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SHORT COMMUNICATION

Nitrate reductase mediates nitric oxide-dependent gravitropic response in *Arabidopsis thaliana* roots

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ABSTRACT

Plant roots respond positively to gravity force and orientate it growth providing anchorage to the soil and gathering water and nutrient sources. The gravitropic response is a complex process wherein nitric oxide (NO) participates as a key signaling molecule. Here, we used genetically impaired genotypes to demonstrate the role of the nitrate reductase (NR) enzyme as a possible source of endogenous NO during gravitropic response in *Arabidopsis thaliana* (*A. thaliana*) roots. *A. thaliana* has two NR genes, *NIA1* and *NIA2*. The single mutants *nia1* and *nia2*, and the double mutant *nia1/nia2* showed perturbed gravitropism. Complementation with the exogenous NO donor, S-nitroso-L-cysteine, partially rescued the wild-type phenotype in *nia2* and *nia1/nia2* but not in the *nia1* mutant. Our findings showed that each *NR* gene differentially contributes to reaching the optimum level of NO during the gravitropic response, suggesting that NIA1 and NIA2 isoforms are not equivalent and have potential regulatory feedback to each other during the gravitropic response in *A. thaliana* roots.

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Text

Plant gravitropism involves the bending of roots in response to gravity. Positive gravitropism enables roots to grow downwards providing anchorage and facilitates soil exploration to take up water and mineral ions required for plant growth and development. Both signaling molecules, auxin and NO, are involved in gravitropism control.[1,2] Gravitropism requires coordinated and asymmetric cell elongation of the lower and the upper sides of roots.[3] A recent study in *A. thaliana* has showed that the asymmetric accumulation of NO between the upper and the lower sides of the root tip is critical for gravitropism.[4] Diminished endogenous NO levels reduced and even reversed gravitropism, resulting in root upward bending.[4] NO is a multifunctional lipophilic signaling molecule able to diffuse through membranes and possess a wide range of physiological functions.[5] The best characterized NO production pathway in plants involves the reduction of NO_2^- to NO via different non-enzymatic or enzymatic mechanisms. The NR enzyme (EC 1.6.6.1) is one of the most important enzyme-based pathways for NO biosynthesis in plants.[5–8] In addition to its reductase activity on nitrate (NO_3^-) to form nitrite (NO_2^-), NR is also able to catalyze the reduction of NO_2^- to NO.[9] Mitochondrial electron transport chain and plasma membrane-bound nitrite-NO reductase (NiNOR) are other enzymatic systems that perform reductive NO production in plants. Oxidative mechanisms include animal NO synthase-like enzyme (NOS like), polyamines and hydroxylamines.[10] The *A. thaliana* NR-impaired lines *nia1*, *nia2*, and *nia1/nia2* have been used to establish the involvement of NR in NO production.[9–12] Double mutant *nia1/nia2* have only 0.5% of wild-type shoot NR activity and display very poor growth on media with NO_3^- as the unique form of nitrogen.[13] Particularly, in seedlings, NIA2 isoform is responsible for 90% of the total NR activity, whereas NIA1 accounts for the remaining 10%. Both genes, *NIA1* and *NIA2*, are expressed in leaves and roots; however, in roots, *NIA1* is expressed at higher levels than *NIA2*.[14] Primary root growth[9] and root hair development[15] is reduced in *nia1/nia2* mutant and can be restored by exogenous NO application.

NR is necessary during early gravitropism in *A. thaliana* roots

To determine the contribution of NR to the gravitropic response, we adopted a genetic approach using single and double NR mutants. Firstly, we confirmed the endogenous NO level in roots using the cell-permeable NO-sensitive fluorophore 3-amino, 4-aminomethyl-2,7-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes, USA) described by París et al. (2018).[4] Roots of *nia2* and *nia1/nia2* displayed an endogenous NO level reduction of 19% and 37.5% in comparison to wt roots, respectively (Figure 1). However, *nia1* roots did not show a statistically significant change in NO-associated fluorescence (Figure 1). These data are in agreement with previous results described in roots of *nia1/nia2* seedlings, and with the decrease of endogenous NO reported in leaves of *nia2* and *nia1/nia2*.[11,16] Next, we took advantage of the decreased NO production mediated by NR in *nia1*, *nia2, and nia1/nia2* to investigate how endogenous NO levels

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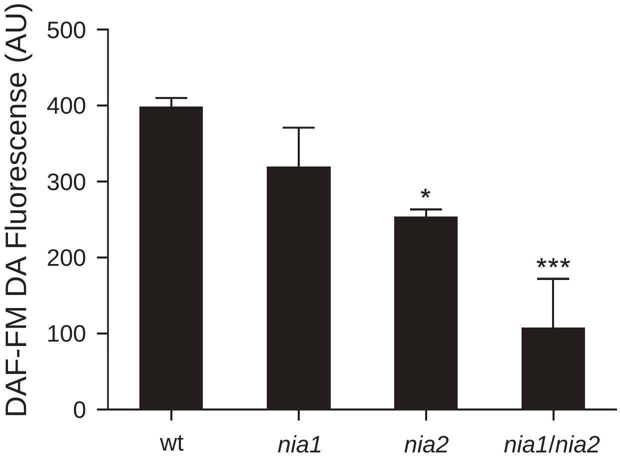

Figure 1. Nitric oxide quantification in roots of NR mutant lines.

Four-day-old wt, *nia1, nia2* and *nia1/nia2 A. thaliana* seedlings were loaded with 10 µM DAF-FM DA probe as described by París et al. (2018)[4]. Roots were observed using a confocal microscope (Zeiss LSM333) 20 x objective, NA = 0.8, with an argon ion laser set at 488 nm; emission was collected between 490 and 561 nm. Associated fluorescence was quantified using FIJI.[17] Means (±SE) are shown. P values in comparison to wt were calculated with two-tailed Mann–Whitney test, **p* < 0.05 and ****p* < 0.0001. The average values of 2 independent experiments were analyzed, *n* = 13.

affect root gravitropism over time. Four-day-old *A. thaliana* seedlings were grown vertically and then rotated 90 degrees to induce gravistimulation. Images were taken sequentially every 30 min for 480 min to calculate the root tip angle relative to the horizontal line. Compared with wt plants, at 120 min after the onset of gravistimulation a statistically significant decrease in the average value of the root tip angle was detected in the three selected NR mutants (Figure 2). At 480 min after gravistimulation, the average values of tip angles were 80°, 60°,

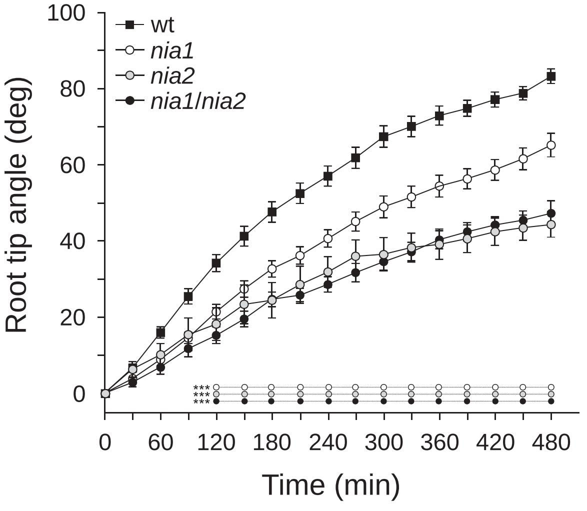

Figure 2. Root tip angle of NR mutants after gravistimulation.

Wild-type, *nia1, nia2,* and *nia1/nia2 A. thaliana* seedlings were grown vertically on 0.5x Murashige and Skoog salt (MS), 0.8% agar and 1% sucrose for 4 days. Seedlings were transferred to fresh MS medium and plates were mounted in front of a photo scanner (Epson Perfection V600) and let set for 60 min before 90-degree rotation. For each time series of individual seedlings, root tip angle was measured on digital images using FIJI.[17] Means (±SE) are shown. P values in comparison to wt were calculated with two-tailed Mann–Whitney test, ****p* < 0.0001. The average values of at least four independent experiments were analyzed, *n* ≥ 20.

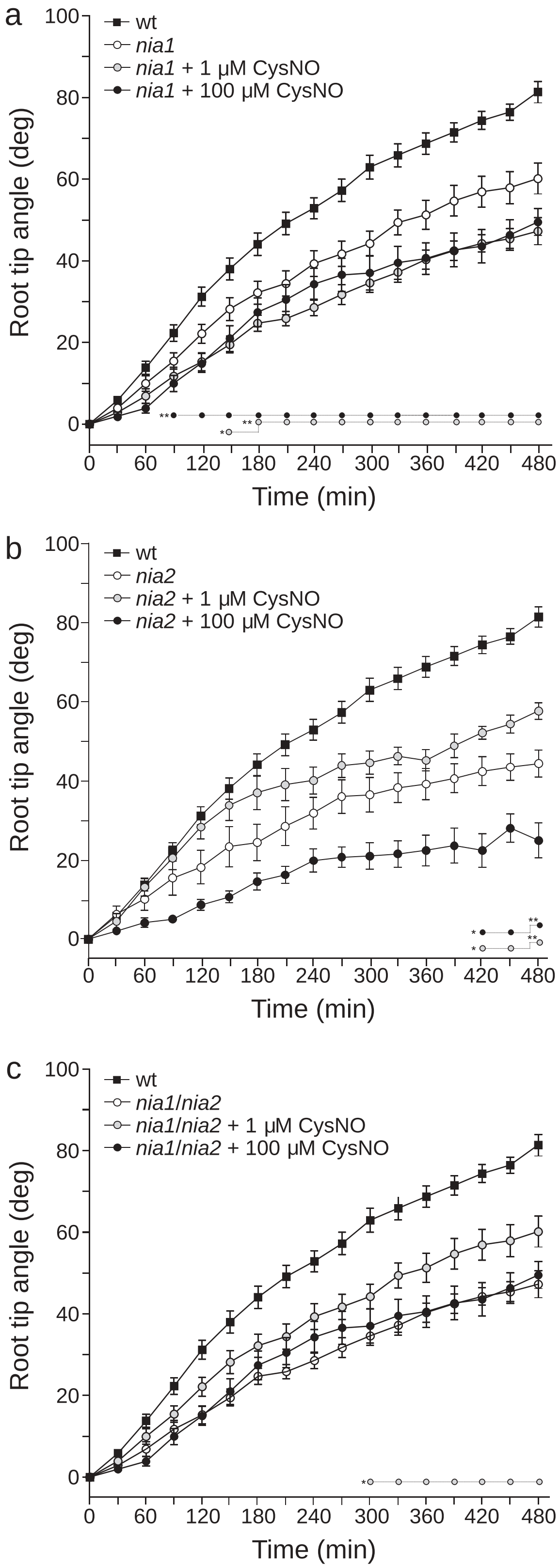

Figure 3. Tip angle of NO-treated roots mutants after gravistimulation.

Four-day-old seedlings (a.) *nia1*, (b.) *nia2* and (c.) *nia1/nia2*, were transferred to fresh MS medium, containing 1 µM or 100 µM of CysNO, or water as a control. Solutions were also poured at the surface of each root to ensure homogeneous absorption and action. Stock solutions of CysNO were prepared as previously described.[18] A gravitropic assay was performed as described in Figure 1. P values in comparison to untreated mutant were calculated with two-tailed Mann–Whitney test, **p* < 0.05 and ***p* < 0.005. The average values of at least four independent experiments were analyzed, *n* ≥ 20.

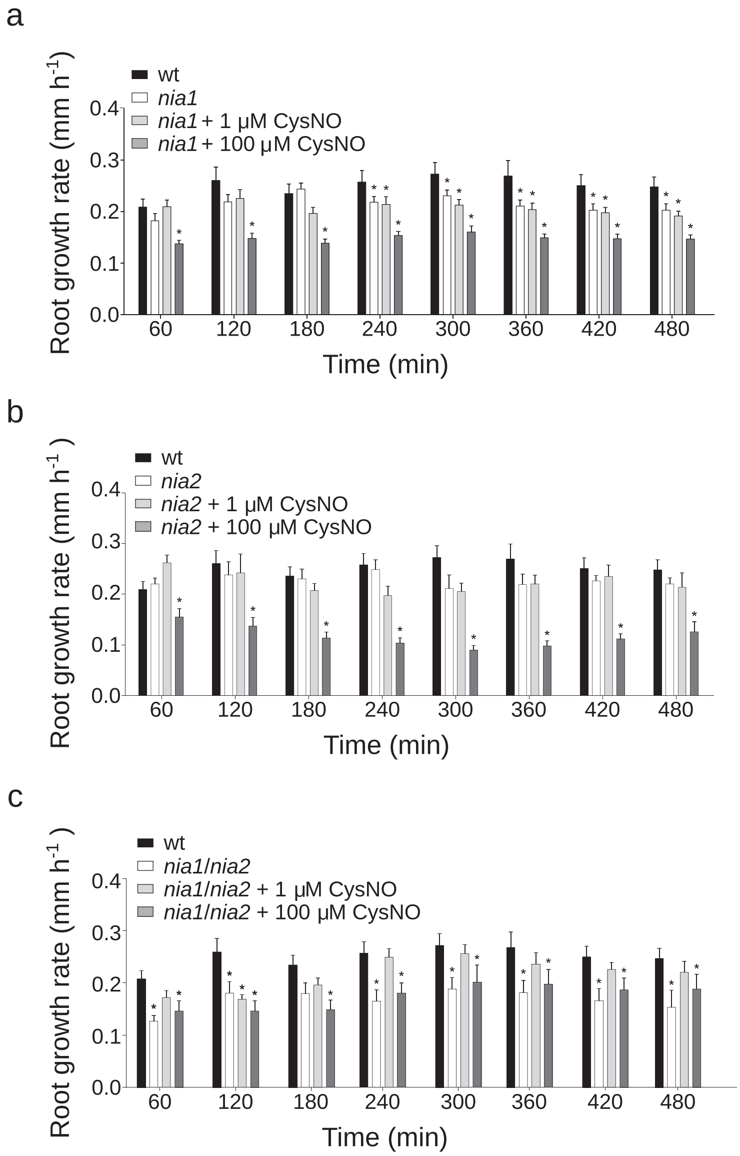

Figure 4. Growth rate of graviestimulated NO-treated root mutants.

Four-day-old seedlings (a.) *nia1*, (b.) *nia2* and (c.) *nia1/nia2*, were grown and treated as described in Figure 3. Roots were monitored every 60 min for 480 min. For each time series of individual roots, root tip position and distance were measured on digital images using FIJI[17] to calculate average values (±SE) of growth rate expressed as mm h^{-1}. P values in comparison to wt were calculated with two-tailed Mann–Whitney test, $^{*}p \leq 0.05$. The average values of at least four independent experiments were analyzed, $n \geq 20$.

43°, and 45° in wt, *nia1*, *nia2*, and *nia1/nia2* roots, respectively.

NO partially rescued the phenotype of NR mutants under gravitropism

Before gravistimiluation, we treated *nia1*, *nia2*, and *nia1/nia2* roots with the physiological NO donor, S-nitrosylated version of L-cysteine (CysNO). Four-day-old seedlings were placed on supplemented plates with 1 or 100 µM CysNO and water as a control, and let to set vertically for 60 min before 90-degree rotation. Later, the root tip angle was recorded every 30 min for each root as described above. We found that both *nia2* and *nia1/nia2* altered gravitropic response was partially rescued by the addition of 1 µM CysNO. At 480 min after gravistimulation, root tip angle increased 14° and 12° for *nia2* and *nia1/nia2* respectively compared with control (Figure 3(b,c)). However, the application of CysNO did not rescue the *nia1* gravitropic deficient phenotype (Figure 3(a)). Finally, 100 µM CysNO did not recover the gravitropic defect of any mutant. Additionally, the *nia1/nia2* mutant has a reduced shoot and root growth rate, suggesting that NO is able to participate in critical physiological processes.[11] To assess whether the NO production by NR is affecting the growth rate during the gravitropism, we measured the position of the root tip every 60 min. Both *nia1* and *nia1/nia2* mutants displayed a significantly lower growth rate during the gravitropic response in comparison to wt plants (Figure 4(a,c)). However, *nia2* mutant did not show a significant decrease in the growth rate (Figure 4(b)). Interestingly, *nia1/nia2* growth was rescued with the addition of 1 µM CysNO (Figure 4(c)); meanwhile, the *nia1* phenotype was not rescued under our experimental conditions (Figure 4(a)). High CysNO concentrations of 100 µM were in detriment of the growth rate for the wt and the three mutant lines tested (Figure 4). Our study demonstrates that NR activity by NIA1 and NIA2 is likely to underlie the endogenous NO level required in *A. thaliana* gravitropism. In addition, this work shows that the functional contributions of each *NR* gene affect the gravitropic response. Thus, we speculate that NR isoforms have potential regulatory feed to each other during gravitropism. In leaves, a comparative study between the single mutants, *nia1* and *nia2* have shown that NIA1 despite being expressed at significantly lower levels than NIA2, is the most prominent NR enzyme in guard cells.[19] Here, we demonstrated that despite *nia1* mutant has a subtle reduction on the endogenous NO level, *nia1* gravitropic response was diminished significantly. Additionally, *nia2* mutant showed a similar reduction than *nia1/nia2* double mutant on the gravitropic response. Both mutant phenotypes were partially rescued by exogenous NO treatment (Figure 3(b,c)), suggesting that mutations on *nia1* and *nia2* did not build an additive effect on this response. In consequence, our results reveal an uncharacterized signaling role for NR in *A. thaliana* during gravitropic response and support the idea that probably a tightly adjusted timing, concentration, and localization is necessary for NO action during gravitropism.

In this work, we showed that simple and double NR mutants are not completely impaired in root bending (Figure 2). In soybean primary roots both NOS-like enzyme and NR have been proposed to regulate the gravitropic root response.[2] If complementarily to NR, NO generation by NOS-like, NiNOR, and mitochondrial nitrite reduction could be participating during the signaling of gravitropic response in *A. thaliana* roots has to be demonstrated. Moreover, we could not rule out the possible participation of other nitrate metabolism-dependent signals during gravitropism. Further biochemical, cellular and genetic investigations on NO metabolism and signaling during the gravitropic response would contribute to elucidate this complex tropic response.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Abbreviations

CysNO	S-nitroso-L-cysteine
DAF-FM DA	3-amino, 4-aminomethyl-2,7-difluorofluorescein diacetate
NO	nitric oxide
NR	nitrate reductase

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