Participation of Nitrate Sensor NRT1.1 in the Control of Cytokinin Level and Root Elongation under Normal Conditions and Nitrogen Deficit

A. V. Korobova*, G. R. Akhiyarova*, V. V. Fedyaevb, R. G. Farkhutdinovb, S. Yu. Veselova,b, and G. R. Kudoyarova*

a Ufa Institute of Biology, Ufa Federal Research Center, Russian Academy of Sciences, Ufa, 450054 Russia
b Bashkir State University, Ufa, 450076 Russia
*e-mail: muksin@mail.ru

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Abstract—NRT1.1 nitrate transporter acts as a nitrate sensor in some plant responses. We tried to check if it may be involved in the control of cytokinin level in the plants known to be involved in the growth responses to nitrate level. The experimental objects were Arabidopsis thaliana plants of the original ecotype Columbia (Col-0) and chl1-5 mutants. The effects of the NRT1.1 gene mutation in chl1-5 plants on hormonal and growth responses to nitrogen starvation were studied. Two types of growing conditions were used: (1) plants were placed on either standard Hoagland–Arnon or modified solution, where potassium and calcium nitrates were substituted with their chlorides; (2) plants were placed on Pryanishnikov medium, where ammonium nitrate serves as the source of nitrogen and nitrogen deficiency being modeled by its withdrawal from the medium. It has been first shown that mutation of the NRT1.1 resulted in a decline in cytokinin level in the roots of chl1-5 mutants, while roots of wild type plants were longer in accordance with lower cytokinin content in them; this hormone is known to inhibit root elongation. Cytokinin content decreased in A. thaliana, Columbia ecotype, paralleled by acceleration of root elongation in response to both variants of nitrogen starvation, while chl1-5 roots responded in this way only when nitrogen was withdrawn from Pryanishnikov solution. Substitution of nitrates by chlorides in the Hoagland–Arnon solution had no effects on either chl1-5 roots' length or cytokinin content in them. The results suggested the involvement of NRT1.1 transceptor in the control of cytokinin level and root elongation rate in the nitrate but not in ammonium starved plants, confirming the specificity of response.

Keywords: Arabidopsis thaliana, NRT1.1 transceptor, root growth, cytokinins, nitrogen starvation, immunohistochemical localization, hydroponics.

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INTRODUCTION

The changes in the growth and development of roots are important adaptive reactions that ensure the optimization of the uptake of the elements of mineral nutrition and water by the plant. When studying the reaction of plants to changes in the level of nitrates, cytokinins attract special attention [1, 2]. The impacts of return of plants to the media with nitrates after their cultivation without nitrogen have been studied in detail [3]. It is known that the expression of the genes controlling cytokinin synthesis is induced, and this leads to an increase in the level of these hormones in Arabidopsis [4] and rice plants [5]. The effect of nitrate elimination from the nutrient medium has received less attention, although some studies have shown a decrease in the level of cytokinins in nitrogen deficiency [6]. Since cytokinin treatment inhibited root growth [7] and the decreased level of cytokinins in transgenic plants was accompanied by their growth acceleration [8], then activation of root growth by nitrate starvation can be explained by a reduction in the content of these hormones.

Since cytokinins are important in plant reactions to nitrogen deficiency, it was necessary to understand how plants perceive nitrate levels by regulating cytokinin content and plant growth. An appreciable role in the perception of nitrate signal is attributed to the carrier of nitrates NRT1.1 [9]. It has been shown that when nitrates are added to the medium without nitrogen in the chl1-5 mutant by NRT1.1 gene, expression of the genes encoding nitrate carriers and enzymes involved in their metabolism was not induced. Based on these results, it is assumed that NRT1.1 combines the functions of a sensor and a nitrate carrier, which makes it possible to call it NRT1.1 transceptor. Its participation in the regulation of lateral root growth is
connected with NRT1.1 functions, such as an NO₃-
dependent carrier of auxins [10]. Under low nitrate
content, it ensures the auxins’ outflow from primor-
dium of lateral roots, which prevents them from elon-
gation. Less attention was paid to the possible involve-
ment of NRT1.1 transceptor in the regulation of cyto-
kinin levels and root growth.

The goal of our study was to identify the effect of
the NRT1.1 gene mutation on the cytokinin content
and phenotype of A. thaliana plants when grown on
full nutrient medium and removed from it, and, thus,
to check whether the NRT1.1 sensor participates in the
hormonal and growth responses to nitrogen starva-
tion.

MATERIALS AND METHODS

Plant Arabidopsis thaliana [L.] Heynh., ecotype
Columbia (Col-0), and mutant by nitrate transceptor
NRT1.1 (chl1-5) were studied. After stratification on
moist filter paper in Petri dishes for 3 days at 4°C, the
seeds were transferred to the pots (100 mL) with sand
saturated with Pryanishnikov solution, in which
ammonium nitrate is the only source of nitrogen, or
Hoagland–Arnon medium containing nitrogen in the
form of potassium and calcium nitrates and grown in a
climate chamber (MLR-350H, Sanyo, Japan), as
described earlier [7]. The water content of the sand
was maintained at 65% of the total moisture capacity.
After 2 weeks following the transfer of the plants to the
climate chamber, they were placed into wells of poly-
styrene microplates with holes where the plants
floated on the surface of the nutrient solution. In pre-
liminary experiments, the plants were grown in liquid
media—tenfold diluted standard Pryanishnikov and
Hoagland–Arnon solutions—that resulted in the plant
maximum mass accumulation by ten times, so the
diluted media were selected for further research.

Two variants of nitrogen removal from the medium
were used. (1) The plants were placed on Pryanish-
nikov medium, and they were transferred to a con-
tainer with Pryanishnikov solution without ammo-
nium nitrate when simulating nitrogen starvation.
(2) Half of the plants received a complete Hoagland–
Arnon solution, and the other half of the plants
received a modified solution in which potassium and
calcium nitrates were replaced by chlorides, as
described earlier [11]. Plants were grown under con-
tinuous aeration. After 2 days of plant growth in
hydroponics, the tissues were taken for analysis of
cytokinins; we measured the root length after 4 days.

To extract cytokinins, plant roots were homoge-
nized in 80% ethanol and incubated overnight at 4°C.
After the ethanol was filtered and evaporated, the
water residue was cleaned on the C18 (Waters, United
States) cartridge as described earlier [12]. After evapo-
ration of the solvent, the dry residue was dissolved in
0.02 mL of 80% ethanol, and cytokinin metabolites
were separated by thin-layer chromatography [12].
Different forms of cytokinins were eluted for 15 h with
0.1 M phosphate buffer (pH 7.2–7.4) from the corre-
sponding zones identified by the markers’ position in
the UV-light. The aliquot was then added to the plate
wells in a series of dilutions, and the enzyme-linked
immunoassay was performed with the help of antibod-
ies against trans-zeatin riboside, highly specific to
trans-zeatin derivatives [12].

Specific rabbit antibodies to the zeatin riboside
were used for immunohistochemical localization of
cytokinins in longitudinal sections of root tips. Plants
were fixed in 4% paraformaldehyde solution (Rie-
del-de Haën, Germany) and 0.1% glutaric aldehyde
(Sigma, Germany). After 2 h, the root tips were
washed from the detergent and enclosed in agarose
blocks, as described earlier [13]. Using a rotary micro-
tome (HM 325, MICROM Laborgerate, Germany),
we prepared 1.5-μm thick histological sections.
Immunolocalization of hormones was carried out, as
described earlier [14]. The preparations were analyzed
using a light microscope Axio Imager.A1 (Carl Zeiss
Jena, Germany) equipped with a digital camera Axio-
Cam MRC5 (Carl Zeiss Jena).

The activity of cytokinin oxidase in the roots was
determined as described earlier [7]. Decrease in the
number of isopentenyladenine (IP) as a result of decay
was determined by enzyme-linked immunoassay as
previously described [12] but with the use of specific
antibodies against isopentenyladenosine.

Statistical analysis was performed using Microsoft
Excel. The figures show the average values of the
parameters and their standard errors. Significant dif-
fferences are marked by different letters (*P < 0.5,
,t-test). When measuring the length of the main root by
biological repetitions (n), one plant was used to mea-
sure the length of the main root, and 30 plants were
used to quantify cytokinins.

RESULTS

At ammonium nitrate removal from Pryanishnikov
medium, the weight of Col-0 plants decreased by 29%-
(from 9.7 ± 0.7 to 6.9 ± 0.4 mg in the presence and
absence of nitrogen in the media, respectively).
Replacement of potassium and calcium nitrates with
chlorides in Hoagland–Arnon solution caused a simi-
lar (31%) decrease in weight of Col-0 plants (from
10.9 ± 0.7 to 7.5 ± 0.3 mg in solutions with and with-
out nitrates, respectively). This indicates that growth
suppression was a consequence of nitrogen deficiency
and chloride toxicity was not apparent, probably due
to the low concentration of these anions (1.5 mM).

The roots’ length measurement showed that they
were longer in chl1-5 mutant compared to Col-0 plant
in both Hoagland–Arnon and Pryanishnikov media
(Fig. 1). Removal of NH₄NO₃ from Pryanishnikov
medium resulted in elongation of plant roots of both
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genotypes in comparison with control plants that received nitrogen. Removal of nitrates from Hoagland–Arnon medium was also accompanied by an increase in the length of Col-0 roots; however, this impact did not affect the root lengthening of chl1-5 roots. We recorded a higher content of cytokinins in the roots of Col-0 plants compared to the roots of chl1-5 mutants (Fig. 2). Removal of NH₄NO₃ from Pryanishnikov solution reduced the total content of zeatin derivatives in plant roots of both genotypes. The reaction to the replacement of potassium and calcium nitrates by chlorides in Hoagland–Arnon solution was different: in Col-0 plants, the content of cytokinins in the roots declined, while it remained at the control level (plants receiving potassium and calcium nitrates) in chl1-5 mutants. Comparison of root length and content of cytokinins in plants of different genotypes with a sufficient supply of nitrogen and its deficiency allows us to notice the correlation between hormonal status and growth reaction of plants to genetic modification and removal of nitrogen from the environment. Between the level of cytokinins and the length of roots (expressed as a percentage of indicator values in Col-0 plants that received nitrogen) revealed a high correla-

Fig. 1. Length of the main root of the 18-day-old A. thaliana plants of the original Columbia ecotype and chl1-5 mutant by NRT1.1 transceptor, which received a tenfold diluted standard Pryanishnikov and Hoagland–Arnon solutions 4 days after nitrogen (N−) removal from the medium. Ammonium nitrate was excluded from Pryanishnikov solution, and potassium nitrate and calcium nitrate were replaced with chlorides in Hoagland–Arnon solution (n = 30).

Fig. 2. Total content of the three forms of cytokinins (zeatin (Z), its riboside (ZR) and nucleotide (ZN)) in the roots of the 16-day-old plants of the original A. thaliana ecotype Columbia and mutant NRT1.1 (chl1-5), which received a tenfold diluted Pryanishnikov or Hoagland–Arnon solutions 2 days after removal of nitrogen (N−) from the medium.
The activity of cytokinin oxidase in Col-0 plants reduced with nitrogen deficiency in the medium (440 ± 36 and 253 ± 25 ng IP/(h g FW) in the roots of Col-0 in the presence and absence of nitrates, respectively), unlike chl1-5 mutants, where the enzyme activity level did not depend on the presence of nitrogen in the medium (243 ± 22 and 216 ± 19 ng IP/(h g FW) in the roots of chl1-5 in the presence and absence of nitrates, respectively). The enzyme activity in the roots of Col-0 plants was higher than in chl1-5 mutants.

Immunohistochemical localization of zeatin showed a decline in the intensity of cytokinin staining at the tips of the roots of Col-0 plants upon the removal of nitrates from the solution of Hoagland–Arnon and the absence of significant differences in the level of coloring in the chl1-5 mutants (Fig. 3). A slight decrease in the coloration of chl1-5 root tips compared to Col-0 ones growing on the unmodified Hoagland–Arnon solution was not statistically significant.

CONCLUSIONS

The reduction in the cytokinin content that we registered in the roots of the ecotype Col-0 at the removal of nitrates from the medium (Fig. 2) corresponds to the literature data on the influence of nitrogen levels on the content of cytokinins [3, 6]. In addition, there are only few studies that have determined the content of cytokinins in nitrogen starvation, though the decrease in the level of cytokinins under this effect is widely discussed [2, 15]. The influence of nitrate levels on the content of cytokinins was mainly studied as a reaction to the increase in nitrate concentration in the nutrient medium, and the activation of cytokinin synthesis was shown in these experiments [3]. Only a relatively recent reduction in the content of cytokinins under the impact of nitrate deficiency in A. thaliana plants has been shown [15].

Since cytokinins are attributed an important role in the adaptation of plants to nitrate levels, it is important to understand which sensor systems are responsible for this reaction. Although the sensory function of the NRT1.1 nitrate transceptor was shown by the example of regulating the activity of the genes controlling the transport and metabolism of nitrates [9], the participation of NRT1.1 in the regulation of cytokinin levels was not revealed. This sensor function was only assumed by Kiba et al. [15] based on the results of the analysis of the transcript level of multiple mutant genes by the chl1-5 nitrate sensor performed by Wang et al. [16]. The second supplement to this work shows the reduced level of expression of AtIPT3 and AtIPT4 genes responsible for cytokinin synthesis. We have shown for the first time that a mutation in the gene encoding NRT1.1 actually leads to a reduced content of cytokinin in plant roots (Fig. 2), which confirms the participation of the transceptor of nitrates in maintaining the level of these hormones. The lower level of cytokinins cannot be explained by the increased level of their oxidative degradation, because the activity of cytokinin oxidase in chl1-5 mutants was not higher but lower than in Col-0 plants. Rather, this phenomenon is associated with a lowered level of cytokinin synthesis in the mutant, which corresponds to the literature data on the level of AtIPT3 and AtIPT4 gene expression in this mutant [16]. It is known that cytokinin oxidase activity increases under the action of cytokinins, providing homeostatic control of their level [17]. Consequently, the low cytokinin oxidase activity may be the result of a reduced level of cytokinins, the content of which has declined due to inhibition of their synthesis.

Of particular interest is our data indicating that there are no changes in cytokinin level when removing nitrates from Hoagland–Arnon medium in chl1-5 mutants, unlike Col-0 plants (Fig. 2). These results support the hypothesis that a functionally adequate
NRT1.1 sensor is necessary to perceive nitrate starvation and to trigger reactions aimed at reducing the cytokinin content in plants under this effect. It is important that the removal of NH₄NO₃ from the nutrient medium (as opposed to the removal of potassium and calcium nitrates) led to a decrease in the cytokinin content in the chl1-5 roots as well as in Col-0 roots. The plants have not only a sensor for nitrates but also for ammonium ions. Thus, in A. thaliana, a receptor-like CAP1 protein kinase was found to be involved in the sensing of ammonium concentration and its homeostatic control, as well as to play an important role in the regulation of polar root hair growth [18]. It is known that ammonium level affects the expression of genes controlling cytokinin synthesis [5]. Although the expression of these genes is influenced not by ammonium itself but by its biochemical derivative glutamine [5], the reduced cytokinin content in a mutant deficient in NRT1.1 can be explained by the ability of plants to react to the ammonium levels and its metabolic products when removing ammonium nitrate from the Pryanishnikov medium.

The data of immunohistochemical localization of zeatin at the root tips (Fig. 3) confirmed the main pattern registered by the immunoenzyme analysis (Fig. 2). Thus, the weaker staining of zeatin in the roots of Col-0 transferred to Hoagland-Arnon solution, which does not contain nitrates, indicates a reduction in the level of this hormone at the tips of the roots and is consistent with a lower total content of cytokinins in the roots of these plants. The absence of significant changes in staining in chl1-5 plant is consistent with the quantitative measurement of the total content of cytokinins in the roots (Fig. 2), indicating that the level of cytokinins in the mutant does not depend on the presence of nitrates in the nutrient solution.

Changes in the cytokinin content in the roots under the influence of both genetic factors and changes in the conditions of plant growth were accompanied by corresponding changes in the rate of root elongation. Thus, (1) in the mutant, the lower cytokinin level in the roots was combined with a longer length of roots compared to the plants of the original genotype; (2) the decrease in the content of these hormones in Col-0 plants was accompanied by an increase in the length of the roots under the influence of nitrogen deficiency; (3) root lengthening was enhanced in accordance with a decline in their cytokinin content in chl1-5 plants when ammonium nitrate was removed from the Pryanishnikov medium; and (4) no change in the cytokinin level in the plant corresponded to no change in the length of their roots when removing nitrates from Hoagland–Arnon medium.

These results are quite consistent with the literature data that lowering the cytokinin level in plant roots enhances their elongation by reducing the inhibitory effect of cytokinins on the division of cells of the root meristem [8]. We described longer roots in NRT1.1 plants for the first time. Obviously, this is due to the possibility that the phenotype of this mutant has been studied so far by growing plants on agar plates with sugar addition, which has a specific impact on the development of the root system [19]. We previously showed that hydroponics more adequately imitates the features of root system growth in soil [20].

It is known that mutations by NRT1.1, in some aspects, deprive plants of the ability to feel nitrates, and cytokinins serve as indicators of the availability of nutrients [21]. The data presented above indicate the participation of the nitrate sensor NRT1.1 in the regulation of the level of cytokinins in plants, i.e., in the maintenance of their content in the presence of nitrates and in its decrease in the starvation of these ions. Changes in cytokinin content in the roots correlate with the length of the roots and obviously regulate the rate of their elongation.

**COMPLIANCE WITH ETHICAL STANDARDS**

The study was carried out without using animals and without involving people as subjects.

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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