

Abstract of Ph.D. Thesis

**ROLE, GENERATION AND SOURCE OF NITRIC OXIDE (NO) IN
ROOTS UNDER AUXIN TREATMENT AND OSMOTIC STRESS**

ZSUZSANNA KOLBERT

Supervisor: Prof. Dr. László Erdei

University of Szeged
Faculty of Science and Informatics
Department of Plant Biology

Biology Ph.D. School

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INTRODUCTION AND AIMS

A marvelous property of plants is the rapid and efficient adaptability to dynamically variable environment. Plants regulate growth and development of their organs (e.g. root system) acted on the actual status of the environment (e.g. water supply). Beside environmental factors, the endogenous hormonal system (auxin and other hormones) of the root is the major regulator of developmental processes. In the rich signal transduction network between the external and internal regulatory elements, signal molecules make contact between the components to ensure the phasing of signals.

The main goal of our laboratory was to investigate this binary regulatory system manifested itself under root development. We compared the effects of a notable stress factor, osmotic stress and an endogenous regulator, auxin on root growth and development. We paid special attention on investigation of nitric oxide (NO) in this system, since we supposed that this novel, bioactive molecule can be a key component of the signal network regulating root development.

In my thesis I present the results which intend to answer the following questions:

- Is there a similarity between the effects of exogenously applied auxin and osmotic stress on root morphology?
- Is NO synthesis induced by auxin or osmotic stress in roots?
- Are there any differences between the degree and the temporal properties of NO production induced by auxin or osmotic stress?
- Which enzyme can be the possible candidate for generating auxin- or osmotic stress-induced NO?

MATERIALS AND METHODS

Plant material

- *Pisum sativum* L. cv. Rajnai törpe
- *Triticum aestivum* L. cv. GK Öthalom
- *Arabidopsis thaliana* L. wild type (Col-1 and Col-0)
- *Arabidopsis thaliana* L. *pxa1* mutant
- *Arabidopsis thaliana* L. *Atnoa1* mutant
- *Arabidopsis thaliana* L. *nia1*, *nia2* mutant

Determination of root growth parameters (lateral root number, primary root length)

Detecting NO by fluorescent microscopic method with 4,5-diaminofluorescein diacetate (DAF-2DA)

RESULTS

Among several NO detection manners one of the most frequently used is the fluorescent microscopic determination of NO by DAF-2DA, since it makes possible *in vivo* and *in situ* visualization of it in plant tissues. Root segments and cross-section were dyed with DAF-2DA and they were investigated under Zeiss Axiovert 200M-type fluorescent microscope.

I. The effects of exogenous auxin and osmotic stress on root development and NO content of roots

The basis of our experimental results is constituted by the recognition, that the developmental responses of roots to auxin and osmotic stress are similar. In both cases, the reduction of primary root (PR) length reached approximately 50% of the control and the increase of number of lateral roots (LRs) were 180% under osmotic stress and about 280% at the highest indole-3-butyric acid (IBA) concentration. Despite the similarities in characteristics of root growth and development under osmotic stress and auxin load, there is a significant difference in the intensity of the accompanying NO generation: while the maximal value is three-fold in the roots with the high auxin load (at 10^{-5} M IBA), osmotic stress response resulted in 14-fold increase of NO level at 400mOsm (-0.98 MPa) polyethylene glycol (PEG 6000) concentration. In both cases LR initiation was followed by an intensive enhancement of NO production. The wide concentration range of exogenously applied IBA made possible to investigate the binary effect of it on root growth and development: low concentrations of IBA (10^{-9} M- 10^{-8} M) were favorable for PR elongation, which was not followed by NO accumulation. Higher IBA concentrations (10^{-7} M- 10^{-3} M) were responsible for LR development and induction of NO generation. The increase in LR number was directly proportional to the auxin concentration, but NO fluorescence showed a maximum in 10^{-5} M IBA-treated roots.

We found that the intensive NO formation is closely linked to LR development, since transferring plants from a solution containing high concentration of IBA to low hormone

containing nutrient solution, elongation of LR primordia and decrease of NO fluorescence were detected. In the case of a conversely treatment NO levels increased in roots and new primordia appeared on PRs. According to these results we established that there is a close relationship between auxin content (modified by exogenous IBA treatments) and NO formation in roots.

As we stated, under osmotic treatments root development was similar to that of IBA treated plants. The question arose if under osmotic stress acropetally transported auxin was responsible for the intensified formation of LR and NO synthesis. Using the auxin polar transport inhibitor, naphthylphthalamic acid (NPA), the number of LR initials and the NO level were decreased, while the growth of PR practically was not affected. The relative low inhibition of LR development by NPA can be explained by the fact that NPA is not an inhibitor of transport of IBA but it indicates that at least for a low extent, indole-3-acetic-acid (IAA) can also be responsible for LR formation.

We investigated the time dependence of PR elongation, LR initiation and NO formation under osmotic stress or exogenous auxin. The elongation of primary root proved to be equal in time, and this constant, linear growth of PR was effected neither by auxin nor by PEG treatment. The lateral root initiation is more interesting, because as the effect of IBA treatment the short LR initials appeared only after the 48th hours of treatment. Interestingly, the situation is similar under osmotic stress, namely the number of the 400 mOsm PEG induced LRs increased after 48 hour- treatment. The most exciting result was that under osmotic stress a rapid phase of NO generation was distinguishable which is followed by a slower but more significant NO accumulation. In exogenous auxin treated roots this fast NO generation could not be found. In these plants NO formed only after the 48th hour of treatment parallel with appearance of laterals. The osmotic stress induced transient NO accumulation was named as “stress-NO”. We found this early NO burst also in PEG-treated *Arabidopsis* and wheat roots, which refers to the general nature of this phenomenon.

To answer the question, whether the early NO burst has a role in PEG-induced LR development, we suppressed the PEG-induced transient NO burst with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid (cPTIO) in the first 36 h. These samples showed decreased LR number compared to control and 400 mOsm PEG-treated roots in the 120th h of treatment. This strongly suggests that “stress-NO” is needed to PEG-induced LR development.

Indole-3-butyric acid is a natural auxin form in several plant species (pea, maize, tobacco, *Arabidopsis*). However there are contradictory results in literature concerning that

IBA acts via IAA, or it has its own auxin effect. This question has great importance in our experimental system, namely the discovered NO accumulation in LR primordia is the direct effect of IBA or not. For investigation of this we used peroxisomal fatty acid β -oxidation-deficient *pxa1 Arabidopsis* mutant, in which IAA is not able to convert IBA. In these control mutant roots higher NO fluorescence was found compared to wild type (Col-0). The reason of this difference can be that mutants contain more IBA than wild type roots, because conversion of it to IAA does not happen (however literature data are not available). Treatment with 10^{-5} M IBA resulted in enhancement of NO formation in mutant laterals comparably to wild type, which suggests the own auxin effect of IBA on NO production.

The effects of exogenous auxin and osmotic stress on root development and NO content of roots can be summarized as follows:

1. Osmotic stress and exogenous auxin treatment induced similar changes in root morphology of pea: i.e. increasing concentrations of both exogenous auxin and polyethylene glycol caused shortening of PRs and enhancement of LR number.
2. Both factors induced NO accumulation in roots, however temporal properties of NO formation were different. As the effect of osmotic stress, a two-phase NO generation was detectable, where a rapid, transient NO formation (“stress-NO”), could be distinguished followed by a slower, but more significant NO accumulation. These two periods of PEG-induced NO synthesis was found also in roots of wheat and *Arabidopsis thaliana* L. Transient NO peak was not observed in auxin-treated roots.
3. “Stress-NO” is required for osmotic stress-induced LR formation of pea.
4. IBA directly (not through its conversion to IAA) induces NO formation in LR initials of *Arabidopsis thaliana* L.

II. Possible enzymatic source of auxin- and osmotic stress-induced NO

After we worked out the details concerning involvement of NO in root developmental processes, we tried to identify the possible source of NO. For these experiments wild type (Col-1), *Atnoa1* mutant (deficient in mitochondrial GTP-ase activity) and nitrate reductase (NR)-deficient double mutant (*nia1, nia2*) *Arabidopsis* plants were used. Our results clearly show that exogenously applied IBA caused 4-fold increase in lateral root frequency of wild type and *Atnoa1* plants. The same concentration of auxin resulted in only 2-fold enhancement of LR frequency in the case of NR-deficient mutant. In wild type *Arabidopsis* IBA-induced NO fluorescence was restricted to the LR initials in contrast to PR sections, where it remained at the control level. The role of NR in auxin-induced NO accumulation was firstly proved by the fact that tungstate, a potent inhibitor of the enzyme decreased NO levels in LR primordia and N^G-monomethyl-L-arginine (L-NMMA) and the inhibitor of mammalian NOS enzyme had no effect on it. Beside this, LNMMA treatment did not influence the basal NO level of root cells, while tungstate decreased it. Mutant plants showed different basal levels of NO in their control state (without treatment). In *nia1, nia2* mutant roots lower NO fluorescence compared to the other two groups was detected. This suggests NR-dependent NO synthesis in roots of *Arabidopsis*. Exogenous IBA was able to induce NO generation in wild type and *Atnoa1* LR initials, but it failed to do so in *nia1, nia2* roots. Since two phases of NO generation were distinguishable in PEG-treated *Arabidopsis*, we had to separately examine the sources of NO generated in the two processes. Within 24 hours after osmotic treatment, LNMMA did not inhibit NO formation in wild type laterals, and “stress-NO” appeared in NR-deficient mutant. This led us to the conclusion that generation of early NO transient is independent from the activities of NOS and NR enzymes. The second phase of PEG-induced NO formation is NR-associated, because in roots of *nia1, nia2* double mutant IBA treatment did not result in enhanced NO level in contrast to wild type and *Atnoa1* roots, where NO fluorescence significantly increased after auxin treatment.

Our results obtained in association with possible enzymatic source of auxin- and osmotic stress-induced NO can be concluded as follows:

1. Exogenous auxin-induced NO synthesis is NR-associated in *Arabidopsis thaliana* L. root primordia.

2. Generation of “stress-NO” under osmotic stress is independent from NOS or NR activities. Presumably, early NO is formed by non-enzymatic pathways.

3. The second phase of PEG-induced NO formation is obviously related to the activity of nitrate reductase enzyme.

Our results detailed above were published in international, referred papers (see publication list). According to our best knowledge this is the first dissertation, in which details are worked out regarding to the role and enzymatic source of NO in root developmental processes regulated by auxin or osmotic stress.

LIST OF PUBLICATIONS

(Present thesis is based on articles marked by an asterisk)

***Kolbert Zs**, Bartha B, Erdei L. (2008) Exogenous auxin-induced NO synthesis is nitrate reductase-associated in *Arabidopsis thaliana* root primordia. *Journal of Plant Physiology* 165: 967-975 IF: 2,239

***Kolbert Zs**, Bartha B, Erdei L. (2008) Osmotic stress- and indole-3-butyric acid -induced NO generations are partially distinct processes in root growth and development in *Pisum sativum* L. *Physiologia Plantarum* 133: 406-416 IF: 2,192

***Kolbert Zs**, Bartha B, Erdei L (2005) Generation of nitric oxide in roots of *Pisum sativum*, *Triticum aestivum* and *Petroselinum crispum* plants under osmotic and drought stress. *Acta Biologica Szegediensis* 49(1-2):13-16

Bartha B, **Kolbert Zs**, Erdei L. (2005) Nitric oxide production induced by heavy metals in *Brassica juncea* L. Czern. and *Pisum sativum* L. *Acta Biologica Szegediensis* 49(1-2):9-12

***Kolbert Zs**, Erdei L. (2008) Involvement of nitrate reductase in auxin- induced NO synthesis. *Plant Signaling & Behavior* 12: 1-2

*Erdei L, **Kolbert Zs**. (2008) Nitric oxide as a potent signaling molecule in plants. *Acta Biologica Szegediensis* 52:1-5

***Kolbert Zs**, Sahin N, Erdei L. (2008) Early nitric oxide (NO) responses to osmotic stress in pea, *Arabidopsis* and wheat. *Acta Biologica Szegediensis* 52: 63-65

Csiszár J, Pintér B, **Kolbert Zs**, Erdei L, Tari I. (2008) Peroxidase activities in root segments of wheat genotypes under osmotic stress *Acta Biologica Szegediensis* 52: 155-156

Posters

Bartha B, **Kolbert Zs**, Sági B, Erdei L. (2005) Nitric oxide production induced by Cd, Cu and Zn in *Brassica juncea* and *Pisum sativum*. Cost Action 859 Phytotechnologies to promote sustainable land use and improve food safety, 14-16 June 2005, Pisa, Italy

Kolbert Zs, Erdei L. (2006) Relationship between nitric oxide (NO) and auxin during root processing of *Pisum sativum* L. III. EPSO Conference, 28 May-1 June 2006, Visegrád, Hungary

Erdei L, Bartha B, Vashegyi Á, **Kolbert Zs**. (2006) Interaction between heavy metals and macroelements: review and a case study. Cost Action 859 Phytotechnologies to promote sustainable land use and improve food safety, 22-24 June 2006, Santiago de Compostela, Spain

Kolbert Zs, Erdei L. (2006) Time -and concentration dependence of exogenous auxin- effect on NO generation and root development properties in pea. XV. FESPB Congress, 17-21 July 2006, Lyon, France

Kolbert Zs, Bartha B, Erdei L. (2007) Root development under osmotic stress and in the presence of exogenous auxin in *Pisum sativum* L.: The role of nitric oxide. World Conference of Stress, 23-26 August, 2007, Budapest, Hungary

Kolbert Zs, Erdei L (2008) Osmotic stress- induced nitric oxide (NO) in drought tolerant and sensitive wheat cultivars and its source in *Arabidopsis* mutants. XVI. FESPB Congress, 17-22 August 2008, Tampere, Finland

Conference lectures

2005 8th Hungarian Congress of Plant Physiology

2008 Congress of National Federation of PhD Students

2008 9th Congress of the Hungarian Society of Plant Biology

Grants

Regional Committee in Szeged, Hungarian Academy of Sciences Year 2006 **I. Award**

Frank-Helianthus Non-Profit Foundation, Cereal Research Company, Szeged Year 2007

III. Award

National Association of PhD Students, “*Pro Patria et Scientia*”, Hungary, Year 2008

I. Award

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