International Scientific Journal
Medical and Biological Sciences
http://bioscience.scientific-journal.com

Exogenous 5-aminolevulinic acid increases the expression of Nar1 gene and nitrate reductase protein accumulation in barley seedlings.

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Abstract— The information on mechanisms and pathway of plant nitrogen assimilation by 5-aminolevulinic acid (ALA) is still limited. In addition, the molecular mechanism of nitrate reductase (NR) regulation in response to ALA treatment in plants has not fully been elucidated. In this study, we investigate the effect of different concentrations of exogenous ALA on expression, protein content and enzyme activity of NR in 7-day-old barley (*Hordeum vulgare* L.) seedlings grown in the presence of its substrate, KNO_3. Our data indicate that the inducible *Nar1* gene is up regulated and protein content and enzyme activity also increase in leaves of barley seedlings treated with ALA. We suggest that ALA influences growth and development of barley plants by means of nitrate reductase regulation at the transcriptional and post-translational levels.

Keywords– 5-Aminolevulinic acid, Hordeum vulgare L., nitrate reductase, plant growth regulator, transcriptional and post-translational regulation

I. INTRODUCTION

Nitrogen is one of the most important inorganic nutrients and a major component of proteins, nucleic acids, secondary metabolites and various cofactors. Assimilation of nitrogen is a fundamental biological process that has a noticeable effect on plant development [1]. Plants have evolved to utilize nitrate as a source of environmental nitrogen, and nitrate functions as a potent signal that regulates nitrogen and carbon metabolism [1-3]. Nitrate reductase (NR – EC 1.6.6.1-2) is the first and key enzyme of the nitrate assimilation. Barley (*Hordeum vulgare* L.) has two, differently regulated, NR genes, one encoding the NADH-specific NR (*Nar1*) and the other encoding the NAD(P)H-bispecific NR (*Nar7*) [4]. The NADH NR (EC 1.6.6.1) has been well defined structurally, biochemically and genetically in barley [5, 6] and is a homodimer of 110 kD [7]. NADH NR is found in highest concentrations in leaves while the NAD(P)H NR (EC 1.6.6.2) is located primarily in the roots [8]. NR is inducible, labile and prone to transcriptional and post-translational regulation by endogenous and exogenous factors, including the nitrate and nitrite salts, amino acids, light, circadian rhythms, antibiotics, temperature, the Ca^{+2} level and others [9-13]. In the higher plants NR is under influence of plant growth regulators such as auxin [14], gibberellin [15], salicylic acid [16] which modify enzyme activity [13]. The role of cytokinins on NR has been studied more extensively in comparison to other growth regulating substances. Cytokinins were shown to act at all levels of NR gene expression: They increase mRNA content, protein amount and enzyme activity [13]. Treating plants with plant growth regulators is an effective way to increase efficiency of nitrate utilization and improve agricultural production.

On the other hand, recent studies are focused on the unique precursor of chlorophyll and heme – 5-aminolevulinic acid (ALA). In low concentrations, it acts as plant growth regulator promoting very effectively growth, development and productivity of plants [17]. However, the mechanisms of exogenous ALA influence on plant growth are still poorly understood [18]. Previous studies have suggested that ALA has an ability to promote plant nitrogen assimilation. It was found that ALA stimulates NR activity in the presence of potassium nitrate (KNO3), which leads to significantly higher levels of total nitrogen and proteins in maize leaves [19] as well as decreases nitrate content in plants [20, 21]. Iwai et al. [22] suggested that addition of a fertilizer containing nitrate and exogenous ALA would increase the yield of paprika and strawberry because the plants could absorb more nitrate from the hydroponic solution. However, the authors did not analyze the relationship between nitrate assimilation and ALA treatment. Recently Wei et al [21] showed that ALA treatment decreased nitrate accumulation in senescent leaves of pakchoi, which was related with higher activity and gene transcript of NR. Nevertheless, the molecular mechanism responsible for enhancement of nitrogen assimilation in monocot plants and plants on early stage of development by exogenous ALA has not yet been studied.

In the present paper, we investigate the effect of low concentrations of ALA on NADH-specific NR in barley leaves at the physiological, biochemical and molecular levels.

II. MATERIALS AND METHOD

Plant growth and treatments

Barley seeds (*Hordeum vulgare* L. cv. Gonar) were soaked in distilled water at room temperature for 2 h. Then the seeds were germinated for 2 d at 25 ◦C in the dark between two layers of blotter paper. After seed germinations, the seedlings were transferred to plastic containers and grown for 5 d under

illumination with white fluorescent lamps LD-40 (160 µmol m^{-2} s^{-1}) at 25 ◦C for 14/10 h light/ dark period. For 5 d, plants were watered with solutions containing different concentrations of KNO_3 (1, 5, 10, 20 mM) or KNO_3 + ALA (5 mM + 0.01, 0.05, 0.1, 0.5, 2.5 mg/L). For comparison purpose, plants were grown with distilled water and KNO_3 (5 mM) used as control, respectively. For all experiments, 1-2 cm sections of leaves were cut, starting 0.5 cm from the tip.

Nitrate reductase assay

For the extraction of NR, 0.1 g of frozen leaves was ground in a chilled mortar with a buffer containing 50 mM Hepes-KOH, 1 mM EDTA, 1 mM mercaptoethanol and 1% bovine serum albumin. The homogenate was centrifuged at 13000g for 10 min at 4 ◦C. In the supernatant the total NR activity was determined using Hepes-KOH buffer supplemented with 5 mM EDTA. For the analysis of the NR actual form, 10 mM $MgCl_2$ was added instead of EDTA. The reaction was run with 0.1 M KNO_3, 5 mM NADH for 5 min at 30 ◦C and stopped with 0.1 mL of 5 mM oxaloacetic acid. The color reaction with nitrite produced in the samples was initiated by adding 0.5 mL of 1% sulfanilamide in 1 N HCl and 0.5 mL of 0.02% naphtylethylenediamine dihydrochloride. After 10 min incubation the stained samples were centrifuged for 10 min at 4000g and the amount of nitrite was determined colorimetrically at 546 nm using standard curve.

Quantitative real time PCR analysis

The Real Time PCR was performed using MiniOpticon real-time PCR system from (Bio-Rad, USA). Total RNA was isolated from 200 mg of fresh leaves tissue with Tri Reagent (Sigma-Aldrich, USA) according to manufacturer's instruction. Total RNA yield was determined using NanoDrop Spectrophotometer ND-2000 (Thermo Scientific, USA). To avoid any DNA contaminations, the RNA samples were treated with RNAse-free DNAseI (Fermentas, Lithuania) and then reversed transcribed into first-strand cDNA with High-Capacity cDNA Reverse Transcription Kit (Fermentas, Lithuania) following manufacturer's instruction. PCR reactions were carried out using Real Time 2×PCR Master Mix SYBR® kit (Thermo Scientific, USA). The expressions were analyzed with the following primers: 5′ AAG GGA TAC GCA TAC TCA GG 3′ (forward) and 5′- TGA GGT TCC AGA TGA GCT TC- 3′ (reverse) for *Nar1* and 5′- TAA GGG ACA TCA AGG AGA AG- 3′ (forward), 5′- AGT TGT AGG TCG TCT CGT G- 3′ (reverse) for *actin* as an internal standard gene. The following conditions of amplifications were applied: 10 min at 95 ◦C; 40 cycles of 15 s at 95 ◦C, 30 s at 59 ◦C and 30 s at 72 ◦C, with final melting for 15 s at 65 ◦C. The amplified transcripts were visualized on 2% agarose gels. Gels were then scanned using Gel Doc™ XR (Bio-Rad, USA).

Protein gel blot analysis

For protein isolation, fresh leaves (400 mg) from plants were homogenized in 500 µL extraction buffer. Proteins (20 mg) were resolved on a 10% SDS-PAGE gel (Invitrogen, USA) according to Laemmli [23]. Proteins were transferred to nitrocellulose membrane using blotting buffer containing 20% methanol, 25 mM Tris buffer pH 8.8, 0.03% SDS. After blocking in TBST buffer containing 4% non-fat dried milk powder for 1 h at room temperature, the membranes were incubated with anti-NR antibody at 1:1000 dilutions (Agrisera, SW). The proteins were detected with anti-rabbit IgG antibody conjugated to alkaline phosphatase at 1:2000 dilutions (Abcam, UK). The alkaline phosphatase assay was conducted using BCIP/NBT (Sigma-Aldrich, USA) according to the manufacturer's protocol.

Protein determination

Determination of the protein amount for SDS-PAGE/protein gel blot analysis was performed by the Bradford assay [24] using bovine serum albumin as a standard.

Statistical analysis

All assays including gene expression were obtained at least in four independent biological samples, each analyzed in triplicate (n = 12), and the means ± SD of these values are presented in the table and figures. In order to test the significant differences between calculated values, equality of variances was tested by an *F*-test followed by Student's *t*–test using a *P*-value of <0.05 as the threshold for significant difference. The quantitative PCR data were analyzed by the ΔΔCT- method [25].

III. RESULTS AND DISCUSSION

The effect of KNO_3 concentrations on the growth parameters, NR activity and NADH NR gene expression have been studied in 7-day-old barley seedlings. The addition of KNO_3 in the range of 1-20 mM led to significant increase of both total (+EDTA) and actual (+$MgCl_2$) NR activities as compared to control plants (Fig 1a). Plants treatment with KNO_3 (20 mM) increased NR activity in them 5 times in comparison with non-treated ones. Plant growth closely correlated with the NR activity. At the highest KNO_3 concentration (20 mM), the average height and width of seedlings increased to 114%, 117% compared with controls, respectively. These data are in agreement with the results from the sunflower and safflower plants [26]. Quantitative RT-PCR measurements also revealed that the accumulation of transcript was clearly up-regulated by the increase of KNO_3 concentration (Fig 1b). The results demonstrated that nitrate flux has an important role in the regulation of *Nar1* expression in barley. In addition, our finding indicated a strict correlation between plant growth, NR activity, expression of inducible gene *Nar 1* and KNO_3 concentrations added in barley seedlings.

At the next step, we analyzed the effect of exogenous ALA on barley plants. Plants were treated with different concentrations of exogenous ALA in the presence of 5 mM KNO_3 (control) for 5 d as described in Materials and Methods. It was found that exogenous ALA caused significant increase in growth of plants (Fig 2) and broadening of leaf blades as compared with control (Table 1). Several studies have reported that low concentration of ALA improves growth and yield of several crop species [27, 28]. Also, our results supported idea that exogenous application of ALA via rooting medium is effective in promoting growth [18].

The stimulation of total (+EDTA) and actual (+$MgCl_2$) NR activities in the presence of ALA concentrations were observed (Fig 3a). In the highest amount, 0.1 mg/L, ALA increased by 99% and 109%, respectively. This effect is in good agreement with previous data [16, 21]. The molecular basis of the ALA impact on NR activity was analyzed by RT-PCR and protein blot techniques. Quantitative RT-PCR measurements indicated that the accumulation of transcripts was distinctly up-regulated by the increase in ALA concentration (Fig 3b). Treatment with 2.5 mg/L ALA led to 4-fold increase of relative transcripts in plants compared with controls. These results indicate that exogenous ALA has a strong impact on nuclear gene expression (NGE) of NR at the initial stage of plant growth. Maruyama-Nakashita et al. [28] showed that exogenous application of ALA increased the transcript level of some sulfur assimilatory genes in *Arabidopsis thaliana*. Two years later Czarnecki et al. [29] proved the contribution of ALA synthesis in plastid-to-nucleus signaling. They showed that inhibited ALA synthesis resulted in the repression of 158 genes and up-regulation of 167 genes in *Arabidopsis* seedlings. They proposed that retrograde signals emitted at the level of ALA formation adjust the NGE. Our data confirm the impact of exogenous ALA on NGE, namely *Nar 1* at the early stages of barley growth.

The data on protein blot analysis using anti-NR antibody showed that a distinct band of NR (~ 100 kDa) could be observed on the membrane (Fig 3c). The band intensity enhanced depending on the ALA concentration. Treatment with 2.5 mg/L ALA led to the maximal protein production (about 120% increases over control). Thus, in barley seedlings treated with exogenous ALA a positive correlation exists between parameters of plant growth, NR activity and protein content, *Nar1* expression, and the ALA concentration. These data indicate that ALA in low concentrations affects growth and development of plants by means of up-regulated expression of their NR.

The present data also revealed positive correlation between ALA concentrations and majority of variable measures. It is a well-known that using ALA over certain concentration is harmful to plant because it acts as photodynamic herbicide [30]. Hence, determination of an efficient concentration of ALA is necessary if it is to be used for improving plant growth under optimum or stress conditions.

IV. CONCLUSION

In summary, we observe that treatment of barley seedlings with exogenous ALA influences on NR at biochemical and molecular levels. We provide evidence that low concentrations of ALA influence on activity of NADH NR enzyme. The mechanisms of ALA on NR enzyme activity involve stimulation of *Nar1* expression and increase NR protein. Furthermore, these results demonstrate the importance of NR regulation with ALA at early stages of ontogenesis that often correlates with plant growth and crop production.

V. ACKNOWLEDGMENT

This work was supported by grant (B11MC-017) from Belarussian Republican Foundation for Fundamental Research. We also thank Dr. N. Kozel and Dr. E. Kabachevskaya for their kind helps and Dr. D. Shcharbin for improving the English of the manuscript.

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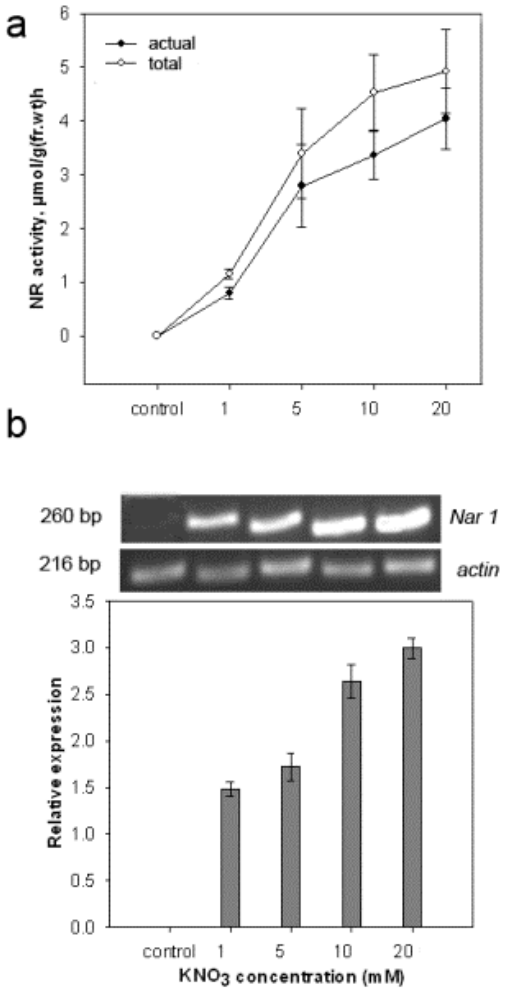

Fig. 1 The effect of KNO_3 in different concentrations on (a) actual (●) total (○) NR enzyme activity, and (b) NR expression. Seedlings were grown in the presence of different concentrations of KNO_3 for 5 d. The *actin* amplification band was shown to confirm equal loading of RNA and RT efficiency in 2% agarose gel. Error bars indicate SD

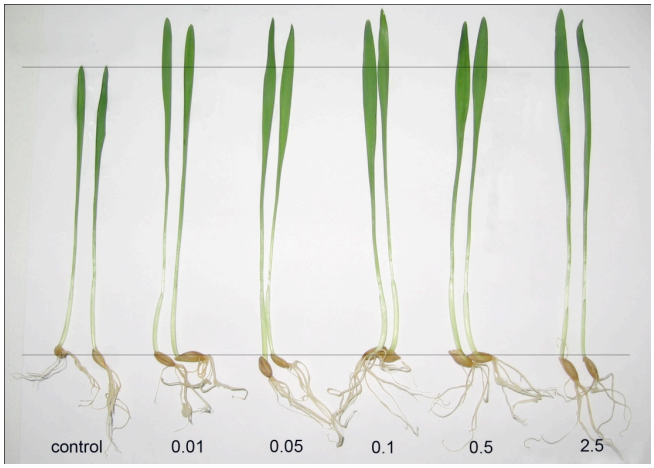

Fig. 2 Effect of different concentrations of exogenous ALA on growth of barley seedlings. Plants were grown in growth chambers under controlled conditions (illumination with white fluorescent lamps LD-40 (160 µmol m^{-2} s^{-1}) at 25 ◦C for 14/10 h light/ dark period) and were treated with exogenous ALA as described in Materials and Methods. Control plants were treated with 5 mM KNO_3. The numbers show the different concentration of ALA (mg/L)

TABLE I. Physiological response of 7-day-old barley seedlings grown under different concentrations of exogenous ALA

Treatments	Height (cm)	Width (mm)
Control (5 mM KNO_3)	11.86 ± 0.63	4.4 ± 0.51
KNO_3 (5 mM) + ALA (0.01 mg/L)	13.03 ± 0.48*	4.6 ± 0.51
KNO_3 (5 mM) + ALA (0.05 mg/L)	13.51 ± 0.41*	5.1 ± 0.56*
KNO_3 (5 mM) + ALA (0.1 mg/L)	13.31 ± 0.41*	5.6 ± 0.51*
KNO_3 (5 mM) + ALA (0.5 mg/L)	13.44 ± 0.37*	5.5 ± 0.52*
KNO_3 (5 mM) + ALA (2.5 mg/L)	13.43 ± 0.4*	5.4 ± 0.31*

Given are means ± SD of four independent experiments. * indicates a significant difference from the control ($P < 0.05$)

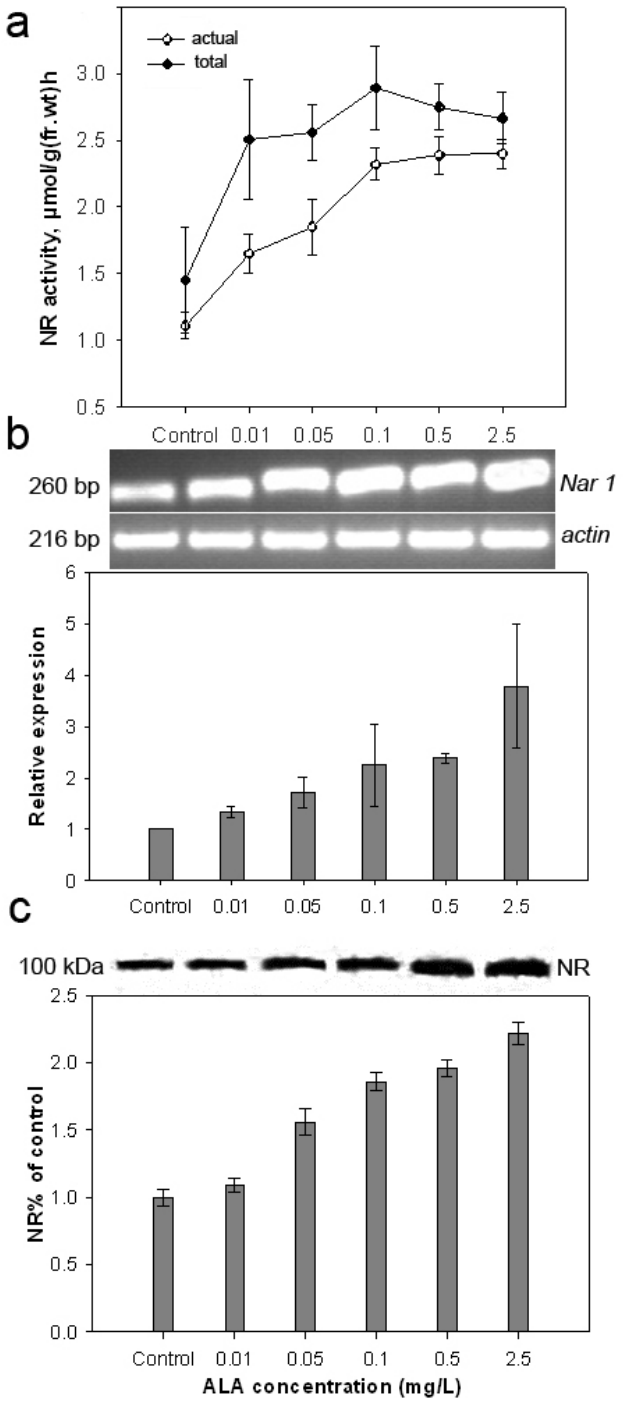

Fig. 3 The influence of ALA on NADH NR enzyme activity, transcript and protein content in barley. Seedlings were grown in the presence of different concentrations of ALA and 5 mM KNO_3 for 5 d. (a) total (●), and actual (○) NR activity. (b) NADH-NR transcripts (c) accumulation of the NADH NR protein. The *actin* amplification band was shown to confirm equal loading of RNA and RT efficiency in 2% agarose gel. Error bars represent SD