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Nitric oxide modifies root growth by S-nitrosylation of plastidial glyceraldehyde-3-phosphate dehydrogenase

Jinzheng Wang*, [1], Yu Wang [1], Qiang Lv, Lei Wang, Jing Du, Fang Bao, Yi-Kun He**

College of Life Sciences, Capital Normal University, Beijing 100048, China

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ABSTRACT

Nitric oxide (NO) plays an essential role in a myriad of physiological and pathological processes, but the molecular mechanism of the action and the corresponding direct targets have remained largely unknown. We used cellular, biochemical, and genetic approaches to decipher the potential role of NO in root growth in *Arabidopsis thaliana*. We specifically demonstrate that exogenous application of NO simulates the phenotype of NO overproducing mutant (*nox1*), displaying reduced root growth and meristem size. Using root specific cell marker lines, we show that the cell in the cortex layer are more sensitive to NO as they show enhanced size. Examination of total S-nitrosylated proteins showed higher levels in *nox1* mutant than wild type. Using an in vitro assay we demonstrate that plastidial glyderaldehyde-3-phosphate dehydrogenase (GAPDH) is one of NO direct targets. The function of GAPDH in glycolysis provide a rational for S-nitrosylation of this enzyme and its subsequent reduced activity and ultimately reduced growth in roots. Indeed, the rescue of the root growth phenotype in *nox1* by exogenous application of glycine and serine, the downstream products of plastidial GAPDH provide unequivocal evidence for mechanism of NO action through S-nitrosylation of key proteins, thereby delicately balancing growth and stress responses.

1. Introduction

Nitric oxide (NO), a conserved *trans*-organismal signaling molecule regulates multiple physiological processes. In plants specifically, NO is involved in a range of physiological processes including seed germination, flowering, stomatal closure and senescence [1–5]. Additionally, NO plays a pivotal role in stress responses against abiotic stresses [6,7], as well as biotic stimuli [8–11]. More recently, through a mutant screen, the function of NO in regulating level of phytohormone cytokinin and potassium uptake was elucidated [12,13].

Two distinct enzymatic pathways of NO synthesis have been extensively studied, namely: the nitrite-dependent nitrate reductase and the arginine-dependent NO synthase-like pathway [10,14,15]. It is well established that nitrate reductase generates NO, whereas the existence and relevance of NO synthase remains controversial.

Plastidial gene, *NOA1*, which encodes a GTPase, can promote NO accumulation indirectly [16,17], suggesting plastids as the likely localization site of NOA1-associated NO production pathway [18]. Interestingly, NO overproducing mutant (*nox1*) with a dysfunctional *PPT1*, originally identified as *chlorophyll a/b binding protein underexpressed1 (cue1)* [19–21], is hyper-sensitive to the NO donor, nitroprusside (SNP), and has higher NO levels in the mutant leaves as compared to wild type [4]. Those reports support the critical role of plastids in NO production.

The cysteine of target proteins can be S-nitrosylated by nitric oxide [22]. S-nitrosylation similarly to phosphorylation shows substrate specificity and reversible posttranslational modification [23–25]. Proteomic approaches have revealed that many proteins are S-nitrosylated, and the modification changes occur in S-nitrosoglutathione reductase (GSNOR) mutant or in wild type under the conditions such as responding to pathogens [26–29].

To understand the physiological function of NO, we focused on the mechanism of action of NO in controlling root growth by using a combination of exogenous NO application, in concert with two alleles of *PPT1* mutant, known as *cue1* and *nox1*, that contain high endogenous levels of NO. These studies reveal that NO reduces

* Corresponding author.
** Corresponding author.
E-mail addresses: jinzheng006@gmail.com (J. Wang), yhe@mail.cnu.edu.cn (Y.-K. He).
[1] These authors contribute equally to this work.

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meristem size but increases cell size predominantly in cortex layer in roots, suggesting some degree of cell specificity. Additionally, we show that NO modifies platidial GAPDH to regulate primary root growth.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana ecotypes mutants *cue1-5* and *nox1* are Columbia (Col-0) background, which were described before [4]. Cellular marker lines are reported as follows:*J0571*(C24) (made by Jim Haseloff) and *SCRpro::GFP-SCR*(Col-0) [30].

The seeds were grown in half-strength MS media in square petri dishes (10 cm side). NO donor, sodium nitroprusside (SNP) was added on the lid with solidified agar to release NO vapors. For NOS inhibitor aminoguanidine (AMG) and amino acid treatment, all these chemical materials were supplemented in the 1/2 MS medium directly, and SNP was added in the lid.

2.2. Root length and meristem size measurement

Seedlings were grown for 5 or 7 days, pictures were taken for analysis of root length using ImagJ software. To measure meristem size, HCG solution [31] was used for mounting samples. The samples were observed with a microscope equipped with Nomarski optics. The number of meristematic cortex cells was measured using microscopic pictures as described [32]. Roots were counterstained with 10 μg/mL PI (Sigma-Aldrich) and analyzed with a Leica SP2 inverted confocal microscope following the protocol of Heidstra et al. (2004) for cell identity analysis.

2.3. Determination of endogenous NO content

Endogenous NO level of roots was detected by NO fluorescent probe -amino-5-methylamino-2′,7'-difluororescein diacetate (DAF-FM DA; Molecular Probes #D23842).

2.4. Saville-Griess assay

SNO content measurement was mainly based on the Saville-Griess assay [33]. SNO content was measured using photometric absorption at 540 nm using 96-well plates with photometric Reader (Thermo). The SNO content was determined according the GSNO concentration standard curve absorption at 540 nm [33].

2.5. Protein extraction, expression and purification of recombinant proteins

The coding sequence of GAPCp1 was cloned into the EcoR I and Hind III sites of pGEX-KG (Amersham Bioscience) Vector. The whole constructs were transformed into *E.coli* BL21(DE3) pLyss Chemically Competent Cell (TransGen Biotech), and expression of the fusion protein was induced by 1 mM IPTG 2 h at 37 °C. The induced

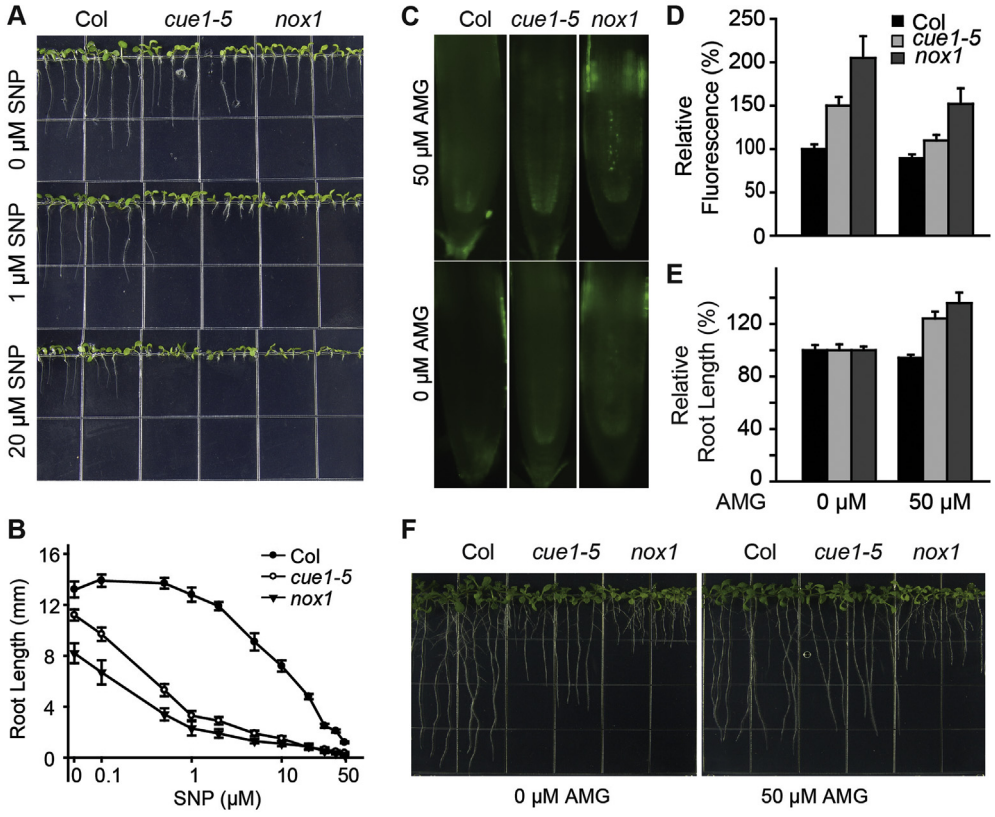

Fig. 1. Exogenous and endogenous NO inhibits root growth. (A) Root growth of Col, *cue1-5* and *nox1* mutants in response to NO donor SNP. (B) The effect of different concentration of SNP on root growth of wild type Col-0, *cue1-5* and *nox1*. All the experiments were repeated for three times. (mean ± SD; n = 30 seedlings). (C) The endogenous NO levels in Col-0 and *cue1-5, nox1* mutants without or with aminoguanidine (AMG) treatment, a NO-synthase inhibitor. Roots were stained with DAF-FM DA. Fluorescence was analyzed with the same exposure time by confocal microscopy. (D) Relative NO fluorescence intensity. The intensity of roots was calculated using wild type Col as control. (mean ± SD; n = 10 seedlings). (E and F) Root growth phenotype after AMG treatment. (mean ± SD; n = 30 seedlings).