above five different membrane voltages.

erties. For example, the activity of a $K^+$ channels was dependent on there being more than one subunit protein in the plasma membrane [17], but the properties of channels can also depend on the amount of protein in the membrane [18]. There are also examples of cotransporters and exchangers which require two proteins for their function [19,20].

The properties of G-protein-coupled receptors have been shown to be modified by the expression of a second protein, a receptor-activity modifying protein (RAMP) [21]. These RAMPs have a single transmembrane domain and they interact with receptors to modify their function. The *Nar2* gene product, which appears to have a single transmembrane domain (Lefebvre, P.L., personal communication), may even have this type of function, switching *Nrt2;1* function from some as yet unknown activity to a functional form that is able to transport nitrate.

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