

THE ROLE OF NITRIC OXIDE (NO), ITS RELATIONSHIP WITH REACTIVE OXYGEN SPECIES (ROS) AND AUXIN AS A RESULT OF COPPER STRESS

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Doctoral (PhD) dissertation theses

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Introduction

Plants are exposed to a number of stress factors in their environment, such as stress caused by accumulated heavy metals (HMs) in the soil. The primary point of attack of metals is the root, as this is where the plant first encounters the metal. That is why HM-induced morphogenetic response phenotype appears primarily in the root system, which manifests itself in three symptoms: the inhibition of the meristem cell division and cell elongation of the primary root, and the re-differentiation of the pericycle cells. As a result of these cell-level processes, the primary root of the root system, manifesting stress-induced morphogenetic response, is short, the number of lateral roots is increased, and root hair develops closer to the root tip. Consequently, plants are able to react to unfavourable environmental conditions by rearranging their organ development, which is called **stress-induced morphological response** (SIMR).

Besides being an essential microelement for plants, **copper** is also a redox-active heavy metal which can be toxic in higher concentration and may cause SIMR. A change in hormonal homeostasis may be found in the background of growth changes. **Auxin** is a major growth regulator that promotes developmental processes both in the shoot and in the root system. That is why changes in auxin homeostasis induced by various stress factors may be partly responsible for morphological changes as well. It is signal molecules that establish a relationship between each of the components in the rich signal transduction network between the outer (environmental) and inner (hormonal) regulatory elements, thus ensuring the synchronization of developmental and growth signals. Similarly to other transitory metals, the generation of various **reactive oxygen species** (ROSs) is catalysed by the redox cycle between the two oxidation states of copper (Cu^+ and Cu^{2+}). ROSs generated as a result of copper stress are known to have a double role, as in higher concentration they may be harmful to the vital processes of cells, while in low concentration they act in the intracellular signal transduction network as a secondary messenger, thus transferring numerous responses in plant cells. **Nitric oxide** (NO) is one of the reactive nitrogen species (RNSs), a diffusible gas signal molecule in plants, which participates in normal developmental processes and plays a role both in biotic and abiotic stress responses. That is why NO may be viewed not as a specific but rather as a general, multifunctional signal molecule.

The important role of auxin and ROSs in heavy metal induced SIMR signal transduction is already known, and as NO, being a multifunctional signal, regulates a number

of root developmental processes and acts closely together with auxin and reactive oxygen species, it can be assumed that NO as a development regulating molecule also appears in the stress-induced morphological response triggered by Cu. In my PhD work, I set out to test this hypothesis. Consequently, in our research, the focus was on the generation, functioning, role and interactions of NO in *Arabidopsis* plants exposed to copper stress. It was examined what effect copper has on the shoots and roots of these plants in relation to time, and what reactions, hormonal and signal transduction background mechanisms may be behind these processes.

Objectives

It was the NO molecule that was in the focal point of the experiments. Its generation, its role played in the signal transduction pathway and its relationship with other reactive molecules (ROS) were examined in the course of morphological responses appearing as a result of copper stress.

During our work, we were looking for the answers to the following questions:

- With relation to time (short and long term experiments), how does the copper-induced morphological response appear with respect to shoot and root?
- Do auxin and nitric oxide play a role in the signal transduction of copper-induced morphological responses?
- What kind of interactions appear between auxin and NO under copper stress?
- What mechanisms can explain the changes appearing in the NO metabolism due to the effect of copper?
- What relationship is there between NO and ROSs in the copper tolerance mechanism, and how do ROSs contribute to the appearance of stress-induced morphological responses?

Materials and methods

Plant materials

- Col-0 wild-type
- *nia1nia2*, *nia1nia2noa1-2* NO-deficient,
- *nox1 (cue1)* and *gsnor1-3* NO-overproducer,
- *vtc2-1*, *vtc2-3* ascorbic acid deficient,
- *miox4* ascorbic acid overproducer *Arabidopsis thaliana* L. plants

- DR5::GUS transgenic *Arabidopsis thaliana* plants were used for the examination of auxin-dependent gene expression

Applied treatments

- 5, 25 and 50 μM copper sulphate (CuSO_4)
- 1mM N^G nitro-L-arginine methyl ester (L-NAME) - NOS inhibitor
- 1mM Na-wolframate - NR inhibitor
- 10 and 100 μM sodium nitroprusside (SNP) - NO donor
- 50 and 100 μM 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxid (cPTIO) -NO scavenger
- 10 μM naphthylphthalamic acid (NPA) - polar auxin transport inhibitor

Applied methods

Morphological measurements: Primary root length, hypocotyl length and petiole length with respect to cotyledon diameter and leaf number. The counting of lateral roots (LRs) was done on DR5::GUS transgenic plants, on which **histochemical staining** was also performed. **Element analysis:** the element content (Cu, Fe, Zn, Mn, Mo, B) of wild-type (Col-0) *Arabidopsis* plants was determined with inductively coupled plasma mass spectrometer (ICP-MS). **Fluorescence spectrometer:** *in vitro* examination of the specificity of DAF-FM, DCF and APF fluorophores with fluorescence spectrophotometer. **Fluorescence microscopy:** detecting RNS and ROS molecules: the NO content was determined with the help of DAF-FM DA, O_2^- with DHE, H_2O_2 with AR, ONOO^- with APF, the intracellular ROS content with $\text{H}_2\text{DCF-DA}$ and viability with FDA fluorescent dye. The meristematic zone (MZ), elongation zone (EZ) and differentiation zone (DZ) of the primary root (PR) and the cotyledon were also examined.

Results

In our work, the copper-induced hormonal (auxin)- and signal (NO) components of growing responses were studied. The examinations were mostly conducted with microscopic methods.

With the usage of wild-type plants, the microelement concentrations (Cu, Fe, Zn, Mn, Mo, B) of the shoot- and the root system and their tissue distribution were determined in case of *Arabidopsis* growing on agar. Based on the results, it can be stated that seedlings are capable of absorbing and accumulating part of the copper present in soil and transfer it to their shoot systems. Exposure to copper significantly modifies the microelement homeostasis of seedlings and the shoot-root distribution of the examined microelements.

The effect of copper exposure on root and shoot development was determined with relation to treatment time, using 7- and 17-day *Arabidopsis* plants. In the **shorter term** experiments, the lowest copper concentration (5 μM) resulted in increased cotyledon area and

increased hypocotyls and PR length, while higher copper exposure (50 μM) caused significant inhibition on shoot and root development because of the accumulated copper. In the **longer term** (17 days), it was observed that copper-induced strong shoot growth inhibition and root elongation inhibition did not occur, which suggests the activation of the acclimatization process in the case of longer term copper exposure. Cell division enabling lateral root initiation proves to be sensitive to copper exposure. In the root systems of 25 μM copper-treated plants, symptoms of SIMR were detected. The appearance of SIMR depends on the concentration and length of time of copper treatment, as it was only longer term, medium-strength (25 μM) stress that generated its appearance.

The change in hormone metabolism and transport plays an essential role in HM-induced developmental responses. As developmental processes are mainly controlled by morphogenes like auxin, we examined *in vivo* the inducible DR5 promoter expression of auxin in DR5::GUS transgenic *Arabidopsis*. In case of **shorter term** copper treatment, compared to the control, a more intense auxin dependent gene expression was found in both the cotyledons and the root of seedlings. In the **longer term** (17 days), copper – similarly to other environmental stress factors (e.g. salt, Cd) – triggered the decrease of DR5 expression in *Arabidopsis* leaves.

Nitric oxide, as a basic component of auxin signal transduction, controls various developmental processes in plants. In our **shorter term** experiments, it was found that as a result of Cu, certain changes also took place in NO levels in both organs. In cotyledons, 5 μM Cu caused significant NO accumulation, while the higher HM load decreased the NO content. In the control situation, the NO-specific fluorescence shows significant tissue specificity in PRs, as much higher NO levels can be detected in the EZ compared to the MZ. In the EZ of the PR, the copper treatment caused a significant NO decrease, while the NO content of the MZ was not affected. The possible mechanisms of changes happening in NO levels were examined with genetic and biochemical methods, and it was found that in cotyledons both the L-arginine and NR dependent biosynthetic pathways can be responsible for copper-induced NO accumulation.

Based on the NO content decrease in the elongation zone of the root, it was hypothesized that Cu-induced O_2^- eliminates NO through the peroxynitrite generating reaction. Our results, however, do not support this hypothesis. The background mechanism of the copper-induced NO level decrease is assumed to be the down-regulation of either or both

biosynthetic pathways (L-arginine and/or nitrate dependent). The **longer term** Cu exposure (for 17 days) resulted in a significant growth regarding the NO content. Apart from the concentration of HM stress, the length of treatment also has an effect on NO generation.

In roots, the main enzymatic source of NO is NR, and this is the enzyme that produces NO under stress conditions. Therefore, the possible participation of this enzyme in copper-induced NO release was examined by genetic method. Contrary to the wild type, in the PR of the NR deficient double mutant *nia1nia2*, copper-induced NO production could not be detected, which refers to the basic role that NR plays in copper-induced NO synthesis.

In our further studies, the objective was to explore the relationship between hormonal (auxin) and signal (NO) components in the signal transduction of copper-induced morphological responses. In the **short term** experiments, it was the effect of auxin on NO levels that was first studied with the application of NPA besides copper treatment and NO levels were detected in both organs. Based on the results, it was concluded that auxin transport is necessary for 5 μ M copper-induced NO accumulation in cotyledons, that is auxin controls the NO synthesis positively in case of low copper exposure. However, in case of higher Cu concentration, it was the lack of auxin that caused NO level increase. Those plants in which NPA decreased the auxin level showed a significantly higher NO fluorescence in the root, compared to those plants that were only treated with copper. This suggests that auxin controls the NO level in the PR negatively. In our experiment, exogenous indol-3-acetic acid (10^{-6} M) induced NO generation neither in cotyledons, nor in the PR. The effect of NO on auxin-dependent gene expression was examined through biochemical methods as well. When the endogenous NO level was enhanced by donor application, auxin sensitive gene expression decreased significantly in cotyledons and PR tips alike. This implies an inhibitory link between the hormonal (auxin) and signal (NO) components of copper-induced morphological changes. These findings were confirmed with genetic examination as well, during which Cu-induced growth response was compared in wild-type, NO over-producer (*nox1*) and NO deficient (*nia1nia2* and *nia1nia2noa1-2*) seedlings. In case of NO excess, smaller cotyledon sizes can be measured, while NO deficient mutants have slightly bigger cotyledons compared to the wild type. What is more, the decrease in the size of cotyledons is more pronounced in case of NO excess. Contrary to this, the elongation of hypocotyl cells in NO deficient mutants shows greater sensitivity compared to wild type, while in *nox1*, there was no morphological response given to copper. However, *nox1* has shorter hypocotyl than the wild type. Regarding PR elongation, the behaviour of mutants is not obvious. Under control conditions, the PR

length of NO over-producer and NO deficient plants is shorter than that of the wild type. Copper treatment did not induce PR shortening in the *nox1* and *nialnia2noal-2* mutants, however, it caused an expressed reduction in the PR length of the NO deficient double mutant. The various root growth responses given to copper in case of NO deficient lines can be explained by the hypothesis that close control NO status is necessary for the regulation of the root architecture. The deviation of NO content from the optimal level results in the inhibition of copper-induced root morphological response.

In our further experiment, the copper tolerance of mutant seedlings (*nox1*, *gsnor1-3* and *nialnia2*) with altered NO metabolism was studied. These plants showed smaller shoot and root sizes and fresh weight (FW) when compared to the wild type, which supposes the necessity of an optimal NO level for proper growth. The *vtc2-3* and *miox4* plants showed wild type-like shoot and root sizes under control conditions. Consequently, the modified ascorbate content and the slightly modified ROS levels resulting from this do not influence significantly the early development of *Arabidopsis*. The lowest CuSO₄ concentration (5 µM) that was applied resulted only in a slight growth in copper content (~80 µg g⁻¹DW), and it did not prove to be toxic for the Col-0 seedlings. On the contrary, the 5 µM copper treatment had a slight growth enhancing effect (non-significant increase of FWs), however, for this effect to manifest, increased NO and ROS levels were necessary in the plants. In case of NO deficient plants, a stronger growth inhibitory effect of copper was observed, compared to the wild type, which reflects its role played in the maintenance of NO growth under abiotic stress conditions.

After that, the vitality of the MZ cells of the root was examined, from which conclusions for copper tolerance could be drawn. In case of mild stress, high NO levels enhanced sensitivity, while in case of higher copper concentration, tolerance was fostered. This means that the signalling function of NO depends on the strength of stress. Under slight copper exposure in *nox1* plants, the negative effect of NO on vitality can be reversed by eliminating high NO content. In case of *vtc2-1* and *vtc2-3* *Arabidopsis*, cell vitality decreased as a result of copper exposure, however, *miox4* root cells proved to be more vital. Based on this, it can be assumed that the higher ROS level, having formed as a result of decreased ascorbate content, leads to copper sensitivity, while the lower ROS content, caused by ascorbic acid overproduction, favours copper tolerance. Higher ROS levels disturb the redox state of cells, which leads to oxidative damage. This presumably increases copper sensitivity.

Biochemical experiments were conducted in order to prove whether NO participates in the formation of copper tolerance. In the course of this, the NO level of plants was modified by applying exogenous NO donor (SNP) and NO scavenger (cPTIO), and the vitality of wild type and NO mutant plants in the root MZ was detected. Based on the results, it can be stated that NO improves copper-induced cell damage under strong copper stress, as NO treated Col-0 plants showed better viability. The high degree of copper tolerance of the NO over-producer mutant and the increased sensitivity of the *nia1nia2 Arabidopsis* also prove the stress mitigating effect of NO. In order for copper tolerance to be formed, a rigidly regulated NO balance is required, as NO content that is higher or lower than the optimal level may lead to cell damage.

As the next step, the possible relationship between NO and ROSs under copper stress was examined by microscopic observation. Higher NO level was accompanied by lower $O_2^{\cdot-}$ level in the control state *Arabidopsis* root tips, and this proved to be true the other way round as well, i.e. in the *nia1nia2* MZ that showed lower NO content, higher $O_2^{\cdot-}$ level could be detected than in the wild type. This refers to the fact that under stress free conditions NO is capable of controlling the $O_2^{\cdot-}$ level negatively, presumably through the chemical reaction leading to $ONOO^-$ generation or through the modification of the activity of antioxidants such as superoxide dismutase (SOD). As a result of Cu, the H_2O_2 levels reduced in the presence of NO excess, based on which it can be assumed that, as a consequence of higher NO level, the expression of antioxidant genes (e.g. glutathione peroxidase or glutathione transferase) increased, so probably it was ROS detoxification that took place. Furthermore, in case of NO deficiency, the $O_2^{\cdot-}$ level reduction was accompanied by H_2O_2 generation, which supposes a possible SOD-dependent detoxification. The altered ascorbate metabolism and, therefore, the altered ROS content (*vtc2-1*, *vtc2-3* and *miox4* mutants) resulted in lower NO content than in the Col-0, which refers to the fact that a rigidly regulated level of ROSs has an effect on the NO metabolism under stress free conditions. Considering the copper-induced, non-significant changes, the effect of ROSs on NO metabolism under copper stress is inessential.

The following conclusions can be drawn from the findings of our work:

- The appearance of copper-induced morphological response in *Arabidopsis* plants depends on the duration and concentration of metal stress. In the long term, the *Arabidopsis* shows copper stress induced developmental induction (increased number of LR).

- Both shorter and longer term copper stress alters the auxin and NO metabolism. In the short term, copper causes excess auxin level increase, while the NO level decreases in seedling roots; in the longer term, the changes are opposite to this.
- The hormonal and signal component in copper-induced SIMR signal transduction, i.e. auxin and NO, are in a mutually negative relationship.
- In cotyledons, both assumed enzymatic NO biosynthetic pathways (L-arginine and NR dependent) are responsible, while NO decrease happens in the PR independently from the generation of peroxynitrite and superoxide radicals. In the longer term, the activity of the NR enzyme is responsible for NO synthesis generated in copper-treated roots.
- In case of stronger copper stress, NO participates in forming the tolerance by regulating the generation of superoxide radicals and H₂O₂ during copper stress. In contrast with NO, ROSs increase copper sensitivity in *Arabidopsis* seedlings.

List of publications

(The publications marked with * are directly related to the PhD dissertation.)

1. * **Andrea Pető**, Nóra Lehotai, Jorge Lozano-Juste, José León, Irma Tari, László Erdei, Zsuzsanna Kolbert Involvement of nitric oxide (NO) in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings. *Annals of Botany* 108:449-457, 2011
IF: 4,030
2. * Zsuzsanna Kolbert, **Andrea Pető**, Nóra Lehotai, Gábor Feigl, Attila Ördög, László Erdei In vivo and in vitro study of fluorophore-specificity *Acta Biologica Szegediensis* p37-41 Vol 56, Number 1, 2012
3. * Zsuzsanna Kolbert, **Andrea Pető**, Nóra Lehotai, Gábor Feigl, László Erdei Long-term copper (Cu²⁺) exposure impacts on auxin, nitric oxide (NO) metabolism and morphology of *Arabidopsis thaliana* L. *Plant Growth Regulation* 68:151-159, 2012
IF: 1,670
4. * **Andrea Pető**, Nóra Lehotai, Gábor Feigl, Nóra Tugyi, Attila Ördög, Katalin Gémes, Irma Tari, László Erdei, Zsuzsanna Kolbert Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis* *Plant Cell Reports* 32:1913-1923, 2013
IF: 2,509
5. Nóra Lehotai, **Andrea Pető**, Zsuzsanna Kolbert, László Erdei In vivo and in situ visualization of early signaling events induced by heavy metals in pea root meristem *Acta Physiologiae Plantarum* 33:(6) pp. 2199-2207. 2011
IF: 1,639
6. Réka Szöllősi, Erika Kálmán, Anna Medvegy, **Andrea Pető**, Ilona Sz. Varga Studies on oxidative stress caused by Cu and Zn excess in germinating seeds of Indian mustard (*Brassica juncea* L.) *Acta Biologica Szegediensis* p175-178 Vol 55, Number 1, 2011

7. Ágnes Szepesi, Katalin Gémes, Gábor Orosz, **Andrea Pető**, Zoltán Takács, Mária Vorák, Irma Tari Interaction between salicylic acid and polyamines and their possible roles in tomato hardening processes *Acta Biologica Szegediensis* p165-166 Vol 55, Number 1, 2011
8. Nóra Lehotai, **Andrea Pető**, László Erdei, Zsuzsanna Kolbert The effect of selenium (Se) on development and nitric oxide levels in *Arabidopsis thaliana* seedlings *Acta Biologica Szegediensis* p105-107 Vol 55, Number 1, 2011
9. Zsuzsanna Kolbert, **Andrea Pető**, Réka Szöllősi, László Erdei, Irma Tari Nitric oxide (NO) generation during vegetative/generative transition of the apical meristem in wheat *Acta Biologica Szegediensis* p95-97 Vol 55, Number 1, 2011
10. Nóra Lehotai, Zsuzsanna Kolbert, **Andrea Pető**, Gábor Feigl, Attila Ördög, Kumar Devanand, Irma Tari, László Erdei Selenite-Induced Hormonal and Signalling Mechanisms during Root Growth of *Arabidopsis thaliana* *L. J. Exp. Bot.* 63:(15) 5677-5687, 2012 **IF: 5,242**

Conference papers and posters

1. **Andrea Pető** Studying of copper-induced shoot and root morphological responses in *Arabidopsis* plants Young Plant Biologist Lecture Series of Hungarian Plant Biological Society, 2011. január 17. Szeged SZBK
2. **Andrea Pető** Studying of relationship of reactive oxygen- and nitrogen species as well as auxin in *Arabidopsis* plants for the effect of copper treatment Biologist PhD Conference in Szeged, 21-22. May 2012
3. **Andrea Pető** The role of nitric oxide (NO) during copper stress and its relationship with reactive oxygen species (ROS) Young Plant Biologist Lecture Series of Hungarian Plant Biological Society, 25. January 2013. Szeged
4. **Andrea Pető**, Andrea Farsang, László Erdei Biological indicators for the studying of urban pollution: The heavy metal content analysis of *Taraxacum officinale* Weber ex Wiggers letter in Szeged, 8th Hungarian Ecological Congress 26-28 August 2009, Szeged, Hungary p181
5. Zsuzsanna Kolbert, **Andrea Pető**, László Erdei In vivo imaging of reactive oxygen (ROS) and nitrogen species (RNS) during copper-induced root morphological responses in *Arabidopsis*. 3rd International Plant NO Club, 15-16 July, 2010. Olomouc, Czech Republic p33
6. **Andrea Pető**, Nóra Lehotai, László Erdei, Zsuzsanna Kolbert Metal content and nitric-oxide (NO) production in the roots of heavy metal-treated pea plants. 11th International Symposium Interdisciplinary Regional Research (ISIRR), 13-15 October, 2010 Szeged, Hungary p105