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Response of nitrate reductase activity and *NIA* genes expression in roots of *Arabidopsis hxk1* mutant treated with selected carbon and nitrogen metabolites

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ABSTRACT

In plants sugar sensing and signal transduction involves pathways dependent or independent on HXK1 as a glucose sensor. Research was conducted to determine which pathway is responsible for regulation of the nitrate reduction. The effect of selected carbon and nitrogen metabolites on nitrate reductase (NR) activity in *Arabidopsis thaliana* wild type (WT) and *hxk1* mutant roots was studied. Exogenously supplied sugar, sucrose (Suc) and organic acid, 2-oxoglutarate (2-OG) led to an increase in the total and actual activity of NR. It was due to both the increase in expression of *NIA* genes and NR activation state. The stimulatory effect of Suc and 2-OG on nitrate reduction was less pronounced in *hxk1* mutant roots with T-DNA insertion in the *AtHXK1* gene encoding hexokinase1 (HXK1) and characterized by reduced hexokinase activity and root level of G6P and F6P. On the other hand, it was shown that exogenous glucose did not mimic Suc-mediated NR activation in *Arabidopsis* roots. Taken together, this data suggest that the Suc signaling pathway might be independent from hexose's sensor dependent mechanism.

1. Introduction

Nitrate ions are the main nitrogen source for higher plants growing on well-aerated soils [1]. After being taken up by roots and before assimilation into organic compounds nitrate is reduced to ammonia during a two-step process. The initial and key reaction is reduction of nitrate to nitrite catalyzed by nitrate reductase (NR – EC 1.7.1.1) [2]. Activity of nitrate reductase is subjected to extensive control at both the genetic (transcriptional) and post-translational level in response to various environmental and endogenous factors [3]. The transcriptional control involves alterations of expression of NR encoding genes. The post-translational regulation comprises modifications of enzyme proteins, mostly through the rapid and reversible phosphorylation of NR protein by CDPK and SNF1-related protein kinases (SnRK1). This is followed by binding to 14-3-3 protein which inhibits the enzyme activity (reviewed by [4–6]). Besides reversible phosphorylation also reduction/oxidation of enzyme protein and hysteretic modifications are considered to be involved in posttranslational regulation of NR [7,8]. In *Arabidopsis thaliana*, two genes, *NIA1* and *NIA2* encode the two NR apoproteins. The two genes are approximately 70% identical at the nucleotide level and NIA1 and NIA2 apoproteins share 76% amino acids identity [9]. It was shown, using *Arabidopsis nia1* and *nia2* mutants, that *NIA2* encodes the main isoform of NR responsible for nearly 90% of enzyme activity [9]. Expression of *NIA* genes is not equal in *Arabidopsis* leaves and roots. Recently it was established that NIA2 protein is the main isoform in leaves. In turn, *NIA1* transcript exceeds *NIA2* in roots [10,11].

Expression and activity of NR in leaves and roots depends on many external factors, especially including nitrate and light [12,13]. Induction of *NIA* genes by nitrate is very fast and is observed already after 30 min in maize and *Arabidopsis* [12,14]. However, the increase of NR specific transcript in response to nitrates occurred only in the light when photosynthetic activity was present. Moreover, the presence of sugars, glucose (Glc) or sucrose (Suc), in the environment can replace the light in the induction and regulation of NR activity and sustains it at a high level [3,15]. Gene expression and modifications at the posttranslational level are involved. Nitrate assimilation closely interacts also with organic acids, especially with 2-oxoglutarate (2-OG). This molecule is the primary carbon acceptor of reduced nitrogen during amino acid synthesis in the GS-GOGAT pathway [16]. Moreover, experiments performed previously in tobacco suggested that 2-OG may also act as a signal molecule regulating N assimilation [17,18].

Abbreviations: NR, nitrate reductase; HXK1, hexokinase1; 2-OG, 2-oxoglutarate.

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Carbon metabolites such as sugars and organic acids, usually cause stimulation of various steps of nitrogen assimilation. On the other hand, the intermediate products of nitrogen metabolism, such as amino acids, glutamine (Gln) and glutamate (Glu), are signal molecules responsible for feedback inhibition of nitrate uptake and reduction. Negative action of amino acids concerns mainly reduction of gene expression [1,19]. Transduction of Gln and/or the Glu signal is still being studied and putative Glu receptors, AtGLR proteins encoded by a 20-gene family, were identified in *Arabidopsis* ([20] and references therein).

The role of C metabolites in NR regulation may be due to their necessity as carbon skeletons and energy sources during nitrogen assimilation. Moreover, both glucose and sucrose have been recognized as important signal molecules that control gene expression [21]. It is now well established that hexokinase1 (HXK1), the first enzyme in glucose catabolism, is also a major glucose sensor, implicated in signaling pathways regulating expression of various genes. The role of HXK1 in sugar sensing and signaling is independent of its metabolic function [22]. In the past few years numerous studies on the sugar signal transduction have indicated three glucose signal transduction pathways: (i) a HXK1-dependent pathway involving its sensor function, (ii) a glycolysis-dependent pathway associated with catalytic function of HXK and also (iii) a HXK1-independent pathway [23]. Studies using mutants showing impaired HXK1-mediated glucose signal transduction and/or glucose insensitivity suggest that different pathways may be involved in control of different processes. Evidence has been provided that the HXK1-signaling pathway is involved and responsible for the sugar-mediated feedback regulation of photosynthesis and interactions with hormone signaling (ABA or ethylene) [22]. On the other hand, sugar-induced increase of nitrate uptake and *AtNRT2.1* expression regulation do not depend on sensor function of HXK1 [24]. Also, our recent studies carried out on leaves of *Arabidopsis* homozygous transformed plants with T-DNA insertion in *AtHXK1* (*hxk1*, SALK_034233C) showed that stimulation of nitrate reductase actual activity and *NIA* genes expression is not related to transduction of the sugar signal dependent on HXK1 sensor function [19]. However, the results obtained in leaves did not allow for a clear statement whether catalytic action of HXK was involved in the process. Although hexokinase enzymatic activity was significantly lower in the tissues of *hxk1* mutant, the level of hexose phosphates in the mutant leaves was similar to that indicated in the WT [19] probably due to photosynthetic activity occurring in green tissues [25]. In *hxk1* roots reduced HXK activity is accompanied by a significantly lower level of endogenous G6P and F6P. So in order to elucidate involvement of catalytic activity of HXK in regulation of NR by C and N metabolites, the effect of sugars (sucrose and glucose), 2-OG and amino acids on NR activity and *NIA* expression in *hxk1* mutant roots was investigated.

2. Materials and methods

2.1. Plant material and growth conditions

Roots of seven-week-old *A. thaliana* plants, ecotype Columbia (Col-0) and *hxk1* mutant (SALK_034233C), were used for all experiments. The seeds were provided from Nottingham Arabidopsis Stock Centre, University of Nottingham. Before sowing, the seeds were surface sterilized with 3% sodium hypochlorite for 10 min followed by double flushing with 96% ethanol. Dry seeds were sown on top of cut, dark Eppendorf tubes filled with 0.25× MS medium with 0.8% agar and stratified at 4 °C for 2 days. The tubes were then transferred to nutrient solution containing 3.5 mM nitrate, pH 5.7–6.0 [19]. Plants were grown in nonsterile hydroponics in a growth chamber with an 8 h light (180 μmol m^{-2} s^{-1})/16 h dark cycle at 22 °C/20 °C, respectively. The nutrient solution was continuously aerated and renewed every week to avoid deficiencies. At 42 days after sowing the plants were transferred for 8 days to medium devoid of nitrate for N starvation. Then plants were resupplied with 3.5 mM nitrate solution and incubated for induction of N metabolism. To test the effect of C metabolites and amino acids plants were treated for 8 h with the same medium supplemented with sugars (2 mM glucose, 1 mM sucrose), organic acid (1 mM 2-oxoglutarate), and amino acids (1 mM glutamine or 1 mM glutamate). After incubation roots were cut, frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Nitrate reductase assay

Frozen root tissue (1 g) was ground in a chilled mortar with 2 mL of extraction buffer as described previously by Reda et al. [26]. Crude supernatants were used for measurement of nitrate reductase activity in the absence of $MgCl_2$ ($-Mg^{2+}$, total NR activity) and/or in the presence of $MgCl_2$ ($+Mg^{2+}$, actual NR activity) according to Kaiser and Huber [7] with some modifications. The reaction was carried out in 50 mM Hepes-KOH (pH 7.5) containing 5 mM EDTA ($-Mg^{2+}$) or 5 mM $MgCl_2$ ($+Mg^{2+}$), and 10 mM KNO_3. It was started by addition of 0.2 mM NADH. After 10 min of incubation at 27 °C 1 M zinc acetate was used to stop the reaction. Samples were centrifuged and the amount of nitrite formed was measured colorimetrically [27]. Also the activation state of NR was calculated as the ratio NR_{act}/NR_{total} activity expressed as a percentage.

2.3. Expression of NIA genes

To determine the expression of *NIA1* and *NIA2* genes, real-time PCR was performed using the Light Cycler 480 system (Roche). As an internal standard required for normalization of each *NIA* gene expression of α-tubulin encoding gene *AtTUA4* (AT1G04820) was used. Total RNA was isolated from frozen root tissue (50 mg) with Tri Reagent (Sigma) as described in the manufacturer's instructions. The amount and purity of isolated RNA were measured using NanoDrop Spectrophotometer ND-1000 (Thermo Scientific) and samples showing the 260/280 ratio between 1.8 and 2.0 were used for further analysis. To avoid any DNA contamination RNase-free DNase I (Fermentas) was used. Purified RNA samples were immediately reverse transcribed into first-strand cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. The cDNA was then used as the template for real-time PCR amplification with the Real-Time 2× PCR Master Mix SYBR® kit (A&A Biotechnology). The specific primers for PCR were used as follows: 5′-ATCGTCAAAGAAACCGAAGTC (forward), 5′-ACGGAGCATGGATGAGTT (reverse) for *NIA1*, 5′-GGTTACGCATATTCCGGAG (forward), 5′-CATGCACGAACAGCAATC (reverse) for *NIA2* [28] and 5′-TCCTATGCCCCAGTCATCTC (forward), 5′-TAGTGCGCTTGGTCTTGATG (reverse) for *AtTUA4*. The real-time PCR was conducted using a LightCycler 480 (Roche) with the following conditions: 30 s at 95 °C; 40 cycles of 10 s at 95 °C, 10 s at 56 °C and 12 s at 72 °C, with final melting for 15 s at 65 °C. Melting curve analysis was performed to confirm the specificity of the amplicon and to identify putative unspecific products. Dilution of the samples of lower crossing point (Cp) was used as a standard curve with amplification efficiency around 2.

2.4. Hexokinase activity measurement

Activity of hexokinase was measured according to Wiese et al. [29] in extracts prepared as described by Cho et al. [30] and it was expressed as the changes in absorbance at 340 nm corresponding to the formation of NADPH measured with a spectrophotometer.