

Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport

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Nitric oxide (NO) is considered a key regulator of plant developmental processes and defense, although the mechanism and direct targets of NO action remain largely unknown. We used phenotypic, cellular, and genetic analyses in *Arabidopsis thaliana* to explore the role of NO in regulating primary root growth and auxin transport. Treatment with the NO donors S-nitroso-N-acetylpenicillamine, sodium nitroprusside, and S-nitrosoglutathione reduces cell division, affecting the distribution of mitotic cells and meristem size by reducing cell size and number compared with NO depletion by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Interestingly, genetic backgrounds in which the endogenous NO levels are enhanced [chlorophyll a/b binding protein underexpressed 1/NO overproducer 1 (*cue1/nox1*)] mirror this response, together with an increased cell differentiation phenotype. Because of the importance of auxin distribution in regulating primary root growth, we analyzed auxin-dependent response after altering NO levels. Both elevated NO supply and the NO-overproducing *Arabidopsis* mutant *cue1/nox1* exhibit reduced expression of the auxin reporter markers *DR5pro::GUS::GFP*. These effects were accompanied by a reduction in auxin transport in primary roots. NO application and the *cue1/nox1* mutation caused decreased PIN-FORMED 1 (PIN1)-GFP fluorescence in a proteasome-independent manner. Remarkably, the *cue1/nox1*-mutant root phenotypes resemble those of *pin1* mutants. The use of both chemical treatments and mutants with altered NO levels demonstrates that high levels of NO reduce auxin transport and response by a PIN1-dependent mechanism, and root meristem activity is reduced concomitantly.

cell division and elongation | plant growth regulator | root development

Nitric oxide (NO) is a signaling molecule involved in a variety of physiological processes during plant growth and development and also is an important modulator of disease resistance. Extensive research has shown that NO is involved in the promotion of seed germination, photomorphogenesis, mitochondrial activity, leaf expansion, root growth, stomatal closure, fruit maturation, senescence, and iron metabolism (as reviewed in ref. 1). NO also is important for defense response, playing key roles in the activation of defense genes (e.g., pathogenesis-related protein 1), in phytoalexin production, and in modulation of programmed cell death (1–3). The mechanism for NO signal transduction, plant resistance to pathogens and cell death, cellular transport, basic metabolism, and photosynthesis frequently occurs through an NO-induced change in transcription (4).

Additionally, NO is produced in plant tissues by two major pathways, one enzymatic and the other nonenzymatic (5). The enzymatic pathway of NO production is being studied thoroughly, and much information about the type and subcellular localization of the enzymes involved is available. Different enzymes have been identified that catalyze the synthesis of NO from two different substrates, nitrate and arginine. The first enzyme identified was nitrate reductase, which usually reduces nitrate to nitrite but also is able to reduce nitrite to NO using NADPH as a cofactor. Another key enzyme in NO biosynthesis is *Arabidopsis thaliana* NO-associated (AtNOA1), previously described as catalyzing the

conversion of L-arginine to L-citrulline (6). Recent biochemical reports demonstrate a GTPase activity for this enzyme (7, 8). AtNOA1 is localized in the plastids and has a putative role in ribosome assembly (9). Other enzymes, including xanthine oxidase/dehydrogenase and cytochrome P450, have been suggested occasionally as sources for NO (10). Experimental evidence also suggests a nonenzymatic pathway to produce NO based on the reduction of nitrite to NO at acid pH, mainly in the apoplast of the aleurone cell layer during seed germination (11).

One mechanism of NO action in plant tissues may be the redox-based posttranslational modification of target proteins through S-nitrosylation. NO is able to modify thiol groups of specific cysteine residues in target proteins reversibly and thereby alter protein function. Previous proteomic profiling in plants has identified a number of S-nitrosylated proteins (12). Recent results support the S-nitrosylation of key proteins such as nonexpressor of pathogenesis-related genes 1 (13) and the *Arabidopsis thaliana* salicylic acid (SA)-binding protein 3 (14), both of which are involved in SA-dependent defense responses. The stability of peroxiredoxin II (15) and iron regulatory protein 2 is regulated by S-nitrosylation via the ubiquitin–proteasome pathway (16). In animals, this posttranslational modification also has been shown to cause protein degradation via the ubiquitin-dependent proteasome pathway.

Despite its relevance as a plant growth and stress regulator, our current knowledge about the mechanism of NO action is still limited. Therefore, the identification and characterization of NO targets at the molecular level is essential for deeper insight into this pathway. Here we uncovered a role for NO on primary root growth in *Arabidopsis thaliana*. We found that NO treatment affects meristem size in the primary root mainly by decreasing cell-division rates and promoting cell differentiation. A reduction in meristem size also is observed in the NO overaccumulating mutant chlorophyll a/b binding protein underexpressed 1/NO overproducer 1 (*cue1/nox1*). Interestingly, the root apical auxin maximum is altered after NO addition. Auxin transport and the level of the auxin efflux protein PIN-FORMED 1 (PIN1) are reduced significantly in the *cue1/nox1* background. Consistently, the distorted organization of the quiescent center and surrounding cells of *cue1/nox1* mutants mimics to some extent the phenotype of *pin1*-mutant roots, suggesting a link between NO and auxin signaling in maintaining the size and activity of the root apical meristem.

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Results

Effect of NO on *Arabidopsis* Primary Root Growth. Exogenous application of NO donors in tomato (17) and genetic mutants with altered endogenous NO levels in *Arabidopsis* (18) indicated that NO affects root architecture, reducing overall primary root growth. However, our knowledge of the molecular mechanisms by which NO regulates growth and development in *Arabidopsis* is still fragmentary.

To investigate the role of NO in the regulation of primary root growth in *Arabidopsis*, WT (*Arabidopsis thaliana* ecotype Columbia-0, Col-0) plants were germinated on plates containing different concentrations of NO released by the specific NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). As shown in Fig. 1A, the inhibition was dose dependent because a gradual decrease in the length of the primary root (from 0.8 ± 0.1 to 0.3 ± 0.1 cm) was observed as SNAP levels increased from 0–1 mM. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) partially blocked this NO effect, clearly establishing that NO is the major contributor to the effect of SNAP on root growth (Fig. 1A). To validate our results by using other NO donors, we tested sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), delivery of NO gas, and mutants with high levels of endogenous NO (*cue1/nox1*). The observed inhibition of primary root growth seemed to be independent of the NO source, because different NO donors and the *cue1/nox1* mutant had similar effects

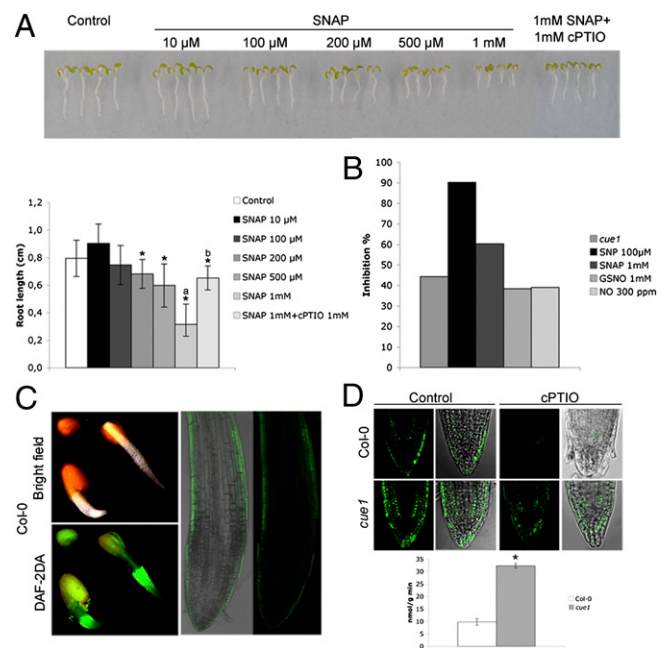


Fig. 1. Effect of NO in the regulation of *Arabidopsis* primary root growth. (A) (Upper) Photograph showing the length of the primary root of WT (Col-0) seedlings grown for 7 d on Murashige and Skoog (MS) agar plates that were untreated (Control) or supplemented with 10 μ M, 100 μ M, 200 μ M, 500 μ M, or 1 mM of the NO donor SNAP or with 1 mM SNAP plus 1 mM of the NO scavenger cPTIO. (Lower) Measurements were obtained 4 d after the treatment of 3-d-old seedlings. Values represent the mean of 30 measurements $\pm$ SD. Asterisks indicate significant differences compared with the untreated control ($P < 0.05$) (a) and with 1 mM SNAP ($P < 0.05$) (b). (B) Inhibition of primary root growth after delivery of NO gas (300 ppm), by treatment with the NO donors SNP (100 μ M), SNAP (1 mM), or GSNO (1 mM) and in mutants with high levels of endogenous NO (*cue1/nox1*). Measurements were taken 4 d after the treatment of 3-d-old seedlings ($n = 25$). (C) Detection of endogenous NO production using DAF-2DA. Plants were grown for 2 d (Upper and Lower Left) or 7 d (Right) on agar plates and then subjected to DAF-2DA incubation. (D) (Upper) Detection of endogenous NO production using DAF-2DA in WT (Col-0) and *cue1/nox1* seedlings in control conditions and after NO scavenging by cPTIO. (Lower) Measurement of NO levels in WT (Col-0) seedlings and the *cue1/nox1* mutant. Asterisk indicates a statistically significant difference from the WT.

(44.3% inhibition in the *cue1/nox1* mutant and 90.2%, 60.2%, and 38.3% inhibition under 100 μ M SNP, 1 mM SNAP, and 1 mM GSNO treatments, respectively) (Fig. 1B).

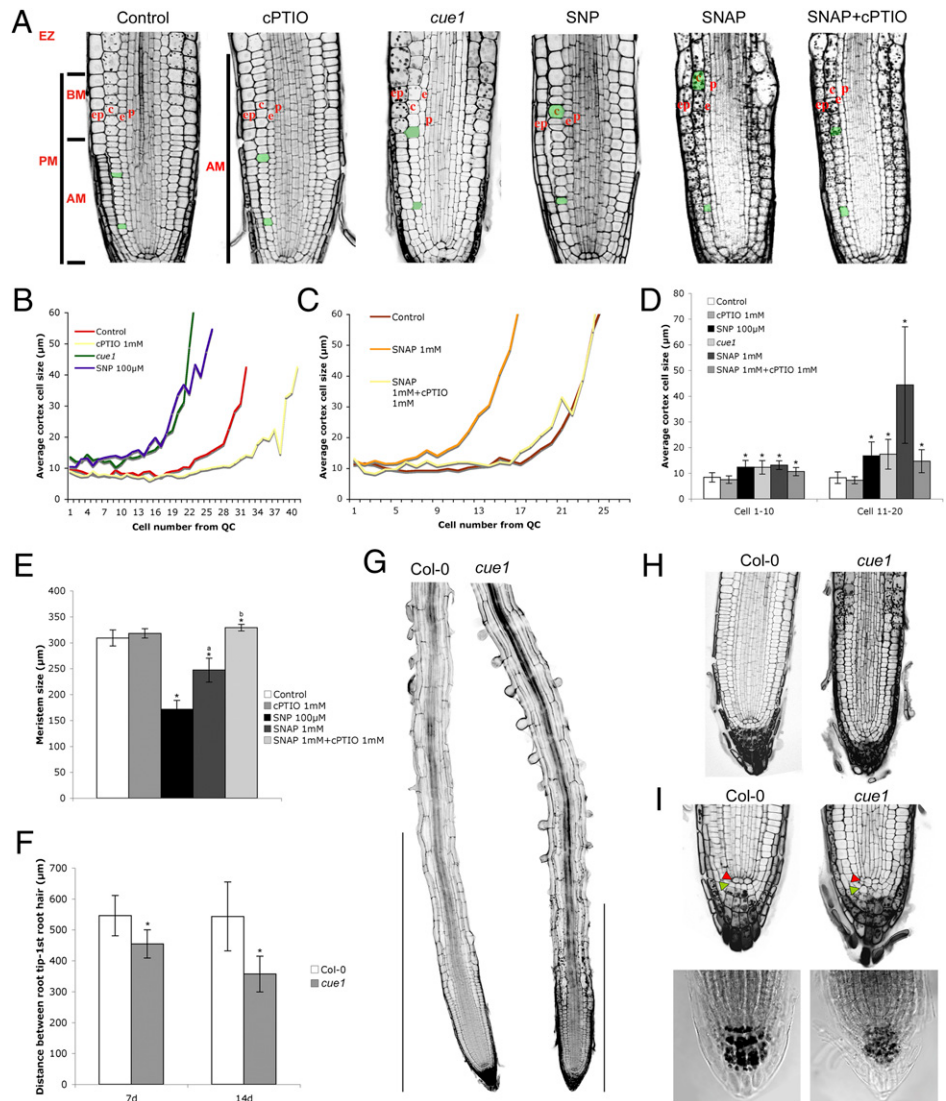
Determination of Endogenous NO Abundance and Distribution in *Arabidopsis* Roots. Sites of NO and other reactive oxygen species production in plant tissues can be identified by using the fluorescence indicator 4,5-diaminofluorescein diacetate (DAF-2DA) (17, 19). DAF-2DA is a cell-permeable compound hydrolyzed inside the cells that emits fluorescence when nitrosylated by endogenous NO. By examining the endogenous NO levels in multiple 2-d-old WT roots with DAF-2DA, we identified NO-dependent fluorescence in the basal meristem and rapid elongation zone in young primary roots (Fig. 1C). DAF-2DA staining of 7-d-old WT roots revealed that NO production was localized mainly in the root apex, as described previously (17). In addition, the maximum NO level was restricted mainly to epidermal cell files closest to the basal meristem, lateral root cap, and cortex/endodermal initial cells (Fig. 1D). Application of the NO scavenger cPTIO reduced NO-dependent DAF-2DA fluorescence (Fig. 1D and Fig. S1). In the *cue1/nox1* background NO accumulation in these tissues was increased threefold or more.

Increases in NO Concentration Reduce the Number of Dividing Cells and Promote Early Differentiation in the Primary Root Meristem. To determine whether the inhibition of primary root growth after NO treatment might be related to differences in the number of cells and/or cell size in the root meristem, we measured the size and number of root cells in a cortical cell file, determining the length of cells from the initials adjacent to the quiescent center (QC) to the rapid elongation/differentiation zone. We found that the total number of cells between the QC and the start of the rapid elongation zone in the cortex layer is affected significantly by altered NO levels. The inflection point on the cell-length curve marking the transition to the rapid elongation zone occurs around cell numbers 17, 33, and 28 in SNP-treated, cPTIO-treated, and control plants, respectively (Fig. 2A and B). Analysis of the *cue1/nox1* mutant revealed results similar to those obtained after SNP treatment with an inflection point around cell numbers 17–18 (Fig. 2A and B), suggesting that NO levels are correlated inversely with the number of cells in the meristematic zone. Interestingly, significant differences in cell sizes in the root apical meristem also were detected at this stage (cells 1–10 and cells 11–20 in Fig. 2D). As shown in Fig. 2A and C, application of the NO scavenger cPTIO partially blocked the action of the NO donor SNAP, clearly establishing that NO levels are correlated positively with cell elongation (Fig. 2A). Hence, in the initial phases of root growth after germination, increases in NO concentration reduce the size of the primary root meristem (309.2 ± 15.6 μ m in control plants vs. 318 ± 9.1 μ m in cPTIO-treated, 171.3 ± 17.4 μ m in SNP-treated, and 247.2 ± 22.9 μ m in SNAP-treated plants, and 329.0 ± 6.55 μ m in plants treated with SNAP plus cPTIO; Fig. 2E) by promoting cell elongation in the root meristem and concurrently decreasing the number of dividing cells. Remarkably, long-term treatment (up to 5 d) with the NO donor SNP almost abolished the pool of dividing cells and enhanced cell elongation in all cell types of the root meristem (Fig. S2).

Early-differentiation phenotypes also are present in the *cue1/nox1*-mutant background. In agreement with the reduced size of the primary root meristem, development of epidermal root hairs and premature vacuolization commence much closer to the initials in *cue1/nox1* than in WT (Col-0) roots (Fig. 2F–H and Fig. S3). A distorted organization of QC and columella stem cells (CSC) is clearly visible in the *cue1/nox1* mutant along with a different pattern of starch granule accumulation, suggesting that the increase in NO levels causes abnormal differentiation in this genetic background as compared with WT (Col-0) (Fig. 2I).

High Levels of NO Reduce the Overall Distribution of Mitotic Cells in *Arabidopsis* Primary Roots. Because NO affects primary root growth by reducing the pool of dividing cells, we marked cells in

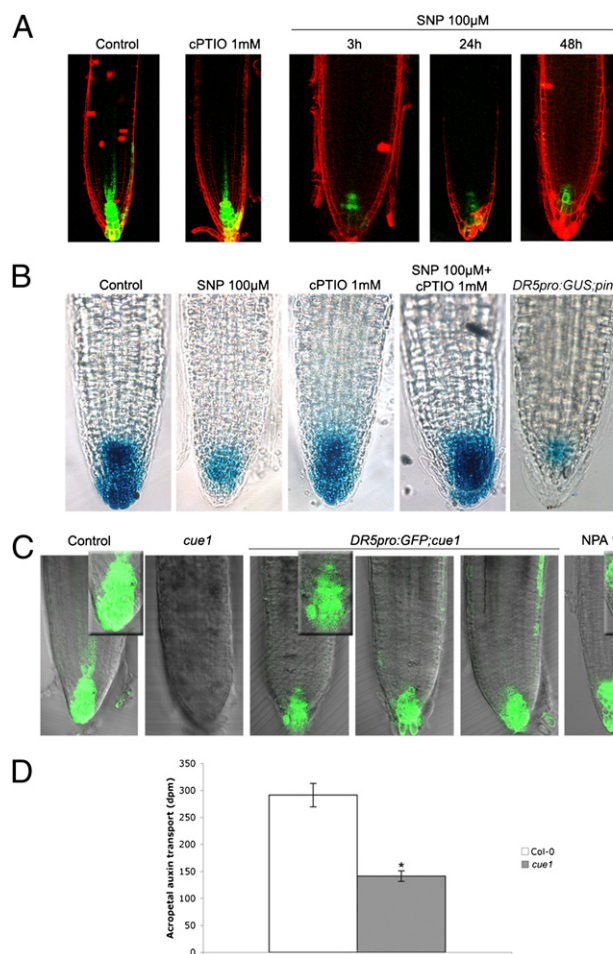
Fig. 2. Effect of NO on the *Arabidopsis* root meristem. (A) Confocal images of roots from seedlings grown for 5 d on unsupplemented MS agar plates (Control), *cue1*-mutant seedlings, or WT seedlings supplemented with 100 μ M of the NO donor SNP, 1 mM of the NO scavenger cPTIO, 1 mM SNAP, and 1 mM SNAP plus 1 mM cPTIO. Vertical lines indicate apical (AM) and basal regions (BM) of the primary meristem region (PM). Cells 1 and 15 from the QC are highlighted in green. Note that meristematic cells are enlarged in the presence of NO and that the start of net elongation is further from the QC in cPTIO-treated seedlings than in untreated controls. (B and C) Cell sizes in the cortical layer of the root. (B) Average cell size in the cortical cell layer (cells 1–40 from QC) of untreated WT seedlings, WT seedlings treated with SNP or cPTIO, and untreated *cue1*-mutant seedlings. (C) Average cell size in the cortical layer (cells 1–40 from QC) of WT (Col-0) seedlings grown as described above and seedlings that were untreated (control) or treated with SNAP or with SNAP plus cPTIO. Measurements were taken 2 d after the treatment of 3-d-old seedlings. (D) Average cortical cell sizes are shown for cells 1–10 and 11–20 (counted from the QC) of the seedlings in A. (E) Size of root meristem in WT (Col-0) seedlings grown for 5 d on unsupplemented MS agar plates (Control) or on medium supplemented with 1 mM of the NO scavenger cPTIO, 100 μ M of the NO donor SNP, 1 mM of the NO donor SNAP, or 1 mM SNAP plus 1 mM of the NO scavenger cPTIO. A minimum of five roots per treatment was analyzed. Asterisks indicate significant differences compared with untreated control ($P < 0.05$). (F) Quantification of the distance between the root tip and the first root hair formed in WT (Col-0) and *cue1*-mutant seedlings. A minimum of 8–10 roots per genotype was analyzed. Asterisks indicate significant differences compared with untreated WT ($P < 0.05$). (G) Representative images of the root tip and the first root hair formed in WT Col-0 and *cue1*-mutant seedlings. (H and I) Representative images of the root meristem of 7-d-old WT (Col-0) and *cue1*-mutant seedlings stained with modified pseudo-Schiff propidium iodide (mPS-PI) (Upper) or Lugol's solution (Lower) highlighting vacuolization (H) and QC/CSC disorganization and starch accumulation (I). Red and green arrowheads indicate QCs and CSCs, respectively.



the G2 stage of the cell cycle with the reporter *CycB1;I_{pro}:GUS-DB* (β -glucuronidase-destruction box) to analyze further the effect of NO on cell division. We observed the effect of modulating NO accumulation on the expression of this reporter. SNP and SNAP treatments reduced the number of cells expressing the *CycB1;I_{pro}:GUS-DB* reporter (Fig. 3A). However, the overall distribution of mitotic cells was not affected significantly by cPTIO treatment. In contrast, cPTIO partially rescued the effect of NO donors on the zone of *CycB1;I_{pro}:GUS-DB* expression (Fig. 3A). Furthermore, we observed a decrease in *CycB1;I_{pro}:GUS-DB* expression in the *cue1/nox1* background, where endogenous NO levels are enhanced (Fig. 3B and Fig. S4). To determine whether an increase in NO level was able to diminish the normal rate of cell division in the root apical meristem, we measured the number of mitotic events in the root apical meristem in *cue1/nox1*-mutant and WT plants. DAPI staining followed by microscopic analysis revealed a significant reduction in the mitotic activity of *cue1/nox1* (Fig. S4). Taken together, these results led us to conclude that high levels of applied or endogenous NO cause a reduction in cell-division rates and an increase in cell lengths consistent with NO positively regulating the exit of cells from the primary root meristem into the elongation and differentiation zones (Fig. 2).

Increasing NO Levels Affects Auxin Response and Reduces Auxin Transport. Spatial patterns of auxin response based on auxin gradients are important factors in the regulation of many plant developmental processes, including cell division, elongation, and differentiation during primary root growth. Using the auxin response reporter *DR5_{pro}:GUS/GFP*, we observed that increasing NO levels after application of the NO donor SNP attenuated DR5 activity in the QC and CSC at 3, 24, and 48 h after treatment (Fig. 4A). The NO scavenger cPTIO partially rescued the depletion of *DR5_{pro}:GUS* expression in roots treated with SNP (Fig. 4B). Similar to the expression pattern of *DR5_{pro}:GUS* in the *pin1* mutant (Fig. 4B), the *DR5_{pro}:GUS/GFP* spatial pattern was altered in *cue1/nox1* mutants, where endogenous NO levels are enhanced (Fig. 4C and Fig. S5), clearly establishing that increasing NO accumulation depletes auxin-dependent reporter expression in the apical auxin maximum. This alteration of auxin-dependent response in the meristematic zone is different from that produced by application of the auxin transport inhibitor naphthylphthalamic acid (NPA) (Fig. 4C).

To determine whether the effect of high levels of NO on the auxin distribution and root meristem activity could be related to NO regulation of polar auxin transport, we tested acropetal



mulation of PIN1 in NO-treated *PIN1^{pro}:GFP-PIN1* seedlings after the 26S proteasome was inhibited by MG132 (Fig. 5B). Therefore, we propose that the PIN1 level is regulated by NO and that high levels of endogenous or applied NO promote reductions in PIN1 protein levels by a proteasome-independent mechanism.

***cue1/nox1* Mutant Root Phenotypes Resemble Those of *pin1* Mutants.** To analyze further the relevance of PIN1 reductions in root responses to NO, we analyzed the root phenotype of *pin1* mutants under standard growth conditions and in response to NO donors (Fig. 5 D–F and Fig. S8). The distorted organization of the QC, surrounding cells, and CSC of the NO-overproducing *cue1/nox1* mutant is similar to that of *pin1* mutants (Fig. 5D). Additionally, the root meristem size (Fig. 5E) and total primary root length (Fig. 5F) of *pin1* mutants resembles plants treated with NO donors and the *cue1/nox* mutant. Interestingly, *pin1* mutants do not display a visible NO-resistant phenotype, instead exhibiting a hypersensitive phenotype under low concentrations of SNAP (200 μ M) (Fig. S8). Conversely, supplying exogenous auxin (IAA or

We also examined fluorescence of GFP fusions to the auxin efflux carriers PIN1 and PIN2 in the presence of the NO donors SNP, SNAP, and GSNO and the NO scavenger cPTIO (Fig. 5A and Fig. S6). Confocal time-course analysis showed that PIN1-GFP fluorescence was decreased clearly in the stele and primary root meristem upon treatment with the NO donors SNP, SNAP, and GSNO. Using immunoblot analysis with anti-GFP antiserum, we also detected a reduction of GFP protein in *PIN1_{pro}::GFP-PIN1* seedlings after treatment with NO donors (Fig. 5B). In agreement with these results, *PIN1_{pro}::GFP-PIN1* fluorescence clearly was reduced in genetic backgrounds where endogenous NO levels are enhanced (Fig. 5C), consistent with the conclusion that increases of NO levels reduce PIN1 levels. Interestingly, PIN1 levels were not altered significantly in lateral root primordia after treatment with cPTIO or SNP or in the *cuel1/nox1*-mutant background, suggesting that regulation of PIN1 levels by NO is restricted exclusively to the primary root (Fig. S6). PIN2 levels in the *PIN2_{pro}::GFP-PIN2* line were not altered significantly in any of the previous treatments (Fig. S6), suggesting that NO regulation of auxin transport in the primary root is specific to the acropetal transport stream and is mediated by changes in PIN1 protein levels.

NO-Dependent Reductions in PIN1 Protein Level Do Not Require Proteasome Activity. We conducted quantitative RT-PCR (qRT-PCR) and proteasome inhibitor experiments to understand the mechanism of NO-dependent reductions in the levels of PIN protein. qRT-PCR analysis showed that *PIN1* transcript levels were not altered significantly after treatment with the NO donor SNP or by mutations in *CUE1/NOX1* (Fig. S7). To determine if PIN1 protein was degraded by the proteasome after NO treatment, we treated *PIN1_{pro}:GFP-PIN1* seedlings with the known proteasome inhibitor MG132, both in the presence and absence of NO (Fig. 5 A and B). PIN1 and PIN2 levels in MG132-treated plants did not differ significantly from that in untreated plants or in plants treated with the NO scavenger cPTIO (Fig. 5 A and B and Fig. S6). Interestingly, *PIN1_{pro}:GFP-PIN1* plants treated both with an NO donor (SNP or SNAP) and with MG132 displayed a low GFP signal not different from that in plants treated only with the NO donor. Furthermore, using immunoblot analysis with anti-GFP antiserum, we could not detect changes in the accu-

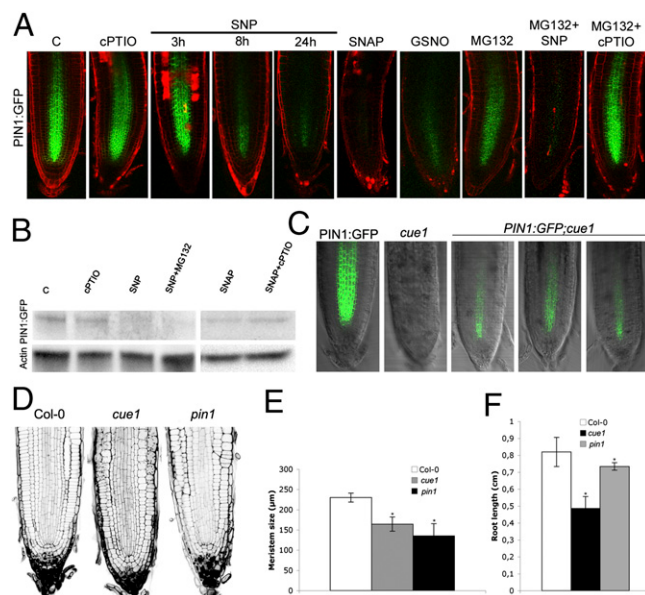


Fig. 5. Disappearance of PIN1 after NO treatment and comparison of *cue1* and *pin1* root phenotypes. (A) Distribution of *PIN1_{pro}:GFP-PIN1* protein is shown in untreated control plants (C), in plants treated with the NO scavenger cPTIO (1 mM; 8 h), and in plants treated with the NO donors SNP (100 μM; 3, 8, and 24 h), SNAP (1 mM; 24 h), or GSNO (1 mM; 24 h), with or without the proteasome inhibitor MG132 (100 μM; 24 h). Root tissues were stained with propidium iodide. (B) Immunoblot analysis with anti-GFP antiserum of in vivo levels of PIN1 protein in root extracts of *PIN1_{pro}:GFP-PIN1* seedlings, in the absence or presence of NO donors and scavengers together with MG132. Actin protein levels also were determined as a loading control. (C) Confocal images of the *PIN1_{pro}:GFP-PIN1* line in the *cue1* background. (D) Confocal images after mPS-PI staining. (E and F) Root meristem size (E) and primary root length ($n = 25$) (F) of roots from WT (Col-0) and *cue1*- and *pin1*^{-/-}-mutant seedlings grown for 7 d on MS agar plates. A minimum of 8–10 roots per genotype was analyzed. Asterisks indicate significant differences compared with WT ($P < 0.05$).

1-naphthaleneacetic acid) or the auxin transport inhibitor NPA to the *cue1/nox1* mutant does not normalize the primary root growth defect present in this genetic background (Fig. S9).

Discussion

The understanding of the importance of NO as a regulator of plant growth and response to environmental stress has increased considerably despite limited information on its signaling. A physiological role for NO in the regulation of root growth has been described (17). NO diminishes primary root growth and promotes lateral root development in tomato (17) and *Arabidopsis* (20). Furthermore, a requirement for NO in auxin-induced adventitious (21) and lateral root (17) development has been reported. NO-hypersensitive mutants isolated in genetic screens let the identification of the NO-overproducing mutant *nox1* and *CUE1* as the mutated gene (18). However, the mechanisms of the precise cellular responses to NO are not yet well understood.

Using different NO donors (SNAP, SNP, GSNO, and NO gas) as well as NO-specific scavengers (cPTIO), we confirmed that high levels of NO inhibit primary root growth in *Arabidopsis* (Fig. 1). The elevation of NO using releasing compounds can cause secondary effects (22, 23), prompting us to test NO-overaccumulating mutants also. Analysis of mutants with high levels of endogenous NO in roots (*cue1/nox1*) showed results similar to those obtained after exogenous NO application (Fig. 2). Thus, exogenously applied NO phenocopies mutants with increased endogenous NO levels (18, 24), mirroring the response of these mutants after specific stress stimuli (25, 26).

To elucidate the cellular modifications and molecular basis of the NO response in *Arabidopsis* roots, we observed cell organiza-

tion within the root apex. Microscopic analysis of 5-d-old seedlings confirmed that the organization of primary root meristem is very sensitive to changes in NO levels (Fig. 2). Our results suggest that increases in NO concentration decrease primary root growth by reducing the number of dividing cells in the meristem. This reduction in number causes fewer division cycles to take place within the root apical meristem (Fig. 3 and Fig. S4). As the seedlings age, the number of cells in the root apical meristem decreases until, after 7 d, the root apical meristem becomes practically exhausted, exhibiting enlargement of all meristematic cells (Fig. S2). Primary root meristem activity is reduced concomitantly with the occurrence of differentiation phenotypes (i.e., development of epidermal root hairs and vacuolization) that are much closer to the initials in *cue1/nox1* than in WT (Col-0) roots (Fig. 2 and Fig. S3). Our data also demonstrate that during early seedling root development, endogenous NO accumulates mainly in a zone situated between the apical meristem and the elongation zone (also called “basal meristem”) (Fig. 2). Interestingly, sites of NO synthesis also have been described in roots (19) where three local centers of NO production were detected: one at the root cap statocytes, another one at the QC and distal portion of the meristem, and the third, the most prominent, at the distal part of the transition zone. The different localization patterns of NO may mirror the diverse effects of NO on plant growth and development. The use of the DAF-2DA fluorescence indicator has the advantage of following NO production in live cells undergoing development or in response to a stimulus and allowed us to localize the sites of NO accumulation in roots (Fig. 2A). Our results define synthesis of NO in the sites where a physiological effect is observed (i.e., the elongation zone of primary roots).

Interestingly, scavenging endogenous NO reduces cell elongation in the basal meristem without significantly affecting the number of dividing cells in the apical meristem (Figs. 2 and 3). Remarkably, in 7-d-old treated seedlings, we found no significant differences between cPTIO-treated plants and controls in the total number of cells between the QC and the start of the rapid elongation in the cortex. This location marking the transition into the rapid elongation zone makes the inflection point on the cell length curve occur around cell number 33 in both control and cPTIO (Fig. S2). During root meristem establishment, NO might affect this transition between the basal meristem and the rapid elongation zone by modifying the number of cells in the apical meristem and changing its axial position relative to the QC. Cells in the basal meristem are important in the response of roots to a variety of environmental signals, such as gravity and thigmostimulation, electrotropic stimulation, water stress, and responses to auxin (27).

Previous studies have described the growth-modulating properties of NO and its interaction with auxin in modulating root growth and developmental processes (28 and references herein). Using *Arabidopsis* lines containing the DR5 auxin-responsive promoter linked to GFP or β-GUS, we observed that increases in NO concentration [either by application of the NO donor (SNP) or the use of *cue1/nox1*-mutant background] attenuates auxin-dependent reporter expression in the QC and CSC. The auxin-induced maximum gene expression in the root apex, which is necessary for meristem maintenance, is normally observed in these cells, but this maximum is diminished in the presence of elevated NO (Fig. 4 and Fig. S5) (29). Additionally, we found that NO reduces the frequency of cell division in the root apex as judged by expression of the mitotic marker *CycB1;1_{pro}:GUS-DB* (Fig. S4) and that chronically high NO levels eventually cause meristem collapse. Taken together with the several lines of evidence that support the hypothesis that perturbing auxin distribution reduces mitotic activity in the root apical meristem, with loss of the QC (30, 31), our results suggest that NO influences the maintenance of the apical auxin maximum, presumably by changing auxin sensitivity, transport, or both.

The process of root organogenesis is controlled by auxin (30, 32, 33). Our data confirm that high levels of endogenous or applied NO specifically attenuate auxin response (Fig. 4 and Fig. S5). In-

terestingly, conditional loss of function of ABP1, a key regulator for auxin-mediated responses (34), and NO show similar effects on the root meristem, exhibiting arrest of cell division and elongated cells next to partially collapsed root meristems. Similarly, the phenotypes of the *pin1* mutant (30), impaired in the regulation of polar auxin transport in vascular tissue (35), resemble those of the NO overaccumulator *cue1/nox1* mutant in regards to the organization of the QC/CSC and starch accumulation. Our results suggest that polar auxin transport is impacted negatively by overaccumulation of NO because PIN1 protein levels are reduced dramatically after delivery of exogenous NO and in the *cue1/nox1* background, which produces increased endogenous NO levels (Fig. 5). Consistent with the PIN1 disappearance with NO exposure, the *pin1* mutant is not resistant to NO. Because qRT-PCR analysis revealed that *PIN1* expression is not influenced by NO (Fig. S7), we hypothesized that the disappearance of PIN1 protein may be regulated posttranslationally. The MG132 proteasome-specific inhibitor was used to determine if the decrease the level of PIN1 protein was caused by proteasome-dependent degradation, but it did not affect PIN1-GFP fluorescence. Our observations indicate that NO regulates PIN1 levels posttranscriptionally, and we propose that reductions in the levels of PIN1 protein are caused either directly or indirectly by a proteasome-independent mechanism. This result stands in contrast to PIN2, which is degraded in a proteasome-dependent manner during the gravitropic response (36).

Recently, the intracellular redox homeostasis regulated by thioredoxin (TRX) and glutathione has been shown to modulate auxin signaling and thus affect key plant developmental processes (37). Thus, auxin transport is impaired after specific inhibition of glutathione synthesis, in the triple mutant of the TRX reductases (*ntra;ntrb*), and the cadmium sensitive 2 (*cad2*) glutathione bio-

synthetic mutant. This reduced auxin transport may result from a decrease in PIN1 (and other PIN auxin efflux proteins). Additional factors other than PIN mRNA down-regulation (i.e., post-transcriptional or protein localization modifications) may affect the regulation of auxin transport (37). These findings also are in agreement with the precise effect of NO on auxin transport, further supporting our conclusions.

We have provided evidence that the disturbance of auxin transport (PIN1 reduction) and auxin response (alteration of DR5 expression pattern) by high levels of NO in the primary root meristem leads to an initial reduction of root meristem length and a progressive disturbance of root apical meristem maintenance. One of the biggest challenges in investigating NO as a signaling molecule is identifying the targets of NO (4). Results presented in this work suggest that PIN1 is a target of NO signaling and that NO-dependent changes in PIN1 protein levels cause the observed auxin transport, *DR5^{pro}:GUS* localization, meristem collapse, and differentiation phenotypes.

Materials and Methods

Plant materials, treatments, measurements of primary root length, cell size, root hair initiation and acropetal auxin transport in *Arabidopsis* roots and additional details about detection of endogenous NO, GUS staining and other procedures are fully described in *S1 Materials and Methods*.

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