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ORIGINAL ARTICLE

Second nitrate reductase of *Dunaliella salina*: functional redundancy or greater?

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Summary

During evolution, algae have retained a single assimilatory nitrate reductase (NR), which governs the reduction of nitrate to nitrite. The unicellular green alga *Dunaliella salina* is a special case, as its cells have two NRs. This makes *D. salina* attractive to study the mechanisms underlying the nitrate assimilation in photosynthetic unicellular organisms. Here we characterize the expression of the gene encoding the second NR, named as *DsaNIA1*. Low levels of mRNA for *DsaNIA1* are present in ammonia-grown cells. *DsaNIA1* is a nitrate- and nitrite-inducible gene. Using spectrofluorometric assays with NO-sensitive fluorescence dye, we demonstrate nitrite-dependent NO synthesis by *D. salina* cells. Moreover, we found that the transcription of *DsaNIA1*, but not *DsaNIA2*, is under the inducing influence of NO-dependent pathway. Together, our data argue for the two differently regulated NR isoforms in *D. salina*.

Key words: *Dunaliella salina*, nitrate reductase, nitric oxide

Introduction

Nitrate is one of the major sources of nitrogen for unicellular algae growth and development. To be used in the biosynthesis of amino acids, proteins, and other nitrogenous compounds, nitrate must be reduced to ammonium. Nitrate reduction to nitrite catalyzed by nitrate reductase (NR) is followed by nitrite reduction to ammonium.

Nitrate reductase is the first enzyme of the nitrate assimilation in algae, yeasts, fungi and plants. Assimilatory NR is a soluble, multicenter redox enzyme belonging to sulfite oxidase family that catalyzes the two-electron reduction of nitrate to nitrite using pyridine nucleotide as the electron donor (Redinbaugh and Campbell, 1985). NADH NR is the most common form in higher plants and algae, some of which also contain NAD(P)H NR, while NADPH NR occurs in fungi. NR is a homodimer with each subunit composed of about 100 kDa polypeptide and three cofactors, FAD, iron-heme (heme-Fe) and molybdenum (Mo)-pterin, in a 1:1:1 ratio (Crawford et al., 1988; Gowri and Campbell, 1989; Kinghorn and Campbell, 1989; Vaucheret et al., 1989). In photosynthetic organisms, NR is present in cytosol (Castaings et al., 2011) and is regulated by nitrate ions, light, growth conditions, hormones, reduced nitrogen metabolites as well as by phosphorylation (Kaiser and Huber, 2001; Garg, 2013; Nemie-Feyissa et al., 2013).

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Moreover, nitrate can induce gene expression and enzyme activity in fungi and plants (Crawford and Arst, 1993; Marzluf, 1993; Hoff et al., 1994). Apart from NR, nitrate regulates genes encoding nitrate transporters, nitrite reductase (NiR), glutamine synthetase, and ferredoxin-dependent glutamate synthase (Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Redinbaugh and Campbell, 1993; Hoff et al., 1994; Balotf et al., 2016).

Interestingly, many higher plant species express more than one isoform of NR (Cheng et al., 1986; Cheng et al., 1988; Wilkinson and Crawford, 1993; Horchani et al., 2011; Medina-Andrés and Lira-Ruan, 2012; Kabange et al., 2021). For plants with multiple isoforms, differences have been revealed on the level of transcription, on the protein expression level, and in the role of the isoforms in a particular function. For example, isoforms differ in their specificity for the co-substrate NADH or NADPH (Beevers et al., 1964; Dailey et al., 1982); alternatively, they are expressed in constitutive or inducible way (Wu et al., 1995).

Nitrate reductases from a number of higher plants have been characterized to varying degree, and that from *Arabidopsis thaliana* is typical (Crawford et al., 1988). *A. thaliana* has two NR genes, *AtNIA1* and *AtNIA2* (Cheng et al., 1986; Cheng et al., 1988; Wilkinson and Crawford, 1993). In spite of the fact that *AtNIA1* and *AtNIA2* demonstrate high similarity, they are regulated differentially. Notably, the basal levels of expression of *AtNIA1* and *AtNIA2* genes in the absence of NO_3- are quite different. *AtNIA2* is responsible for 90% of the total NR activity in seedlings, whereas *AtNIA1* accounts for the remaining 10% (Wilkinson and Crawford, 1991). Furthermore, the two isoforms exhibit differences in their light induction, and constant light exposure can trigger the upregulation of *AtNIA2,* but not *AtNIA1* (Jonassen et al., 2009). The differential regulation of *AtNIA1* and *AtNIA2* may be probably important for plants in their adaptation to various environments.

Apart from nitrate reduction, NR has another function in nitric oxide (NO) biosynthesis. NO is a regulator of growth, development, and stress responses in living organisms. Two main pathways of NO formation in cells have been described, an oxidative pathway involving L-arginine-dependent NO synthase (substrate L-arginine) (Corpas et al., 2009; Lapina et al., 2022), and a reductive pathway involving nitrate reductase (substrate nitrate/nitrite) (Dean and Harper, 1988; Rockel et al., 2002). NO synthase (NOS)-like proteins have not been identified in higher plants (Santolini et al., 2017). Photosynthetic organisms produce nitric oxide (NO) mainly through reductive pathways from nitrite (Hancock and Neill, 2019). The reductive pathway from nitrite requires the previous reduction of nitrate catalyzed by NAD(P)H and molybdenum (Mo)-dependent nitrate reductases (NRs) (Solomonson and Barber, 1990). NRs, as well as other molibdoproteins, can also reduce nitrite to NO (Rockel et al., 2002; Bender and Schwarz, 2018). Different isoforms of NR gene may participate differently in the biosynthesis of NO. For example, AtNIA1 is involved in the process while AtNIA2 does not have such an ability (Wilson et al., 2009).

Unlike higher plants, algae typically contain a single gene encoding NR (Fernández et al., 1989; Fernández and Galvan, 2007). In the model alga *Chlamydomonas*, NO synthesis is carried out by a dual system comprising NR and NOFNiR (mARC). These two components are located in the cytosol and are closely connected at the transcriptional and activity levels (Chamizo-Ampudia et al., 2016).

Another model alga, *Dunaliella salina*, is one of the most halotolerant photosynthetic unicellular eukaryotes. It grows well under a wide range of NaCl concentrations from 0.05 M to approximately 5.5 M solution (Sadka et al., 1991). This alga can withstand extremely harsh environments such as high light intensities, and nutrients stress (Coesel et al., 2008; Hosseini and Shariati, 2009; Lamers et al., 2012). Under conditions of abiotic stresses, *D. salina* cells can accumulate high amount of β-carotene, which is more than 14% of its dry weight. β-carotene and lutein account for 90% and 5% of total carotenoids, respectively (Prieto et al., 2011; Jayappriyan et al., 2013). Carotenoids are employed in the food, cosmetic and pharmaceutical industries as colorant, antioxidant and anti-cancer agents (Ben-Amotz and Levy, 1996; Zhu et al., 2008; Doddaiah et al., 2013). Nowadays, *D. salina* is the best commercial source of natural β-carotene among all organisms in the world (Ben-Amotz and Avron, 1983; Coesel et al., 2008; Hosseini and Shariati, 2009; Duc et al., 2014).

Dunaliella spp. can use a number of nitrogen-containing compounds as nitrogen sources, including ammonium, nitrate, nitrite and urea (Goldman and Peavey, 1979; Latorella et al., 1981; Fabregas et al., 1989; Giordano et al., 1994; Giordano, 1997; Hellio and Le Gal, 1998).

NR activity in *Dunaliella* has been studied at a number of conditions (Jimenez del Rio et al.,

1994; Giordano et al. 2000; Song and Ward, 2004). In 2007, the first protein sequence of a NR (Q7XYS2, UniProt) was identified (Li et al., 2007). Transcripts of the gene encoding nitrate reductase in *D. salina* have been shown to be induced by nitrate but repressed by ammonium (Li et al., 2007). NR activity was activated in the light upon transfer of *D. salina* cells from ammonium to nitrate medium, and both in the light and in the dark after addition of nitrate to cells in N-free medium (Jimenez del Rio et al., 1994). However, the genome of *D. salina* has a second gene for NR (Lao et al., 2014). Compared to the characterized enzyme Q7XYS2, the second NR has been poorly studied. Up to now, the question of whether unicellular algae can use two NRs has not been experimentally explored. Therefore, *D. salina* is an attractive model system for exploring the evolutionary pressure to maintain two enzymes in the one cell. The present study is the first to address expression of the second NR in the model unicellular alga *D. salina*.

Material and methods

Strain and growth conditions

D. salina IBSS-2 were obtained from A.B. Borovkin (Hydrobionts collection of the World Ocean, A.O. Kovalevsky Institute of Biology of the Southern Seas of RAS). Cells were grown in modified Johnsons medium (Sathasivam and Juntawong, 2013) under continuous illumination by white light at 22 °C with a constant orbital agitation at 90 rpm. At each harvesting time, the number of cells was measured employing a counting chamber. The number of viable cells was counted microscopically using 0.05% (v/v) Evans blue (Dia-M, Moscow, Russia), as previously described (Lapina et al., 2022). The numbers of non-viable (stained) and viable (unstained) cells were determined. Depending on the nitrogen source, four variants of the cultivation medium were used: with 5 mM NH_4, 6 mM urea, 5 mM KNO_3, and 10 mM KNO_2.

To change a nitrogen source, cells in the logarithmic phase of growth at a density of 1.5–2 10^6 ml^{-1} cells were sedimented by centrifugation (2,000g, 5 min) and resuspended in corresponding medium at the same cell density.

2-(N,N diethylamino)-diazenolate 2-oxide sodium salt (DEA-NONOate, Sigma-Aldrich, USA) was used at a final concentration of 100 μM.

Quantitative real-time PCR

The total RNA was isolated with Trizol according to the manufacturer's instructions (Invitrogen, USA). DNA contamination was avoided by treatment of the RNA samples with RNase-Free DNase I (ThermoFisher Scientific, Lithuania). Reverse transcription was performed with Revert Aid H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific, USA). Gene expression analysis was carried out by real-time quantitative RT-PCR (RT-qPCR) on the QuantStudio™ 5 Dx Real-Time PCR System (Applied Biosystems, USA) using SYBR Green I following a previously reported protocol (Zalutskaya et al., 2016). The primer pairs used for RT-qPCR were as follows: 5'-CGATGCCGTCTGTGACCCTC-3' and 5'-TG TCACACAACCGCACTCCC-3' for *DsaNIA1*, 5'-ATGCCCGCACTCGCCAACAA-3' and 5'-CATTCACGGTGGAAGCAG-3' for *DsaNIA2* (Gao et al., 2015), 5'-CCATCACCATCGGCA ACG-3' and 5'-GTCGGCAATA CCATGGGA ACA-3' for β-actin gene (Gao et al., 2015). The relative gene expression ratios were normalized with β-actin gene using the ΔCT method (Livak and Schmittgen, 2001). Values were obtained from at least three biological replicates; each replicate was analyzed three times.

Measurement of NO

D. salina cells (10^6 ml^{-1}) were incubated in the growth medium in the presence of 1 μM (4-amino-5-methylamino-2'7'-difluorofluorescein diacetate) dye (DAF-FM DA, Sigma-Aldrich, USA). After 15 min of treatment, the cells were washed, resuspended in the same medium, incubated for additional 30 min to allow complete de-esterification of the intracellular diacetates, and then intracellular generation of NO was evaluated using a microplate reader CLARIOstar (BMG, Germany). Excitation and emission wavelengths were set at 483±14 and 530±30 nm, respectively. Cells autofluorescence was subtracted from the total fluorescence obtained. Fluorescence levels were expressed as arbitrary units (a.u.) per 10^6 cells. Three technical replicates per condition were included on each plate, and

each experiment was performed three times independently.

Western blotting

The protein content was determined with amido black staining. After separation by SDS-PAGE on a 10% polyacrylamide gel (w/v), the proteins were transferred to nitrocellulose membranes (Carl Roth, Karlsruhe) with use of semidry blotting (Trans-Blot Turbo Transfer System, BioRad, USA). The dilution 1:5,000 anti-NR of the primary antibodies was used (Agrisera, Sweden, Cat# AS08 310). As a secondary antibody, the horseradish peroxidase-conjugated anti-rabbit serum (Sigma, USA) was used at a dilution of 1:10,000. The peroxidase activity was detected with Clarity™ Western ECL Substrate (BioRad, USA).

Enzymatic assay for nitrate reductase activity

NR activity was determined as described previously (Minaeva et al., 2017). NR was assayed by measuring the formation of nitrite from added nitrate and NADH in an incubation mixture containing in 1 ml: 60 mM potassium phosphate (pH 7.5), 50 mM KNO_3, 0.1 mM $NADH_2$, and 0.1 ml sample. Before addition of the other chemicals, cells were lysed with 5% toluene. 1 min before starting the NR activity measurements, 1 mM of the electron acceptor ferricyanide 1% (w/v) was added to activate the enzyme. After incubation for 30 min at 30°C the reaction was stopped by boiling (1 min), and the mixture was cleared by centrifugation (27,000g). For the determination of nitrite the supernatant was mixed with 1 ml sulfanilamide (Sigma-Aldrich, USA) in 2 N HCI and 0.2% (w/v) N-(l-naphthyl)ethylenediamine (NNEDA, Sigma-Aldrich, USA). The absorption of the resulting violet color was measured at 540 nm against a blank. Nitrite concentrations in probes were determined using a calibration curve using KNO_2 as a standard. Initial nitrite concentration in probes where reaction was stopped by boiling immediately after addition of incubation mixture were subtracted from each probe.

Statistical analysis

The values for the quantitative experiments described above were obtained from at least three independent experiments with no fewer than three technical replicates. Data represent the mean ± SE. When necessary, statistical analyses were followed by a Student's t test (p value < 0.05).

Results

The second *D. salina* NR is a canonical plant nitrate reductase

The predicted full-length of the L7X5W3 polypeptide consists of 889 amino acids with a calculated molecular weight of 98375 Da. The conducted bioinformatics analysis showed that the amino acid sequence of NR L7X5W3, (UniProt, Fig. 1) from *D. salina* contains all highly conserved regions, including the N-terminal oxidoreductase molybdopterin binding domain located at the 123–301 amino acid position, Mo-Co oxidoreductase dimerisation domain located at 329–457 amino acid position, the cytochrome b5-like Heme/steroid binding domain situated at the 537–607 amino acid position, oxidoreductase FAD-binding domain at the 639-745 amino acid position and the C-terminal oxidoreductase NADH binding domain situated at the 765-861 amino acid position analyzed by BlastP alignment (Fig. 1, A). Protein sequence demonstrates 73.28% identity with the previously identified protein Q7XYS2 (UniProt, Li et al., 2007, Fig. 1, B).

DsaNIA1 is repressed by ammonium

Since the second identified NR, L7X5W3 protein, demonstrated high level of sequence identity with Q7XYS (Fig. 1), the question arose about expression of the L7X5W3 in media with different nitrogen sources. To study this, we transferred *D. salina* cells grown in nitrate-containing medium into ammonium-containing medium. The gene encoding L7X5W3 protein was expressed in nitrate-containing medium, and the levels of its transcript significantly exceeded the levels of mRNA of previously characterized gene (Fig. 2). Considering this, we designated the gene encoding L7X5W3 as *DsaNIA1*, and the gene encoding Q7XYS2 as *DsaNIA2*.

Expression of *DsaNIA1* markedly decreased after transfer to ammonium-containing medium, by 30% after 0.5 h and by 80% after 6 hours in ammonium-containing medium (Fig. 2). Expression of *DsaNIA2* was also downregulated by ammonium; however,

Fig. 1. Structure of DsaNIA1. A – Functional domains of DsaNIA1. The regions referring to molybdopterin binding domain, Mo-Co oxidoreductase dimerisation domain, cytochrome b5-like Heme/steroid binding domain, oxidoreductase FAD-binding domain, and oxidoreductase NADH binding domain are indicated as 1, 2, 3, 4 and 5; B – multiple amino acid sequence alignment of NRs. The protein sequences were derived from NCBI database. The sequences are derived from NR polypeptides of the *D. salina* (DsNIA1; L7X5W3 and DsNIA2; Q7XYS2), and *A. thaliana* (AtNIA1; P11832 and AtNIA2; P11035). The alignment was done using the ClustalW program and manually refined. Functional domains are colored as in A.

to a lesser extent. It decreased by about 50% after 6 hours after transfer to ammonium.

Nitrate-inducible expression of *DsaNIA1*

To study activation of NR in *D. salina* IBSS-2 by nitrate, we assessed enzymatic NR activity upon transfer of cells grown in medium containing urea as a nitrogen source to nitrate-containing medium. As shown in Suppl. Fig 1, after 1 hour of exposure to nitrate, NR started activating and after four hours reached 8-fold increase.

D. salina are able to grow in a broad range of NaCl concentration. To choose optimal conditions for studying NR activity and expression, we analyzed growth of *D. salina* IBSS-2 cells in media supplemented with different nitrogen sources and NaCl concentrations. As it is shown in Fig. 3 (A), *D. salina* strain IBSS-2 demonstrated the most optimal growth in the interval of NaCl concentrations of 0.5–1.5 M. These conditions were chosen for further comparative analysis of *DsaNIA1* and *DsaNIA2* expression. For all conditions studied, relative steady-state expression of both genes was stronger at

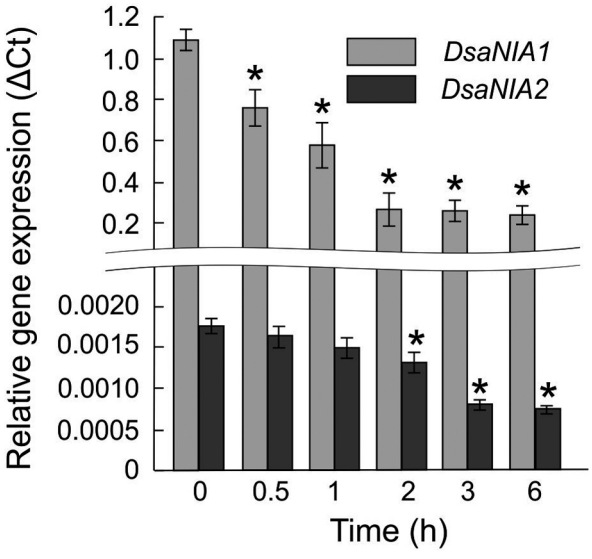

Fig. 2. Effects of ammonium on *DsaNIA1* and *DsaNIA2* expression. Cells were grown on nitrate and transferred to ammonium-containing medium at time point 0. * denotes significant differences between the control at time 0 and test variants according to the Student's t test ($p < 0.05$).

higher concentration of NaCl (Fig. 3, B). This may reflect a possible way of adaptation of *Dunaliella* cells to high salinity. The expression of both *NIA* genes was upregulated when cells were grown on nitrate comparing to growth on urea at both concentrations of NaCl.

Moreover, NR protein levels were higher in nitrate than in urea-containing medium, and the increase was more pronounced at higher salt concentration (Fig. 3, C). In addition, cells grown in nitrate demonstrated levels of NR activity significantly higher than in urea, and NR activity correlated with protein level in media with different nitrogen sources (Fig. 3, D).

Nitrite increases expression of DsaNIA1

Given that nitrite is a product of NR, we wondered whether NO_2 could control the enzyme activity. Unexpectedly, NR activity started growing immediately after exposure of cells to nitrite, and after 2 h reached about 20-fold increase comparing to urea-containing medium (Fig. 4, A). To elucidate how NR activity is controlled by different nitrogen sources, we studied transcription of *DsaNIA1* and *DsaNIA2* genes in cells grown in urea or nitrite at different concentrations of NaCl (Fig. 4, B). Similar to cells grown in nitrate, the levels of *DsaNIA1* expression were higher at 1.5 M than at 0.5M NaCl in nitrite. Upregulation of *DsaNIA1* was registered in nitrite-grown cells compared to urea-grown cells at 0.5 M NaCl, and the increase was more pronounced than in nitrate-containing medium (Fig. 3, B). In contrast, *DsaNIA2* expression could not be registered in nitrite-containing medium.

As shown in Fig. 4 (C), NR protein content was higher in nitrite-containing medium at 1.5 M NaCl than at 0.5 M NaCl. In nitrite-grown cells, NR protein content was increased comparing to urea-grown cells at both NaCl concentrations. NR activity (Fig. 4, D) was correlated with protein content (Fig. 4, C) and was significantly enhanced in nitrite-grown cells at both NaCl concentrations.

We suggest that the observed effects of nitrite may be indirect, caused by NO generation. However, the question of whether NO synthesis occurs in *D. salina* cells has never been explored experimentally. Therefore, we analyzed the potential role of nitrite in NO formation in this alga. As shown in Fig. 5 (A), after 15 min in nitrite-containing medium, NO level started increasing, and after 1 h reached 4-fold increase.

To test whether the expression of *DsaNIA1* is controlled by nitric oxide generated in nitrite-containing medium, we used NO-donor (DEA NONOate) (Fig. 5, B). As shown in Fig. 5 (C), NO strongly increased the *DsaNIA1* transcription. Notably, the pattern of NO-dependent regulation was similar to that of nitrite-dependent control. At the same time, transcription of *DsaNIA2* (Fig. 5, C) was downregulated, and after 1 h of incubation with DEA NONOate, the level of *DsaNIA2* transcription decreased 2.5-fold. Finally, we assessed NR activity in cells after incubation with DEA NONOate (Fig. 5, D). NR activity increased 1.5-fold after 1h of incubation with NO generator.

Discussion

The reduction of nitrate to nitrite is the rate-limiting step of the nitrate assimilation and utilization. Nitrate is a substrate of NR, and in many organisms is known to regulate NR activity and expression (Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Hoff et al., 1994, Balotf et al., 2016). Therefore, the regulation of NR is important for organism' development and growth. Unicellular algae use a single NR for nitrate assimilation (Fernández et al., 1989; Fernández and Galván, 2007). In contrast, the model alga *D. salina* has two

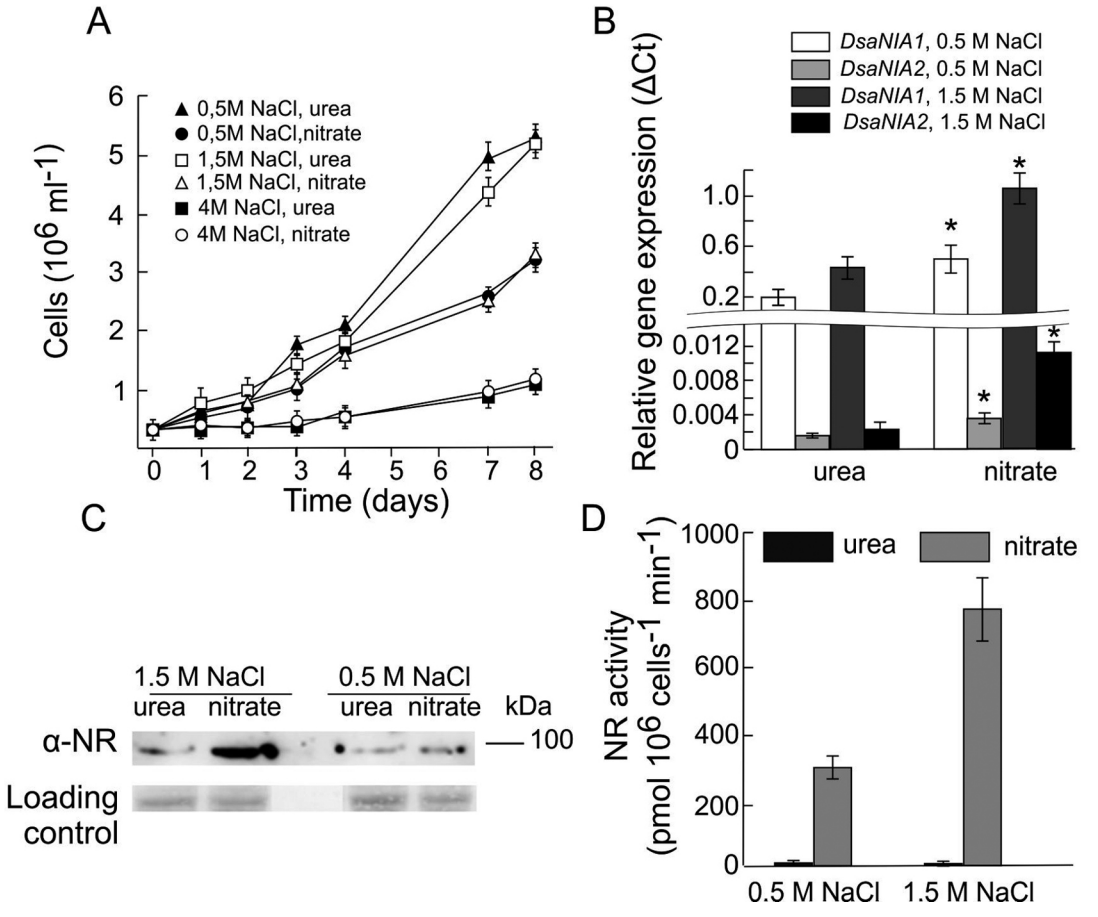

Fig. 3. Effects of nitrate on growth, gene expression, protein abundance and NR activity in *D. salina*. A – Growth of cells with urea and nitrate as nitrogen sources at 0.5 M, 1.5 M and 4 M NaCl. Cell number was analyzed at the indicated times for cultures growing continuously in the light. Data are the means ±SE from three independent experiments; B – relative expression of *DsaNIA1* and *DsaNIA2* genes in urea- and nitrate containing media. Data are the means ± SE from three biological and three technical replicates obtained by RT-qPCR; * denotes significant differences between variants in urea and nitrate according to the Student's t test ($p < 0.05$); C – NR protein abundance in cells grown in urea and nitrate. Each line corresponds to 50 μg of soluble proteins extracted from samples taken from cultures at the time points indicated. Protein loading was normalized by Ponceau staining; D – NR activity in cells grown in urea and nitrate. Data are the means ±SE from three independent experiments.

different isoforms of NR. In this work, we report original insights into expression of the second NR in cells of this halophilic alga.

Multiple sequence alignment of NR sequences shows that all domains in DsaNIA1 are perfectly conserved (Fig. 1). In spite of high identity between two NRs of *D. salina* (73.28%), the transcript levels of *DsaNIA1* were significantly higher than that of *DsaNIA2* (Figs 2–4), hinting on potentially higher abundance of the DsaNIA1. In *Arabidopsis*, AtNIA1 and AtNIA2 also contribute differently to NR activity at different conditions (Cheng et al., 1991; Yu et al., 1998; Konishi and Yanagisawa, 2011).

NR in green algae and diatoms is regulated at the transcriptional level by changing the nitrogen sources in the media. In general, nitrate and ammonium have opposite effects over nitrate assimilation genes. As in many algal species (Fernández and Cardenas, 1982; Fernández et al., 1989; Loppes et al., 1999; Cannons and Shiflett, 2001; Llamas et al., 2002; Imamura et al., 2010), *DsaNIA1* gene is induced in nitrate-containing medium and strongly repressed in ammonium medium (Fig. 2).

In higher plants, nitrite induces activity of NR (Aslam et al., 1987; Aslam and Huffaker, 1989; Lips et al., 1993; VIegas and Silveira, 2002). In contrast

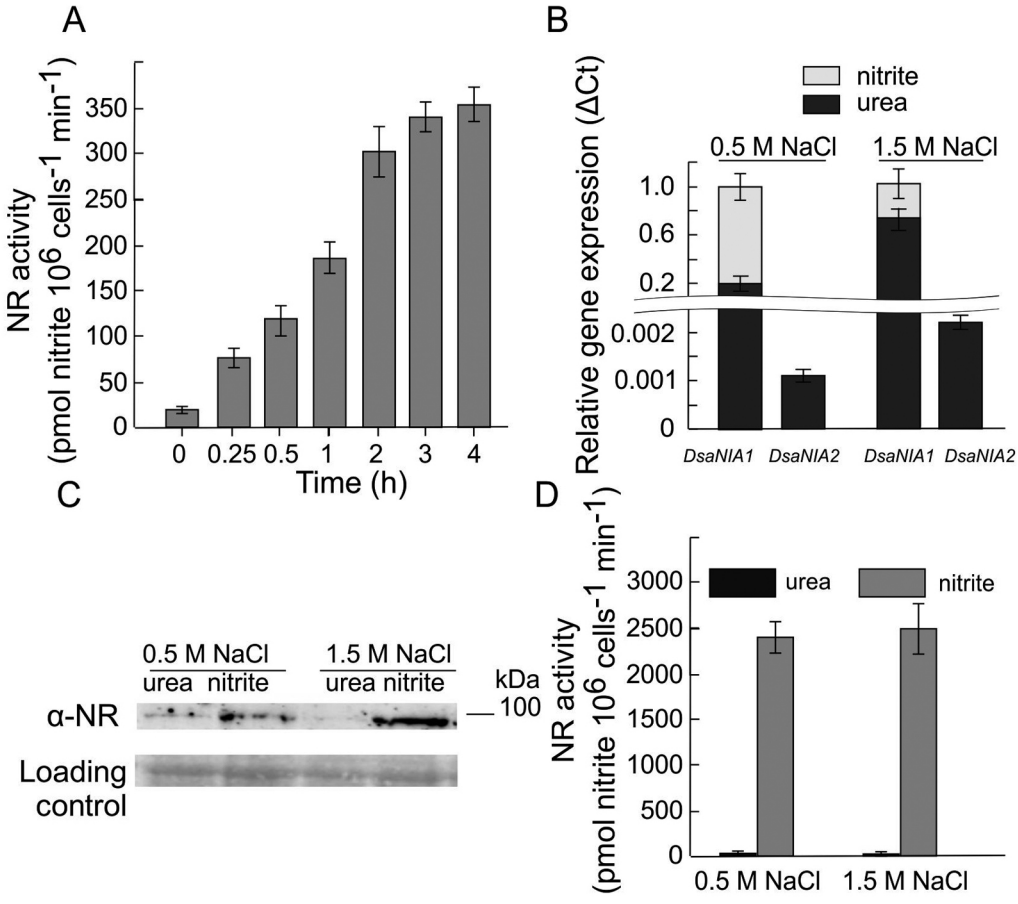

Fig. 4. Effects of nitrite on NR activity, gene expression and protein abundance in *D. salina*. A – NR activity in cells grown in urea and transferred to nitrite at time point 0. Data are the means ±SE from three independent experiments; B – relative expression of *DsaNIA1* and *DsaNIA2* genes in cells grown in urea- and nitrite containing media. Data are the means ± SE from three biological and three technical replicates obtained by RT-qPCR; C – NR protein abundance in cells grown in urea and nitrite. Each line corresponds to 50 μg of soluble proteins extracted from samples taken from cultures at the time points indicated. Protein loading was normalized by Ponceau staining; D – NR activity in cells grown in urea and nitrite. Data are the means ±SE from three independent experiments.

to *DsaNIA2*, expression of *DsaNIA1* was upregulated in cells grown in nitrite-containing medium (Fig. 4), suggesting that NR activity in this medium appears to be performed mainly by DsaNIA1. Here, one scenario would be that nitrite triggers NR expression through NO generation, which, via a signaling cascade, finally induces *DsaNIA1* gene.

We demonstrate that the incubation in nitrite induced the rapid formation of NO in *D. salina* (Fig. 5, A). Given that we observed an induction in the *DsaNIA1* transcript levels by NO generator, DEA NONOate (Fig. 5, B), NO-dependent pathway might be used to regulate the transcription of this gene in nitrite-containing medium. NO is an important signal molecule in many biological plant processes including growth, metabolism, development, and defense processes (Corpas, 2004; He et al., 2004; Neill et al., 2008; de Montaigu et al., 2010; Fernández-Marcos et al., 2011; Yun et al., 2011). Moreover, we still cannot rule out the role of NO in post-translation regulation of DsaNR.

In conclusion, characterization of the DsaNIA1 control expands our understanding of the regulatory aspects of nitrate assimilation in unicellular organisms. We showed that NO is an integral part of DsaNIA1 regulation. This study provides a basis for further research on elucidating the role of two nitrate reductases in one cell.

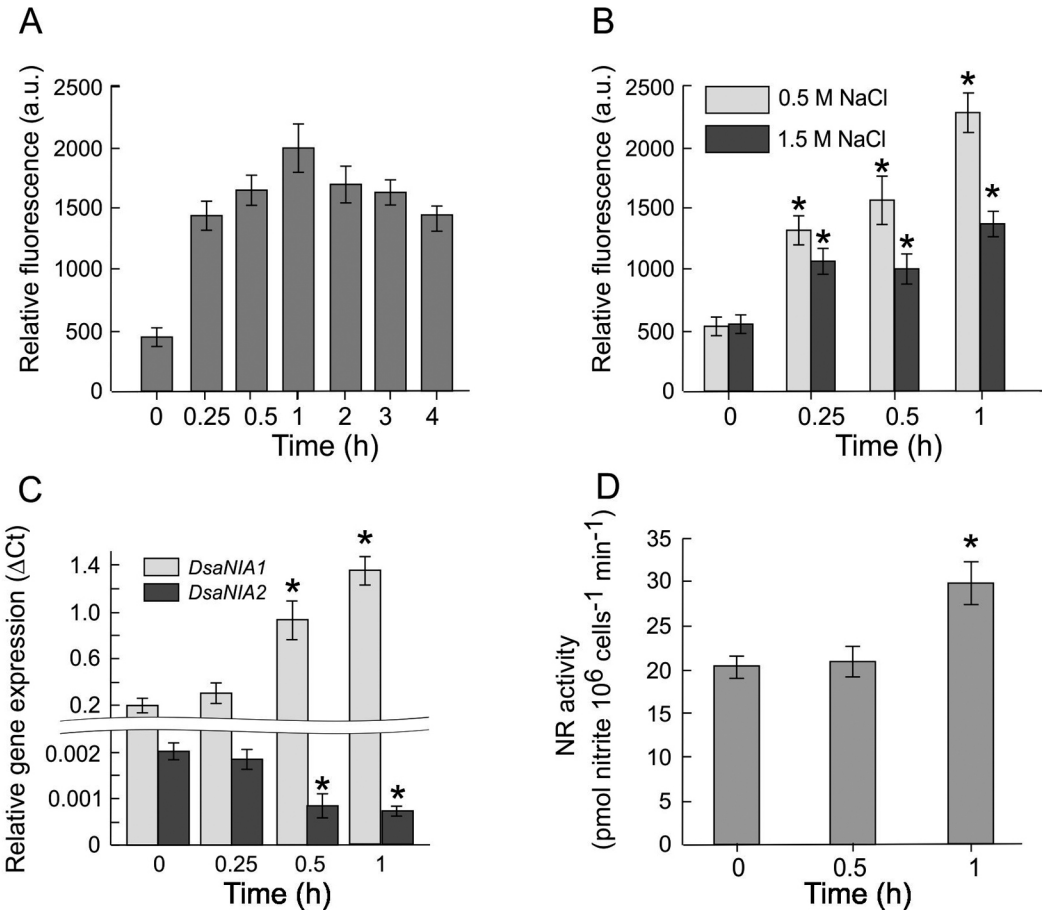

Fig. 5. NO generation and its effects on NR gene expression and enzyme activity. A – Intracellular NO levels. Cells were grown in urea and transferred to nitrite-containing medium at time point 0. Fluorescence intensity due to intracellular NO was determined using DAF-FM DA and is expressed as arbitrary units per 10^6 cells; B – effects of DEA NONOate on NO levels; * denotes significant differences between the control at time 0 and test variants with DEA NONOate according to the Student's t test ($p < 0.01$); C – effects of DEA NONOate on the expression of *DsaNIA1* and *DsaNIA2*. Data are the means ± SE from three biological and three technical replicates obtained by RT-qPCR; * denotes significant differences between the control at time 0 and test variants according to the Student's t test ($p < 0.05$); D – effects of DEA NONOate on NR activity. Data are the means ±SE from three independent experiments. 100 μM DEA NONOate was added to urea-grown cells at time point 0. * denotes significant difference between the control at time 0 and test variant with DEA NONOate according to the Student's t test ($p < 0.05$).

Acknowledgments

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Supplementary materials

Fig. S1. Effect of nitrate on NR activity in *Dunaliella salina*.