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ORIGINAL ARTICLE

Control of arginine biosynthesis in the green alga *Dunaliella salina*

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Summary

Until now, the question of how arginine (Arg) biosynthesis operates in oxygenic phototrophs at high salt concentrations has not been addressed experimentally. This makes a green alga *Dunaliella salina* attractive for studying the molecular events underlying the adjustment of Arg metabolism to high salinity. Enzyme assay and Western blotting show that N-acetyl-L-glutamate kinase (NAGK) activity decreases with increasing salinity and DsPII expression in cells is impaired. Using GC-MS analysis, we also identified that the resulting levels of free Arg are dependent on ornithine flux to putrescine and proline. These observations suggest that NAGKs may have an alternative common PII-independent mode of activation, raising important questions about the relationship between NAGK control and the development of salinity tolerance during evolution.

Key words: arginine, *Dunaliella salina*, N-acetyl-L-glutamate kinase, salinity tolerance

Introduction

Arginine metabolism has been one of the key catabolic and anabolic processes through the evolution of the adaptive responses of both animals and plants (Llácer et al., 2008; Winter et al., 2015; Rodriguez et al., 2017). Among the 21 proteinogenic amino acids, arginine (Arg) has the highest nitrogen to carbon ratio, which makes it especially suitable as a storage form of organic nitrogen.

In plants, arginine regulates its own synthesis via allosteric inhibition of the N-acetyl-L-glutamyl-phosphate formation (Llácer et al., 2008). This rate-limiting step in the ornithine/arginine biosynthesis pathway is catalyzed by N-acetyl-L-glutamate kinase (NAGK), which phosphorylates N-acetyl-L-glutamate to N-acetyl-L-glutamyl-phosphate (Fig. 1; Selim et al., 2020a). In Cyanobacteria and Archaeplastida, NAGK is the target of PII, a signaling protein, which orchestrates metabolic adaptations to nitrogen/carbon abundance (Selim et al., 2020b; Forchhammer et al., 2022). By binding to NAGK when ammonia is abundant, PII relieves NAGK from arginine inhibition and allows nitrogen to accumulate as arginine (Sugiyama et al., 2004; Chen et al., 2006; Beez et al., 2009; Lapina et al., 2018).

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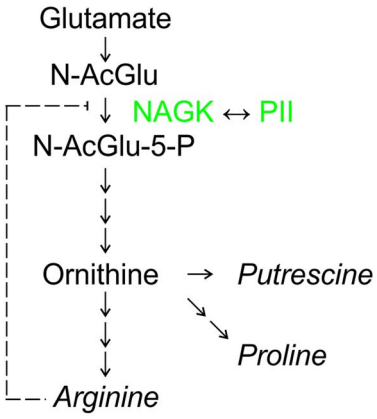

Fig. 1. Abbreviated pathway for the biosynthesis of arginine in *Dunaliella salina* in its connection to the formation of putrescine and proline from ornithine. Dotted blunted line symbolizes the inhibition of NAGK by arginine. NAGK/PII double-headed arrow denotes the regulatory interaction of NAGK and PII proteins in Cyanobacteria and Archaeplastida (Selim et al., 2020a). N-AcGlu, N-acetylglutamate; N-AcGlu-5-P, N-acetylglutamyl-5-phosphate.

Besides protein synthesis, a number of distinct metabolic pathways catabolize Arg, thus serving different functions that frequently are concomitantly active in the same cell. In plants, Arg serves as a precursor for ornithine, proline and polyamines (Majumdar et al., 2016). Animal-type nitric oxide synthases (NOS), which generate nitric oxide (NO) and citrulline from Arg, function only in some algae (Foresi et al., 2010; Jeandroz et al., 2016; Ermilova, 2023).

Among the green algae, *Dunaliella salina* (*Dunaliella hereinafter*) has become a good model system for revealing important facts about the regulation of salt tolerance in the halophytes. Progress has been made in characterizing genes encoding components involved in salt stress response, with an emphasis on transport mechanisms, glycerol and carotenoid metabolism and photosynthetic apparatus remodeling (Polle et al., 2020; Ramachandran et al., 2023). Interestingly, unlike other green algae that use a single nitrate reductase (NR) to assimilate nitrate (Fernández and Galván, 2007), *Dunaliella* has two differently regulated NR isoforms depending on salinity (Zalutskaya et al., 2023), which indicates adaptation of N metabolism to salt stress. Therefore, *Dunaliella* is an attractive model system for exploring the adjustment of N metabolism to high salinity.

Until now, the question of how Arg biosynthesis operates in *Dunaliella* at high salt concentrations has not been addressed experimentally. The apparent gap in information about the mechanisms involved in the control of Arg levels in *Dunaliella* prompted us to investigate the regulation of NAGK activity in cells, which is a key step in the arginine biosynthesis. The present study is the first to address the control of NAGK activity and arginine levels in the halophilic alga, where the signal protein PII is impaired and appeared not involved in the regulatory network.

Material and methods

Strains and cultivation conditions

The *D. salina* strain IBSS-2 was kindly provided by Prof. A. Borovkin (Hydrobionts collection of the World Ocean, A.O. Kovalevsky Institute of Biology of the Southern Seas of RAS). Cells were grown in modified Johnson's medium (Sathasivam and Juntawong, 2013) supplemented with 0.1 M, 1.5 M or 2.5 M of NaCl. The 6 mM urea was used as a nitrogen source. Alga was cultivated in 250 ml flasks in a chamber (KBWF 240, Binder GmbH, Tuttlinger, Germany) at 22 °C under continuous illumination by white light (effluence rate of 45 photons μmol m-2 s-1) with continuous agitation (90 rpm).

At each harvesting time, the number of cells was recorded using a counting chamber. Four hundred cells from each sample were scored for three biological replicates. The number of viable cells was counted microscopically with the use of 0.05% (v/v) Evans blue (Dia-M, Moscow, Russia) as described previously (Baker and Mock, 1994). The number of non-viable (stained) and viable (unstained) cells were determined.

Chlamydomonas reinhardtii strain CC3491, obta-ined from *Chlamydomonas* Resource Center (University of Minnesota, St. Paul, MN, USA), was used as a control in Western blotting. *Chlamydomonas* cells were grown in Tris-acetate-phosphate (TAP) medium (http:// www.chlamy. org/ TAP. html) under continuous illumination by white light (effluence rate of 45 μmol m-2 s-1) at 22 °C with a constant orbital agitation at 90 rpm.

NAGK assays *in vivo*

To determine NAGK activity, *Dunaliella* (2×10^6 cells ml^{-1} in 100 ml) grown at various salinities was used. Cells were harvested from lag, log or stationary phase of growth by centrifuging them at

5000 g for 5 min, and after resuspending in 150 µl of buffer, pH 7.4 (50 mM Tris-Cl, 0.5 mM EDTA, 1 mM DTT and 0.5 mM benzamidine), they were disrupted by glass beads (0.45 mm diameter) using disintegrator (Minilys, Bertin technologies, Montigny-le-Bretonneux, France). The suspension was centrifuged at 20000 g for 15 min at 4 °C, and the resulting supernatant was used as a source of enzyme. All the operations were performed at 0 °C. Protein concentration was assessed by the Pierce™ BCA protein assay kit (No 23227, Thermo Fisher Scientific, Rockford, USA).

The NAGK activity was measured as described earlier (Haas et al., 1995; Heinrich et al., 2004). Briefly, freshly prepared protein extracts (0.4 mg) were added to a reaction mixture (400 µl) containing 400 mM $NH_2OH \cdot HCl$, 400 mM Tris (base), 20 mM $MgCl_2$ and 10 mM ATP. The reaction was started by adding 40 mM N-acetyl-L-glutamate. After incubation at 37 °C for 1 h, the reaction was stopped by the addition of 400 µl of a solution containing 5% (w/v) $FeCl_3 \cdot 6\ H_2O$, 8% (w/v) trichloroacetic acid and 0.3 M HCl. Blank reactions were performed by omitting N-acetyl-glutamate from the assay. The activity was determined spectrophotometrically following the hydroxamate Fe^{3+} complex formation using a molar extinction coefficient 456/M cm at 510 nm. One unit of NAGK refers to the amount of enzyme required to catalyze the conversion of 1 µmol of N-acetyl-glutamate/min. The specific activity of DsNAGK was expressed as units per mg of protein. Measurements were carried out on at least three separate cultures (biological repeats).

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 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 relative gene expression ratios were normalized with β-actin gene using the ΔΔCT method (Livak and Schmittgen, 2001). The primer pairs used for RT-qPCR were as follows: 5'- CCAGGGCGTCACACACAACT -3' and 5'- TCCATGAACGAGGACGCAGC -3' for Dusal.0374s00005, 5'-GCGCATCTGGCAAGAA AGCC -3' and 5'- TTGTAGCCCTGCTCCCC TGT -3' for ArgJ, and 5'- ACCACACCTTCT TCAACGA -3' and 5'-GGATGGCTACATACAT GGCA for β-actin. Values were obtained from at least three biological replicates; each replicate was analyzed three times.

Protein isolation, SDS-PAGE and Western-blotting

Dunaliella (1–2 × 10^6 cells ml^{-1} in 100 ml) and *C. reinhardtii* CC3491 (2–4 × 10^6 cells ml^{-1} in 100 ml) were collected by centrifugation (3000 g, 5 min) and resuspended in 0.1M DTT, 0.1M Na_2CO_3. Then, 0.66 vol of 5% SDS, 30% sucrose was added and suspension was boiled in a water bath for 45 s. The protein concentration was determined by staining with amido black, using BSA as a standard (Popov et al., 1975). After separation of the proteins by SDS-PAGE on a 12% polyacrylamide gel (Laemmli, 1970), they were transferred to nitrocellulose membranes (Carl Roth, Karlsruhe, Germany) by a semidry blotting (Trans-blot SD, Bio-Rad, Bio-Rad Laboratories, Geylang, Singapore). Blots were blocked in 5% non-fat dry milk in Tris-buffered saline solution with 0.1% Tween 20 for 1 h, prior to an incubation in the presence of anti CrPII primary antibodies (1:4,000) (Ermilova et al., 2013). 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).

Determination of Arg and Pro contents

To determine intracellular levels of Arg and Pro, the cells (2 × 10^6 cells ml^{-1} in 100 ml) were pelleted (4000 g, 5 min) and resuspended in 250 µl of MilliQ H2O for 20 min at 95 °C; the supernatant was analyzed after centrifugation (12,000 g, 15 min, 4 °C). Total free Arg was measured as described previously (Sakaguchi, 1950; Vlasova et al., 2023). Briefly, 40 µl of 0.2% 8-hydroxyquinoline and 40 µl of 2 M NaOH were added to 200 µl of the sample, and the reaction mixture was incubated for 10 min on ice. After adding of 40 µl of 19% sodium hypochlorite and vortexing for 30 s, the reaction was stopped with 40 µl of 40% urea. The absorbance was estimated at 500 nm.

Intracellular Pro content was measured as described earlier (Bates et al., 1973). Briefly, 0.16 ml of 5% ninhydrin in 60% acetic acid and 20% ethanol was added to 0.08 ml of the sample and the reaction mixture was incubated for 20 min at 95 °C. Absorbance was measured at 520 nm. All measurements were performed at least in three biological replicates, each analyzed in triplicate.

Determination of Put and Glu content

The measurement and calculations of Put and Glu were conducted as described previously (Zalutsakay et al., 2020). Briefly, 5×10^7 cells were collected for these assays by gentle centrifugation at 4000 g, and each pellet was immediately frozen with liquid nitrogen. The metabolites were extracted and derivatized as previously reported (Puzanskiy et al., 2017). GC-MS analysis was performed by an Agilent 6850 chromatograph (Agilent Technologies, Santa Clara, CA, USA) with an Agilent 5975 mass selective detector. Separation was performed on an HP-5ms column. For accurate target peak identification, standards of Put and Glu (Sigma-Aldrich, St. Louis, MO, USA) were analyzed. Arbitrary quantification was conducted as the normalization of the metabolite peak area by the internal standard (tricosane) peak area. Then, calibration with the putrescine standard was conducted for absolute quantification. Spectra and peak areas were extracted by PARADISE software (Johnsen et al., 2017). Statistical processing was performed in the language environment R 4.1.0 "Camp Pontanezen" (R Core Team 2021).

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 are presented as means ± standard error (SE). When necessary, statistical analyses were followed by the Student's t test ($p < 0.01$).

Results

DsNAKG activity and expression under different salinities

There is evidence that the activity of *Chlamydomonas* NAGK is dependent on the growth phase (Vlasova et al., 2023). We wanted to find out whether the DsNAGK activity also depends on the phase of cell growth. However, *Dunaliella* is able to grow in a broad range of NaCl concentration. To select optimal conditions for studying the activity of NAGK in vivo, we analyzed the growth of *Dunaliella* IBSS-2 cells in the media supplemented with urea as a nitrogen source and various concentrations of NaCl. As shown in Fig. 2 (A), the strain IBSS-2 demonstrated the most optimal growth in the interval of NaCl concentrations of 0.1–2.5 M. These conditions were chosen for further comparative analysis of enzyme activity.

The maximum NAGK activity was observed in cells grown in a medium with 0.1 M NaCl (Fig. 2, B). The enzyme activity increased slightly at the log phase, and then remained at the same level even after entering the stationary phase. When NaCl concentration was increased to 1.5 or 2.5 M NaCl, the enzyme activity at these salt concentrations decreased by 32-14% compared to 0.1 M NaCl. At the same time, there was no significant difference in NAGK activity in *Dunaliella* taken from the media with 1.5 and 2.5 M NaCl (Fig. 2, B). In addition, in both media the enzyme activity was independent of the growth phase. Moreover, there was no obvious difference in the expression of the gene of interest between cultures grown in 1.5 and 2.5 M NaCl. The relative expression level of Dusal.0374s00005.1 was higher in cells from 0.1 M NaCl-supplemented medium compared to those from 1.5 M- or 2.5 M NaCl-supplemented media in log- and stationary phases of growth. Therefore, a decrease in NAGK activity with increasing salinity may be due, at least in part, to a reduction in this gene transcription (Fig. 2, C).

Notably, a rise in enzyme activity in log-phase of cells in 0.1 M NaCl did not correlate with the gene expression (Fig. 2, C, D), hinting the involvement of post-transcriptional control. Since glutamate (Glu) is the precursor of N-acetyl-L-glutamate (NAG), which activates NAGKs, we assumed that Glu levels might differ in *Dunaliella* at different salinities. Indeed, cells exhibited a significantly higher Glu content when grown at 0.1M NaCl than at 1.5 M or 2.5M NaCl (Fig. 2, D). Interestingly, the expression of the gene Dusal.0079s00017 for ArgJ (N-acetylornithine:glutamate acetyltransferase) regenerating NAG (Slocum, 2005) was reduced in 1.5 M and 2.5 M NaCl compared to 0.1 M NaCl (Fig. 2, E).

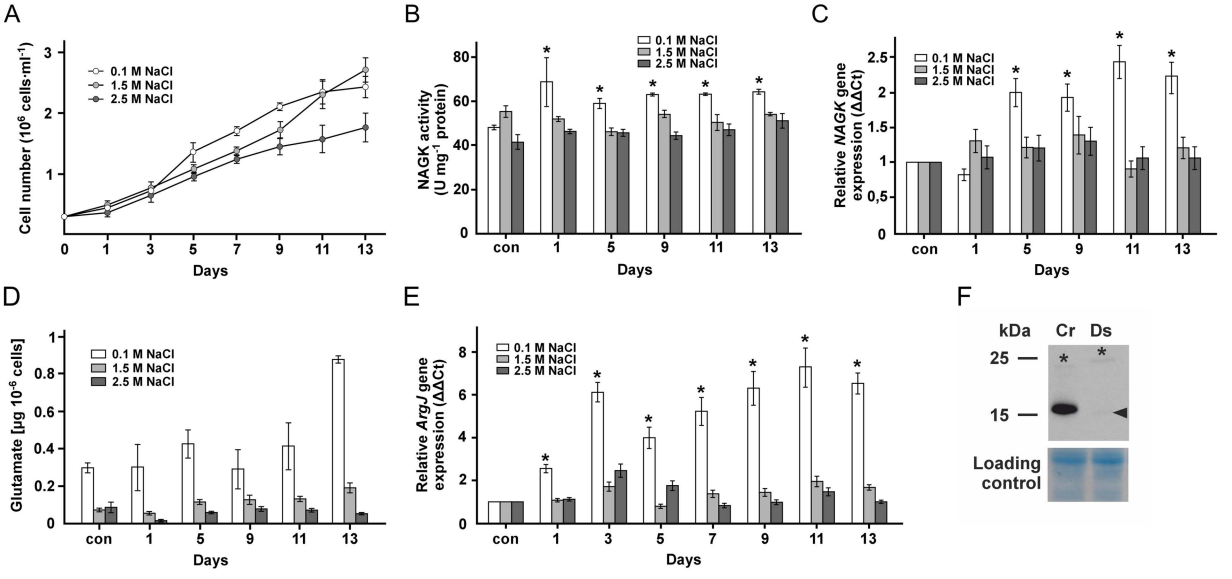

Fig. 2. Effects of salinity on cell growth, NAGK activity and expression of genes, glutamate content and PII protein abundance. A – The growth curves were analyzed in the presence of 0.1 M, 1.5 M or 2.5 M NaCl. Values are means ± SE of three independent experiments; B – time course of the DsNAGK activity during growth of cells at different salinity. Cells were grown as described in (A). Values are means ± SE of three independent experiments. * denotes significant differences between cells in 0.1 M NaCl and in 1.5 M or 2.5 M NaCl according to the Student's t test (*P* value < 0.01); C – Time course of the DsNAGK transcripts accumulation during growth of cells at different salinity. The relative gene expression was normalized to the expression of β-actin gene (Zalutskaya et al., 2023) and calculated using $2^{-\Delta\Delta CT}$. Values are means ± SEs of three biological replicates and three technical replicates. * denotes significant differences between cells in 0.1 M NaCl and in 1.5 M or 2.5 M NaCl according to the Student's t test (*P* value < 0.01); D – time course of Glu content in cells grown at different salinity. The amino acid content is expressed in μg per 10^6 cells. Values are means ± SE of three independent experiments; E – time course of the DsArgJ transcripts accumulation during growth of cells at different salinity. The relative gene expression was normalized as described in (C). * denotes significant differences between cells in 0.1 M NaCl and in 1.5 M or 2.5 M NaCl according to the Student's t test (*P* value < 0.01); F - PII protein abundance in *C. reinhardtii* strain CC3491 (Cr) and *Dunaliella* (Ds). *Dunaliella* cells were grown in 0.1 M NaCl for 5 days. Each line corresponds to 30 μg of soluble proteins extracted from samples. Protein loading was normalized by Coomassie staining.

Expression of PII protein in *Dunaliella* cells

To clarify the role of the DsPII protein in DsNAGK regulation in vivo, we analyzed the levels of the signaling protein in *Dunaliella* cells. We have used a polyclonal antibody against the *Chlamydomonas* PII (CrPII) that reacted with recombinant DsPII (Ermilova et al., 2013). As shown in Fig. 2 (F), the level of mature DsPII (~17.4 kDa) in the total cell extract proteins from cells grown in 0.1 M NaCl-supplemented medium was almost undetectable compared with the level of processed CrPII (~17.5 kDa) in *Chlamydomonas* cells. Moreover, only a faint band corresponding to full-length DsPII (~23.6 kDa) was immunologically detected in the total cell extract proteins. Collectively, these experiments showed that the expression/processing of the full-length PII appears to be noticeably impaired in *Dunaliella* cells compared with that in *Chlamydomonas* and *Chlorella variabilis* (Ermilova et al., 2013; Minaeva and Ermilova, 2015).

Intracellular arginine, putrescine and proline levels are dependent on salinity

Since in this halophilic alga Arg, putrescine (Put) and proline (Pro) are synthesized from ornithine (Orn) (Fig. 1), we hypothesized that Arg levels may be closely dependent on levels of these two protective molecules. Thus, we first compared Arg content in *Dunaliella* grown in the media with different salinity levels. A significant difference was found in the concentration of Arg in cells cultured

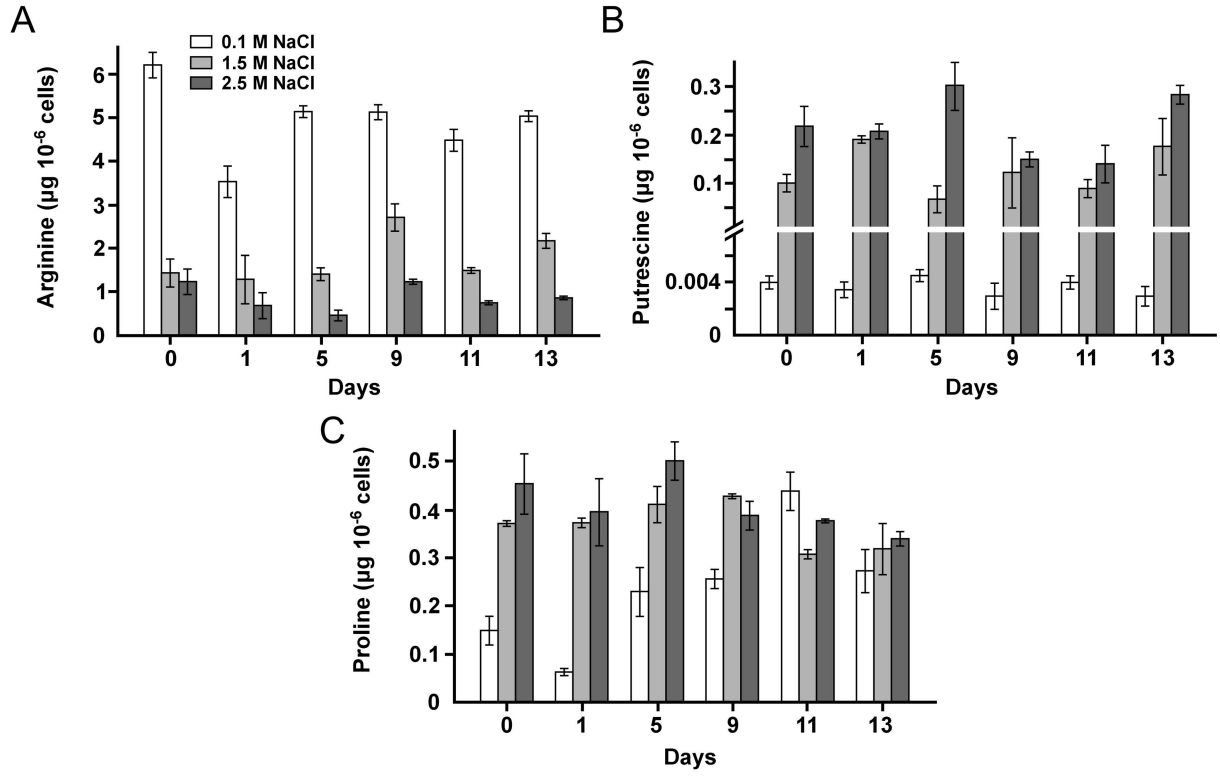

Fig. 3. Effects of salinity on Arg, Put and Pro contents. A – Arg; B – Put; C – Pro. Cells were grown as described in Fig. 2, A. The amino acids and Put content are expressed in μg per 10^6 cells. Values are means ± SE of three independent experiments.

in the media of different salinities: the lowest concentration of the amino acid was present in cells incubated in 2.5 M NaCl and the highest in 0.1 M NaCl (Fig. 3, A).

It was previously shown that polyamines are synthesized by *Dunaliella* to overcome the salinity effects (Liu et al., 2015; Lin and Lin, 2018). The GC-MS analysis demonstrated that Put levels were markedly higher when the cells were grown with 1.5 M or 2.5 M NaCl than with 0.1 M NaCl (Fig. 3, B). In addition to the increase in Put concentration in *Dunaliella* under higher salt concentrations (1.5 M or 2.5 M NaCl), a simultaneous increase in Pro content, a potential osmoprotectant, was registered in cells from lag- and log-phases of growth (Fig. 3, C). Taken together, these data indicate that the concentration of free Arg is negatively correlated with the cellular abundance of the protective molecules Put and Pro.

Discussion

Dunaliella, as the most halotolerant photosynthetic eukaryote, grows in extremely saline environments and exhibits efficient acclimation mechanisms (Polle et al., 2020; Ramachandran et al., 2023). This alga remodels the photosynthesis apparatus in the chloroplast to cope with the hypersaline conditions (Ramachandran et al., 2023). Like non-halophilic algae, the *Dunaliella* chloroplast carries out essential anabolic functions including amino acid biosynthesis. We aimed to understand whether the control of Arg biosynthesis in *Dunaliella* has changed during the adaptation of this alga to high salinity.

The highest levels of DsNAGK activity were seen in cells grown in 0.1M NaCl, where free Arg levels were the greatest, suggesting that concentrations of this amino acid were insufficient to inhibit enzyme in *Dunaliella* cells (Fig. 2, B; 3, A). Western blotting showed that the DsPII expression/translocation into chloroplast was significantly impaired (Fig. 2, F). Here, the scenario possibly would be that gene for DsPII became significantly inactivated once its function was no longer required in highly specialized niche. In accord with an endosymbiotic origin of the PII gene, the plant PII proteins are close relatives of the proteins from cyanobacteria (Sant'Anna et

al., 2009; Uhrig et al., 2009). In cyanobacteria, the PII protein acts as a multitasking signal-integrating regulator (Forchhammer and Selim, 2020; Forchhammer et al., 2022). However, there is no direct evidence of interaction between the PII protein and any target other than NAGK in green or red algae (Minaeva et al., 2015; Li et al., 2017; Lapina et al., 2019; Selim et al., 2020b). Moreover, loss of PII proteins by several green and red algae (Chellamuthu et al., 2013; Selim et al., 2020a) and deterioration of PII expression in *Dunaliella* (Fig. 3, E) support the idea that NAGK may be a single target in red and green algae. Future research in this field may reveal additional actors of this scenario.

In contrast to higher plants and the close relative, *Chlamydomonas, Dunaliella* is not able to synthesize Put from Arg (Fig. 1; Terashima et al., 2011; Majumdar et al., 2016). Importantly, Orn, apart from being a precursor for Arg biosynthesis, serves as a precursor to Put and Pro, which are known to have osmoprotective functions (Winter et al., 2015; Dar et al., 2016; Majumdar et al., 2016; Chun et al., 2018; Lin and Lin, 2018; Khajuria et al., 2018; Zalutskaya et al., 2020). We surmised that the production of Arg is affected by increased utilization of Orn into Orn-Put and Orn-Pro pathways. Indeed, in *Dunaliella*, Arg and Put/Pro are regulated in a reciprocal manner: in higher-salinity medium, the content of free Arg was the lowest and Put/Pro the highest (Fig. 3). Taken together, the results suggest that productions of Arg, Put and Pro are coupled processes, and lowering the concentration of free Arg through promoting Put/Pro formation may bypass the requirement for PII-dependent control of NAGK. However, we cannot rule out the existence of an alternative common mode of NAGKs activation.

Collectively, our findings extend the knowledge of the regulation of Arg synthesis in unicellular algae. Some specific conclusions from this study are: (1) expression of DsPII is impaired; (2) the resulting levels of free Arg are dependent on Orn flux to Put and Pro and are insufficient to inhibit DsNAGK in actively growing cells under high salinity conditions. In the future, it would be of interest to investigate the properties of NAGKs in other marine Chlorophyta and Rhodophyta lacking PII to reveal whether the unique feature of NAGK regulation in *Dunaliella* is related to the development of tolerance to salinity during evolution.

Acknowledgements

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References

Baker C.J. and Mock N.M. 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell Tissue Organ Cult. 39: 7–12. https://doi.org/10.1007/BF00037585

Bates L.S., Waldren R.P. and Teare E.D. 1973. Rapid determination of free proline for water stress studies. Plant Soil. 39: 205-207. https://doi.org/10.1007/BF00018060

Beez S., Fokina O., Herrmann C. and Forchhammer K. 2009. N-acetyl-L-glutamate kinase (NAGK) from oxygenic phototrophs: P(II) signal transduction across domains of life reveals novel insights in NAGK control. J. Mol. Biol. 389 (4): 748–758. https://doi.org/10.1016/j.jmb.2009.04.053

Chellamuthu V.R., Alva V. and Forchhammer K. 2013. From cyanobacteria to plants: Conservation of PII functions during plastid evolution. Planta. 237 (2): 451–462. https://doi.org/10.1007/s00425-012-1801-0

Chen Y.M., Ferrar T.S., Lohmeier-Vogel E.M., Morrice N, et al. 2006. The PII signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid N-acetyl glutamate kinase. J. Biol. Chem. 281(9): 5726–5733. https://doi.org/10.1074/jbc.M510945200

Chun S.C., Paramasivan M. and Chandrasekaran M. 2018. Proline accumulation influenced by osmotic stress in arbuscular mycorrhizal symbiotic plants. Front. Microbiol. 9: 2525.https://doi.org/10.3389/fmicb.2018.02525

Dar M.I., Naikoo M.I., Rehman F., Naushin F. and Khan F.A. 2016. Proline accumulation in plants: roles in stress tolerance and plant development. In: Osmolytes and plants acclimation to changing

environment: emerging omics technologies (Eds: Iqbal N., Nazar R. and Khan N.A.). New Delhi, India, Springer, pp. 155-166.

Ermilova E., Lapina T., Zalutskaya Zh., Minaeva E. et al. 2013. PII signal transduction protein in *Chlamydomonas reinhardtii*: localization and expression pattern. Protist. 164 (1): 49-59. https://doi.org/10.1016/j.protis.2012.04.002

Ermilova E.V. 2023. Nitric oxide (II) in the biology of Chlorophyta. Mol. Biol. (Moskow). 57 (6): 916-924.

Fernández E. and Galván A. 2007. Inorganic nitrogen assimilation in *Chlamydomonas*. J. Exp. Bot. 58 (9): 2279–2287. https://doi.org/10.1093/jxb/erm106

Forchhammer K., Selim K.A. and Huergo L.F. 2022. New views on PII signaling: from nitrogen sensing to global metabolic control. Trends Microbiol. 30 (8): 722-735. https://doi.org/10.1016/j.tim.2021.12.014

Forchhammer K. and Selim K.A. 2020. Carbon/nitrogen homeostasis control in cyanobacteria. FEMS Microbiol. Rev. 44 (1): 33-53. https://doi.org/10.1093/femsre/fuz025

Foresi N., Correa-Aragunde N., Parisi G., Caly G. et a. 2010. Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell. 22 (11): 3816–3830. https://doi.org/10.1105/tpc.109.073510

Haas D. and Leisinger T. 1975. N-acetylglutamate 5-phosphotransferase of *Pseudomonas aeruginosa*. Purification and ligand-directed association-dissociation. Eur. J. Biochem. 52 (2): 365–375. https://doi.org/10.1111/j.1432-1033.1975.tb04004.x

Heinrich A., Maheswaran M., Ruppert U. and Forchhammer K. 2004. The *Synechococcus elongatus* PII signal transduction protein controls arginine synthesis by complex formation with N-acetyl-L-glutamate kinase. Mol. Microbiol. 52 (5): 1303–1314. https://doi.org/10.1111/j.1365-2958.2004.04058.x

Jeandroz S., Wipf D., Stuehr D.J., Lamattina L. et al. 2016. Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. Sci. Signal. 9(417): re2. https://doi.org/10.1126/scisignal.aad4403

Johnsen L.G., Skou P.B., Khakimov B. and Bro R. 2017. Gas chromatography—Mass spectrometry data processing made easy. J. Chromatogr. A. 1503: 57–64. https://doi.org/10.1016/j.chroma.2017.04.052

Khajuria A., Sharma N., Bhardwaj R. and Ohri P. 2018. Emerging role of polyamines in plant stress tolerance. Curr. Prot. Pept. Sci. 19 (11): 1114–1123. https://doi.org/10.2174/1389203719666180718124211

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227 (5259): 680–685. https://doi.org/10.1038/227680a0

Lapina T., Kochemasova L.Yu., Forchhammer K. and Ermilova E. 2019. Effects of arginine on *Polytomella parva* growth, PII protein levels and lipid body formation. Planta. 250 (4): 1379–1385. https://doi.org/10.1007/s00425-019-03249-5

Lapina T., Selim K.A., Forchhammer K. and Ermilova E. 2018. The PII signaling protein from red algae represents an evolutionary link between cyanobacterial and Chloroplastida PII proteins. Sci. Rep. 8 (1): 790. https://doi.org/10.1038/s41598-017-19046-7

Li Y., Liu W., Sun L.P. and Zhou Z.G. 2017. Evidence for PII with NAGK interaction that regulates Arg synthesis in the microalga *Myrmecia incisa* in response to nitrogen starvation. Sci. Rep. 7(1): 16291. https://doi.org/10.1038/s41598-017-16644-3

Lin H.Y. and Lin H.J. 2018. Polyamines in microalgae: something borrowed, something new. Mar. Drugs. 17 (1): 1. https://doi.org/10.3390/md17010001

Liu J., Zhang D. and Hong L. 2015. Isolation, characterization and functional annotation of the salt tolerance genes through screening the high-quality cDNA library of the halophytic green alga *Dunaliella salina* (Chlorophyta). Ann. Microbiol. 65: 1293–1302. https://doi.org/10.1007/s13213-014-0967-z

Livak K.J. and Schmittgen T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta CT}$ method. Methods. 25 (4): 402–408. https://doi.org/10.1006/meth.2001.1262

Llácer J.L., Fita I. and Rubio V. 2008. Arginine and nitrogen storage. Curr. Opin. Struct. Biol. 18 (6): 673–681. https://doi.org/10.1016/j.sbi.2008.11.002

Majumdar R., Barchi B., Turlapati S.A., Gagne M. et al. 2016. Glutamate, ornithine, arginine, proline, and polyamine metabolic interactions: The pathway is regulated at the post-transcriptional level.

Front. Plant Sci. 7: 78. https://doi.org/10.3389/fpls.2016.00078

Minaeva E. and Ermilova E. 2015. Sequencing and expression analysis of the gene encoding PII signal protein in *Chlorella variabilis* NC64A. J. Plant Biochem. Physiol. 2 (1): 1000142. https://doi.org/10.4172/2329-9029.1000142

Minaeva E., Forchhammer K. and Ermilova E. 2015. Glutamine assimilation and feedback regulation of L-acetyl-N-glutamate kinase activity in *Chlorella variabilis* NC64A results in changes in arginine pools. Protist. 166 (5): 493–505. https://doi.org/ 10.1016/j.protis.2015.08.001

Polle J.E.W., Calhoun S., McKie-Krisberg Z., Prochnik S. et al. 2020. Genomic adaptations of the green alga *Dunaliella salina* to life under high salinity. Algal Res. 50: 101990. https://doi.org/10.1016/j.algal.2020.101990

Popov N., Schmitt M., Schulzeck S. and Matthies H. 1975. Reliable micromethod for determination of the protein content in tissue homogenates. Acta Biol. Med. Ger. 34: 1441–1446.

Puzanskiy R., Tarakhovskaya E., Shavarda A. and Shishova M. 2017. Metabolomic and physiological changes of *Chlamydomonas reinhardtii* (Chlorophyceae, Chlorophyta) during batch culture development. J. Appl. Phycol. 30: 803–818. https://doi.org/ https://doi.org/10.1007/s10811-017-1326-9

Ramachandran P., Pandey N.K., Yadav R.M., Suresh P. et al. 2023. Photosynthetic efficiency and transcriptome analysis of *Dunaliella salina* under hypersaline: a retrograde signaling mechanism in the chloroplast. Front. Plant Sci. 14: 1192258. https://doi.org/10.3389/fpls.2023.1192258

Rodriguez P.C., Ochoa A.C. and Al-Khami A.A. 2017. Arginine metabolism in myeloid cells shapes innate and adaptive immunity. Front. Immunol. 8: 93. https://doi.org/10.3389/fimmu.2017.00093

Sakaguchi S. 1950. A new method for the colorimetric determination of arginine. J. Biochem. 37: 231–236.

Sant'Anna F.H., Trentini D.B., de Souto Weber S., Cecagno R. et al. 2009. The PII superfamily revised: A novel group and evolutionary insights. J. Mol. Evol. 68 (4): 322–336. https://doi.org/10.1007/s00239-009-9209-6

Sathasivam R. and Juntawong N. 2013. Modified medium for enhanced growth of *Dunaliella* strains. Int. J. Curr. Sci. 5: 67–73.

Selim K., Ermilova E. and Forchhammer K. 2020a. From cyanobacteria to Archaeplastida: New evolutionary insights into PII signalling in the plant kingdom. New Phytol. 227(3): 722–731. https://doi.org/10.1111/nph.16492

Selim K.A., Lapina T., Forchhammer K. and Ermilova E. 2020b. Interaction of N-acetyl-l-glutamate kinase with the PII signal transducer in the non-photosynthetic alga *Polytomella parva*: Co-evolution towards a hetero-oligomeric enzyme. FEBS J. 287 (3): 465-482. https://doi.org/10.1111/febs.14989

Slocum R.D. 2005. Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiol. Biochem. 43(8): 729-745. https://doi.org/10.1016/j.plaphy.2005.06.007

Sugiyama K., Hayakawa T., Kudo T., Ito T and Yamaya T. 2004. Interaction of N-acetylglutamate kinase with a PII-like protein in rice. Plant Cell Physiol. 45 (12): 1768–1778. https://doi.org/10.1093/pcp/pch199

Terashima M., Specht M. and Hippler M. 2011. The chloroplast proteome: a survey from the *Chlamydomonas reinhardtii* perspective with a focus on distinctive features. Curr. Genet. 57 (3): 151–168. https://doi.org/10.1007/s00294-011-0339-1

Uhrig R.G., Ng K.K.S. and Moorhead G.B.G. 2009. PII in higher plants: a modern role for an ancient protein. Trends Plant Sci. 14 (9): 505–511. https://doi.org/10.1016/j.tplants.2009.07.003

Vlasova V., Lapina T., Statinov V. and Ermilova E. 2023. N-acetyl-L-glutamate kinase of *Chlamydomonas reinhardtii*: *In vivo* regulation by PII protein and beyond. Int. J. Mol. Sci. 24 (16): 12873. https://doi.org/10.3390/ijms241612873

Winter G., Todd C.D., Trovato M., Forlani G. and Funck D. 2015. Physiological implications of arginine metabolism in plants. Front. Plant Sci. 6: 534. https://doi.org/10.3389/fpls.2015.00534

Zalutskaya Z., Derkach V., Puzanskiy R. and Ermilova E. 2020. Impact of nitric oxide on proline and putrescine biosynthesis in *Chlamydomonas* via transcriptional regulation. Biol. Plant. 64: 653–659. https://doi.org/10.32615/bp.2020.094

Zalutskaya Z., Korkina S. and Ermilova E. 2023. Second nitrate reductase of *Dunaliella salina*: functional redundancy or greater? Protistology. 17 (1): 16-29. https://doi.org/10.21685/1680-0826-2023-17-1-2

Zalutskaya Z., Ostroukhova M. and Ermilova E. 2016. The *Chlamydomonas* alternative oxidase 1 is regulated by cadmium stress: New insights into control of expression. Environ. Exper. Bot. 130: 133–140. https://doi.org/10.1016/j.envexpbot.2016.05.015