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Application Article

Heuristic aspect of the lateral root initiation index: A case study of the role of nitric oxide in root branching[1]

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- *Premise of the study:* Lateral root (LR) initiation (LRI) is a central process in root branching. Based on LR and/or LR primordium densities, it has been shown that nitric oxide (NO) promotes LRI. However, because NO inhibits primary root growth, we hypothesized that NO may have an opposite effect if the analysis is performed on a cellular basis. Using a previously proposed parameter, the LRI index (which measures how many LRI events take place along a root portion equivalent to the length of a single file of 100 cortical cells of average length), we addressed this hypothesis and illustrate here that the LRI index provides a researcher with a tool to uncover hidden but important information about root initiation.
- *Methods and Results: Arabidopsis thaliana* roots were treated with an NO donor (sodium nitroprusside [SNP]) and/or an NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [cPTIO]). LRI was analyzed separately in the root portions formed before and during the treatment. In the latter, SNP caused root growth inhibition and an increase in the LR density accompanied by a decrease in LRI index, indicating overall inhibitory outcome of the NO donor on branching. The inhibitory effect of SNP was reversed by cPTIO, showing the NO-specific action of SNP on LRI.
- *Conclusions:* Analysis of the LRI index permits the discovery of otherwise unknown modes of action of a substance on the root system formation. NO has a dual action on root branching, slightly promoting it in the root portion formed before the treatment and strongly inhibiting it in the root portion formed during the treatment.

Key words: lateral root initiation; nitric oxide; root branching; root system architecture.

Root architecture is plastic and is modulated by a number of internal and external stimuli, such as the hormonal and nutritional status of the plant, and availability of water and nutrients in the soil (Malamy, 2005, 2010). Despite the accumulated knowledge about lateral root (LR) formation from the cellular, genetic, and physiological points of view, the mechanisms that determine root branching are not completely understood (Casimiro et al., 2003; Péret et al., 2009; Dubrovsky and Rost, 2012). The root system shape is determined by two main components: root growth and branching. These processes are dependent on plant hormones, among other factors.

During the past decade, nitric oxide (NO) has been extensively studied and recognized as a new plant growth regulator with important functions in physiology and development along the plant life cycle from germination to senescence (Lamattina et al., 2003; Wilson et al., 2008; Santner and Estelle, 2009; Moreau et al., 2010). In different species, it has been demonstrated that NO plays an important role in various aspects of root development, affecting primary root growth as well as adventitious and lateral root formation.

In their seminal work, Correa-Aragunde et al. (2004) found that NO is required for LR formation in tomato plants. In tomato and *Arabidopsis thaliana* (L.) Heynh. it was shown that NO mediates auxin-induced LR formation (Correa-Aragunde et al., 2004; Kolbert et al., 2008). In some other studies, a positive effect on LR formation by NO was found for *A. thaliana* (Kolbert et al., 2010), rice (Chen and Kao, 2012), *Lupinus albus* L. (Meng et al., 2012), and maize (Zandonadi et al., 2010), and this conclusion is currently widely accepted (Lamattina et al., 2003; Neill et al., 2003; Crawford and Guo, 2005; Grün et al., 2006; Osmont et al., 2007; Palavan-Unsal and Arisan, 2009; Baudouin, 2011). Lateral root formation comprises LR initiation (LRI), LR primordium formation, root emergence, and root growth where LRI is a key process of root branching (Laskowski et al., 1995; Charlton, 1996; Nibau et al., 2008; De Smet, 2012; Dubrovsky and Rost, 2012). In most studies, the conclusion about the promoting effect of NO in LR formation is based on the analysis of the number and/or density of only the LRs; no quantitative analysis of both LRs and LR primordia is usually performed. This prompted us to re-examine the effect of NO on LR formation with special attention on the LRI process and to identify important factors that have to be taken into consideration when LRI is studied.

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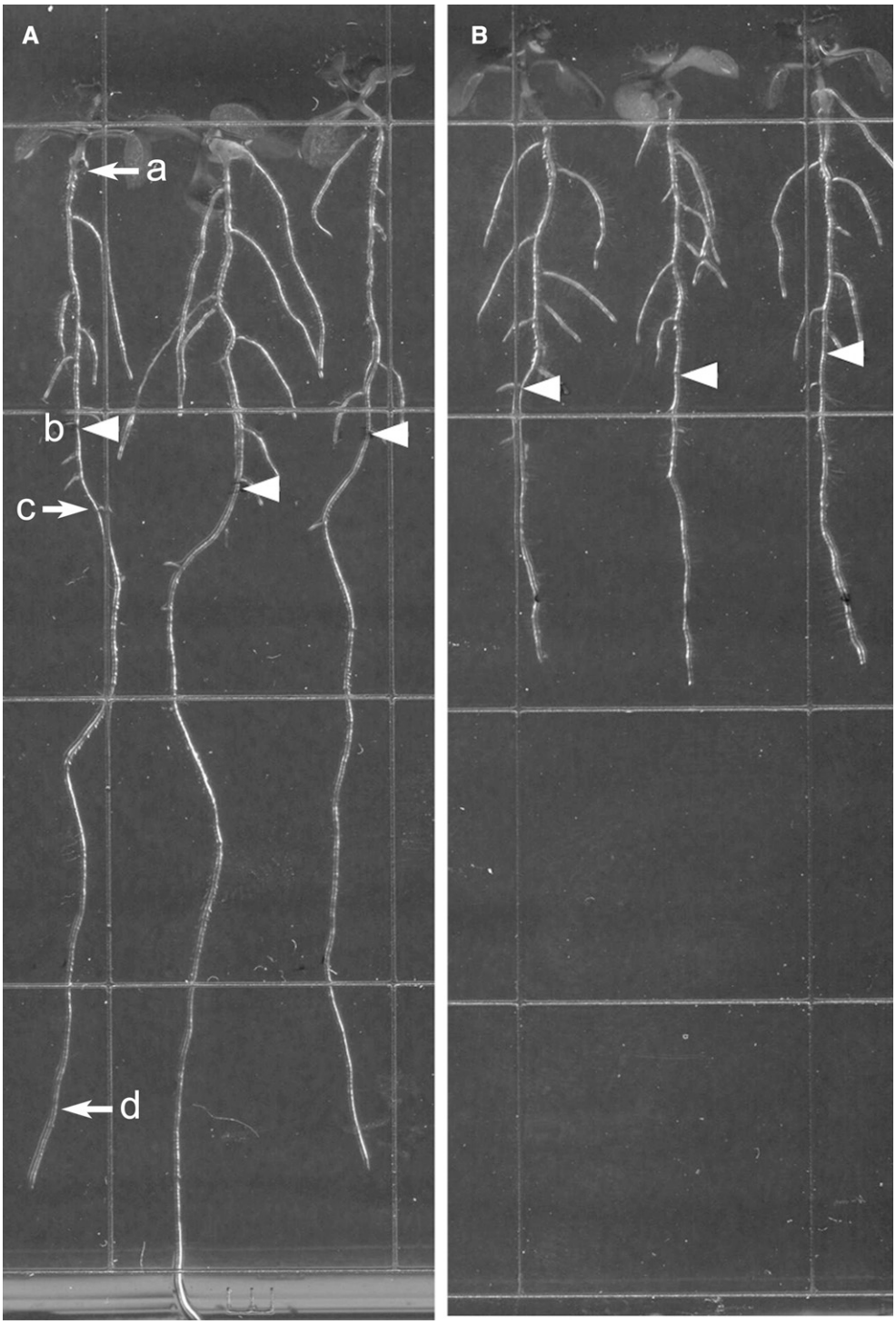

Fig. 1. Inhibitory effect of a nitric oxide (NO) donor on root system formation. Roots grew for five days on growth medium and then were transferred either (A) to the same medium or (B) to the same medium supplemented with 10 μM of an NO donor (sodium nitroprusside [SNP]) and grew for five additional days. Points marked on (A) are identified as follows: (a) root base, (b) length of the primary root on day of root transfer, (c) distalmost lateral root, and (d) approximate position of the distalmost lateral root primordium exactly defined on cleared root preparations. Arrowheads indicate the length of primary root on day of root transfer. Distance between horizontal lines is 13 mm.

We have previously described the limitations of measurements based on numbers of LR primordia (LRP) or LRs per seedling to understand LRI, and have explained why the density of LRI events is a better parameter (Dubrovsky and Forde, 2012). Here we analyzed the effect of NO on root branching to demonstrate why, in some cases, even density of LRs and LRP may not be a sufficient parameter to evaluate the role of a substance in root branching. We focus our attention specifically on

Table 1. An example of the primary data of the parameters collected in untreated seedlings and seedlings treated with 50 μM SNP in one representative experiment.[a,b]

Parameter	No treatment		50 μM SNP	
	Proximal root portion	Distal root portion	Proximal root portion	Distal root portion
Length of the portion, mm	14.0 ± 0.5	45.6 ± 0.6	14.5 ± 0.4	4.4 ± 0.1
Distance from the root tip to the distalmost LRP, mm	—	4.6 ± 0.2	—	2.0 ± 0.4
Cell length, μm	157 ± 3	166 ± 4	158 ± 5	67 ± 5
No. of LRs	7.9 ± 0.5	20.0 ± 0.9	10.3 ± 0.7	1.4 ± 0.4
No. of LRP	2.3 ± 0.4	20.2 ± 1.1	3.2 ± 0.6	1.1 ± 0.3

Note: LR = lateral root; LRP = lateral root primordia; SNP = sodium nitroprusside.

[a] n = 7–12, mean ± SE.

[b] Cell length is given for fully elongated cortical cells; for each root an average of 10 cells was determined.

advantages of the use of the LRI index, a parameter that we introduced previously (Dubrovsky et al., 2009). The LRI index represents an integrative measurement of LRI because it evaluates how many LRI events take place along the length of the root portion that comprises 100 cortical cells. More precisely, it evaluates the number of identified LRI events along a root portion equivalent to the length of a single file of 100 cortical cells of average length. This parameter permits normalization of the density data if there are differences in cell length resulting from a treatment or a genotype. Also, it was shown that growth substances may have differential effects on root branching depending on whether the treated root portion was already formed before the beginning of the treatment or grew during the treatment (Ivanchenko et al., 2008). This factor is not considered in many studies; in our analysis of NO effect on root system formation, we show it is also very significant.

METHODS

***Plant material, growth conditions, and treatments*—**Wild-type *A. thaliana* Columbia-0 (Col-0) and mutant line *cue1* (Salk_116454; Alonso et al., 2003) in Col-0 background were obtained from the Arabidopsis Biological Resource Center, Ohio State University (Columbus, Ohio, USA). The *cue1* (chlorophyll *a/b* binding protein underexpressed) mutant is allelic to *nox1* (nitric oxide overproducer 1), with an inactive phosphoenol pyruvate/phosphate translocator gene that is expressed in chloroplasts and has elevated levels of endogenous NO (Streatfield et al., 1999; He et al., 2004), and was used to verify whether it can phenocopy the NO effects on the wild type. The *A. thaliana* Col-0 accession is widely used in plant biology, and the first complete angiosperm genome was sequenced in the Col-0 accession (Arabidopsis Genome Initiative, 2000). The seeds were surface-sterilized using a 20% commercial bleach solution supplemented with 0.08% Triton X-100 for 10 min, followed by four 10-min washings with distilled sterile water, and maintained at 4°C for 48 h. Seeds were sowed on 0.2× Murashige and Skoog (MS) medium (pH 5.7), 1% sucrose, and 0.8% agar. Sucrose is commonly used to grow *A. thaliana* seedlings in vitro (Weigel and Glazebrook, 2002: p. 12). The MS medium contained salts formulated by Murashige and Skoog (1962) and vitamins formulated by Linsmaier and Skoog (1965) and was prepared based on Linsmaier and Skoog (LS) medium from PhytoTechnology Laboratories (Lenexa, Kansas, USA). Plates were kept in a vertical position in a growth chamber at 21°C under 16-h photoperiod for five days. Light intensity was 105 μmol $m^{-2} \cdot s^{-1}$. After five days, the seedlings were transferred to the same media either supplemented or not with the NO donor sodium nitroprusside (SNP) (Fluka, Buchs, Switzerland) at final concentrations of 10 and 50 μM. As an NO scavenger, 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Sigma-Aldrich, Saint Louis, Missouri, USA) was used. The position of the root tip on day 5 was marked on the Petri dish surface. The seedlings were kept in vertical position for five more days under the same growth conditions. At the end of the experiment, on day 10 after sowing, the primary root was cut at the marked position, and the proximal (the portion that grew during the first five days) and the distal (the portion that grew after transfer) root portions were collected separately. Because we have shown previously that other growth regulators such as an ethylene precursor (Ivanchenko et al., 2008) and auxin (Ivanchenko et al., 2010) have differential effects in the root portions formed before and during the treatments, we analyzed lateral root initiation separately in the proximal and distal root portions. Roots were cleared and slides were prepared according to a modified Malamy and Benfey method (Dubrovsky et al., 2006). The material was analyzed under a Zeiss Axiovert 200 M microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with differential interference contrast (DIC) optics.

***Estimation of root growth and lateral root formation parameters*—**All measurements were performed on cleared root portions. The length of the proximal and the distal root portion of the primary root were measured on images of scanned slides using ImageJ software (http://rsb.info.nih.gov/ij/). To estimate the LR and LRP densities in the proximal root portion, the number of LRs, LRP, or both, was divided by the length of the portion and expressed as number per millimeter. To estimate the densities in the distal root portion, the length of the lateral root formation zone—defined as the zone between the most distal LRP and the most distal LR (Dubrovsky and Forde, 2012)—is usually measured (the distance between points c and d on Fig. 1A). The position of the most distal LRP was marked on a slide with a marker. When the branching zone is extended into the distal root portion (as in Fig. 1A), the length of a fraction of the branching zone within the distal root portion can be determined as a distance between the most distal LR (point c, Fig. 1A) and the proximal (shootward) end of the portion (point b, Fig. 1A). LR density is commonly determined as the number of LRs per branching zone (when the whole branching zone is within the proximal root portion) or its fraction (when only a fraction of a branching zone is present in the root portion) (Dubrovsky and Forde, 2012). In this study, under the highest SNP concentration tested, the distal root portion was very short (see Results and Table 1). Therefore, the LR density was evaluated per length of the distal root portion where both LRs and LRP were present (distance between points b and d, Fig. 1). The length of 10 fully elongated cortical cells in each root portion was measured using an ocular micrometer. To calculate the LRI index, the average cell length (mm) in each of the individual root portions (proximal and distal) was multiplied by the density of all initiation events in each of the portions and by 100 (details of the method are described in Dubrovsky et al., 2009). Because average cortical cell length is expressed in millimeters, when it is multiplied by 100 it gives an estimated root length (mm) that is equal to a length of the cell file comprising 100 cells. Therefore, when the latter length is multiplied by the density of initiation events (expressed as number·mm^{-1}) it gives an estimate of the number of LRI events per root portion equal to the length of 100 cortical cells in a file.

To better evaluate the effect of treatments on LR emergence, the percentage of emerged LRs was obtained from the total number of initiation events (including LRs and LRP) per individual root portion. When an LRP protruded from the parent root epidermis, it was considered an emerged root. The number of initiation events was always determined on cleared preparations. Three independent experiments were performed, each with 7–12 plants. Unless otherwise indicated, statistical analysis was performed on the combined data using Prism (GraphPad Software, La Jolla, California, USA). Statistical analyses were performed using one-way ANOVA, Tukey's multiple comparison test.

***Protocol Notes*—**Some technical details should be taken into account when the data on the densities and LRI index are collected. These are as follows:

1. Care should be taken regarding the number of seeds plated per Petri dish. We noticed that if the number of *A. thaliana* seedlings in a row is greater than 10–12 (in a square Petri dish measuring 9 × 9 cm), root branching can be inhibited. For this reason, no more than 10–12 seeds should be sown per dish of the mentioned size.

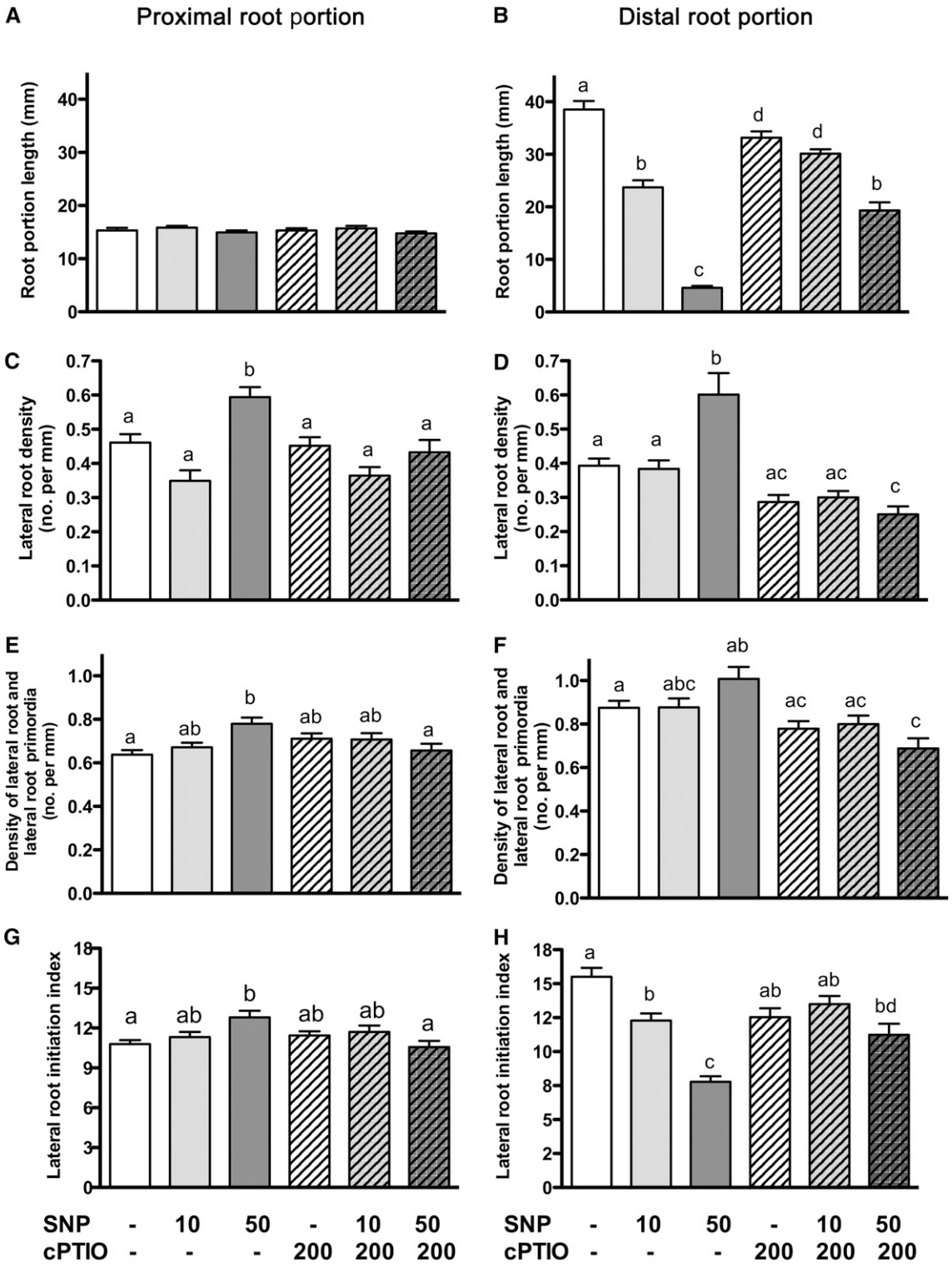

Fig. 2. Nitric oxide (NO) affects root system formation in *Arabidopsis thaliana* seedlings. Roots grew for five days on growth medium and then were transferred either to the same medium or to the same medium supplemented with an NO donor (sodium nitroprusside [SNP]) or an NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [cPTIO]), or both, and grew for five additional days. The data were collected separately for the proximal root portion formed before the beginning of treatments (A, C, E, G) and for the distal root portion formed during treatments (B, D, F, H). (A, B) Length of the root portions (mm). (C) Lateral root density in the proximal root portion. (D) Lateral root density in the distal root portion. (E, F) Density of all initiation events, including lateral roots and primordia, in the proximal (E) and distal (F) root portions. (G, H) Lateral root initiation index estimated as the number of all initiation events along the length of root portion that comprises 100 cortical cells of average length. SNP and cPTIO micromolar concentrations used for treatments are indicated. Combined data of at least three experiments (n = 25–38) were analyzed (mean ± SE). Letters above graph bars indicate statistical differences among treatments at $P < 0.05$ in Tukey's multiple comparison test.

2. Roots or root portions were numbered after being cleared and mounted. The numbering permits the collection of various parameters and subsequent examination of values of derived parameters for the same individual root or root portion.

3. To best visualize LRP in a diarch root such as *A. thaliana*, ideally the root should be positioned so that the protoxylem plane in which lateral roots emerge is parallel to the slide surface. This rarely occurs because, among other reasons, most roots twist at least slightly as they grow. However, when diarch roots are positioned obliquely, it is still feasible for LRP to be visualized. In those cases in which most of the root is positioned with the protophloem plane parallel to the slide surface, many early-stage LRP can be missed and so the LRP number undercounted. Therefore, protophloem-oriented roots should be discarded from the analysis. In our study in *A. thaliana*, less than 10% of roots had to be discarded for this reason.

4. Different clearing procedures can be used; however, it is important not to over-clear the root samples because LRP, especially at the early developmental stages, can be made more difficult to detect and so easily underestimated.

5. After *A. thaliana* roots are transferred to a new growth medium, the roots start to adapt to it. At the base of the distal root portion, formed just after transfer (approximately 200–400 μm from the base of the portion), the cell length can be different compared to the rest of the root portion. Therefore, when cell lengths are measured, this most proximal part of the distal root portion should be excluded from analysis.

6. When cell lengths are measured, it is important to confirm the position of the end walls, which might be in a different focal plane, by adjusting the focus. For this reason, we never measure cell lengths in photos.

7. The length of cells in the same cell layer has to be evaluated throughout the study. When the LRI index is to be evaluated in *A. thaliana*, it is easiest to measure cell length of the cortex proper (external layer of the ground tissue), as was done in this study.

8. The usefulness of the LRI index is not limited to thin roots such as *A. thaliana*. This parameter has been evaluated in tomato (*Solanum lycopersicum* L.; Dubrovsky et al., 2009) and, recently, in maize (*Zea mays* L.) roots (Husakova et al., 2013). When the LRI index is to be evaluated in thick roots, it is advisable to collect the data on cortex cell length of the same tissue layer (e.g., always of the external layer). For cortex cell length data collection, the thick roots can be cleared. If clearing quality is not sufficient, microtome or handmade longitudinal sections can be used for cell length measurements.

RESULTS

Arabidopsis thaliana seedlings were germinated and grown for five days in the standard growth medium and then were transferred to media supplemented with an NO donor (SNP), NO scavenger (cPTIO), or both, and grown for an additional five days. Root portions formed before the treatment (proximal) and those formed during the treatment (distal) were measured. In roots treated with SNP alone, the primary root in the distal root portion, which grew during the treatments, was significantly shorter than in the untreated control (Figs. 1, 2A, 2B).

In the proximal root portion, at 10 μM SNP, there was no change in LR density and in the density of all initiation events including LRs and LRP (Fig. 2C, E). At 50 μM SNP, the LR density and the density of all LRI events increased by 28% ($P < 0.01$) and 22% ($P < 0.05$), respectively, over the values in untreated seedlings (Fig. 2C, E). In this proximal root portion that had formed prior to treatment, no changes in cell length were found under any treatment (data not shown) and, for this reason, LRI index increased to a similar extent as the density of all LRI events (Fig. 2G). Therefore, the increase in both the density of all LRI events (including LRs and LRP) and in the LRI index reflects a real increase of LRI resulting from the SNP treatment. The effect of SNP on LRI was counteracted by 200 μM cPTIO (Fig. 2C), suggesting that the response was NO-specific. Raw data for one representative experiment included in the Fig. 2 are shown in Table 1.

To assess whether LR emergence changed, the percentage of emerged LR out of all LRI events per proximal root portion was analyzed. There was no difference in this parameter in the proximal root portion between treated and untreated roots (Fig. 3A). Therefore, in the root portion already formed by the beginning of the treatment, NO had an inductive effect on LRI and did not affect LR emergence.

In the distal root portion, 50 μM SNP caused a significant decrease in primary root growth compared to the untreated control (Fig. 2B, Table 1). The treatment induced an increase in the LR density (Fig. 2D). The LR density data should be interpreted with care because, as the root portion was short, the number of LRs was low; in 50% of plants the number ranged between three and six LRs per portion, whereas in the other 50% of plants it was less than three LRs per portion (Table 1). The density of all LRI events was not significantly higher at 50 μM SNP compared to the untreated control (Fig. 2F). The increase in LR density induced by SNP was eliminated by cPTIO. However, SNP treatment affected elongated cortical cell length, which at 50 μM treatment was 44% of the untreated control (the complete analysis of the effect of NO on the length of fully elongated cells will be published elsewhere). Therefore, the LRI index significantly decreased at 10 ($P < 0.05$) and 50 μM ($P < 0.005$) SNP and was 21% and 50% of that in the untreated roots, respectively (Fig. 2H). This effect was reversed by cPTIO, showing the NO-specific action of SNP on LRI.

The fact that LR density in the distal root portion treated with 50 μM SNP increased ($P < 0.001$) by 53% compared to the control (Fig. 2D) may indicate that NO promoted LR emergence. Indeed, the percentage of emerged roots increased ($P < 0.001$) by 34% in SNP-treated roots compared to untreated roots (Fig. 3B).

To test further our finding that NO has an inhibitory effect on LRI in the growing root, we hypothesized that in a mutant that has elevated levels of endogenous NO we would expect the inhibition of LRI relative to wild-type plants without any exogenous treatments. Indeed, similar to SNP-treated wild-type seedlings, LR density and the density of all LRI events were greater in the *cue1/nox1* mutant (Fig. 4A, B). However, the primary root growth was significantly inhibited in the mutant due to a decrease in cell production and elongated cell length (data not shown). Consequently, the LRI index was significantly lower in the mutant compared to the wild type (Fig. 4C). In summary, the analysis of *cue1/nox1* root development showed that endogenously elevated NO level in a plant produced a similar effect as NO-donor treatment of wild-type plants. This is true both for the negative effect on lateral root initiation and the negative effect on root length and cortical cell length.

DISCUSSION

It has been demonstrated that NO is an important component in the process of LR and adventitious root formation (Correa-Aragunde et al., 2004, 2006; Pagnussat et al., 2004; Kolbert et al., 2008; Méndez-Bravo et al., 2010). In this work, we showed that the SNP treatments induced LRI in the proximal root portion and inhibited it in the distal portion. In the latter, the LR density was increased by SNP in a dose-dependent manner similar to the study by Méndez-Bravo et al. (2010). However, this effect results both from a decreased length of fully elongated

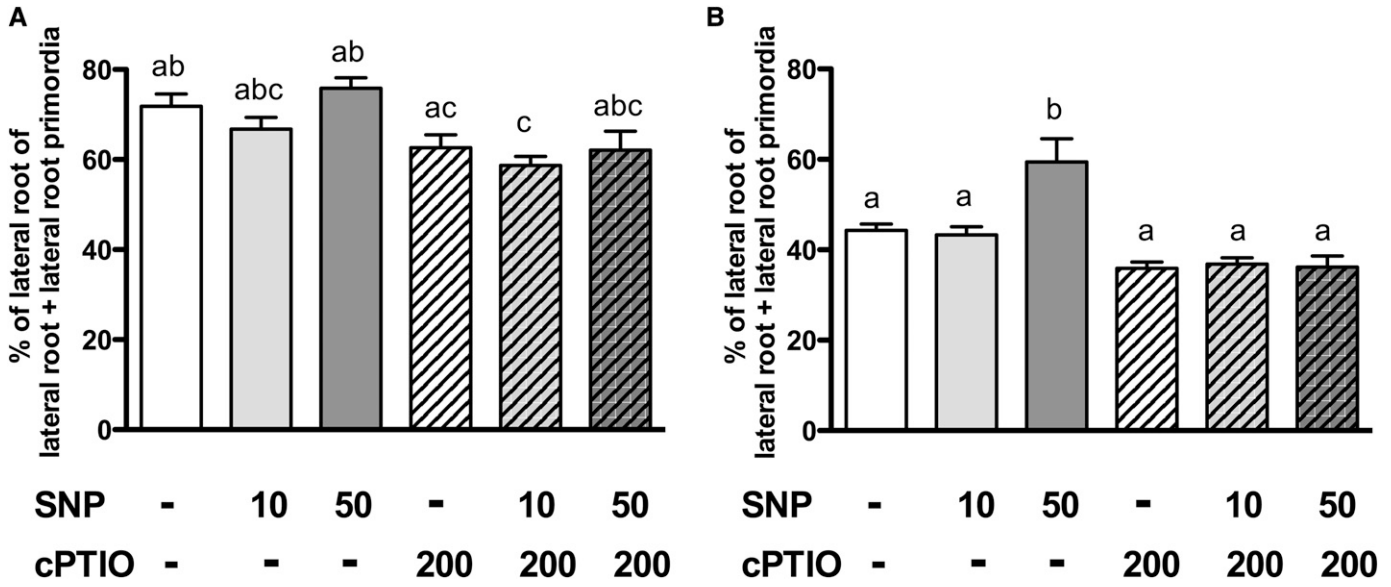

Fig. 3. Nitric oxide affects lateral root emergence in the growing root portion of *Arabidopsis thaliana* seedlings. Experimental design and abbreviations are the same as in Fig. 2. The percentage of lateral roots out of the total number of initiation events, including lateral roots and lateral root primordia per proximal (A) and distal (B) root portion is shown. Combined data of at least three experiments (n = 25–35) are shown (mean ± SE). Letters above graph bars indicate statistical differences among treatments at $P < 0.05$ in Tukey's multiple comparison test.

root cells and from the promotion of LR emergence. When all the effects of NO are considered, including the effect on root initiation and cell elongation, we observed that the LRI index was strongly decreased by NO treatment.

The LR density and the LRI index are distinct parameters (Dubrovsky et al., 2009) and provide different information. Both show the longitudinal spacing of the LRI events, the former per physical distance and the latter per cellular basis. Therefore, when the goal is to understand the developmental processes affecting LRI, the LRI index is a more informative parameter as it evaluates how many LRI events took place per developmentally comparable root portion comprising a file of 100 average cells of the same tissue in the roots of each genotype or treatment. Based on this parameter, we conclude that in the root growing in the presence of the NO donor, the process of LRI in *A. thaliana* is strongly inhibited. This conclusion was confirmed with the analysis of LRI in an NO-overproducing mutant.

Interestingly, in another study using *A. thaliana* seedlings germinated and grown in the presence of SNP, the authors show that the LRI index is not decreased, but increased (Méndez-Bravo et al., 2010). This can be explained by a number of reasons: (1) The LRI index depends on LR and LRP densities and cell length (Dubrovsky et al., 2009); if the density is increased, but the cell length is the same or only slightly decreased under a treatment, the LRI index will also increase. In the study by Méndez-Bravo et al. (2010), the cell length was decreased by only about 15% when treated with 20 μM SNP, but the density increased twofold; therefore, the authors found an increased LRI index. In our study, the cortical cell length decreased by 21% in plants that were transferred to a medium supplemented with only 10 μM SNP, indicating a much stronger effect of SNP on cell length than that reported by Méndez-Bravo et al. (2010). (2) Seedlings in both studies grew in the same growth medium and the same NO donor was used, but in our system the 5-d-old seedlings were transferred to SNP-supplemented media to avoid any effect of SNP treatment during germination that could influence the postgermination root growth. (3) The activity of SNP decreases with storage time and with time after preparation of a solution (personal observations). Therefore, we cannot exclude the possibility that the activity of the compounds used here was different from those used by Méndez-Bravo et al. (2010).

The general analysis of the effect of NO shows that NO has only a slight but significant inducing effect on LRI that is much weaker than the inducing effect of auxin (Kerk et al., 2000; Ivanchenko et al., 2010), even though the known data indicate that the NO-promoting effect is auxin-related (Correa-Aragunde et al., 2004). Nevertheless, our data showed that NO inhibits LRI in the growing root and that this effect is much stronger than the induction of LRI in the root portion formed before the beginning of the treatment.

The effect of NO on LR emergence was first reported in tomato roots where, despite the reduction in primary root length by SNP treatment, the total root length was not affected, because of an increase in LR emergence (Correa-Aragunde et al., 2004). Furthermore, when *A. thaliana* roots were treated with 30 μM SNP, increased density of emerged LRs suggested that NO promotes LR emergence (Méndez-Bravo et al., 2010). Here, it was demonstrated that NO promotes LR emergence of the LRP that were initiated during the treatment in the growing root (distal portion), but not in the already developed root (proximal portion). This suggests that NO may be required during LRP formation for subsequent primordium emergence. It has been demonstrated that NO induces dormancy break in *A. thaliana* seeds by promoting abscisic acid (ABA) catabolism (Liu et al., 2009). On the other hand, it is known that ABA can inhibit LR emergence in *A. thaliana* roots (De Smet et al., 2003). If the NO effect on ABA catabolism is a general one, the promoting effect of SNP in LR emergence in growing roots could be explained by a possible decrease in ABA level.

In conclusion, the application of such a parameter as the LRI index permits the evaluation of whether initiation of LRs is

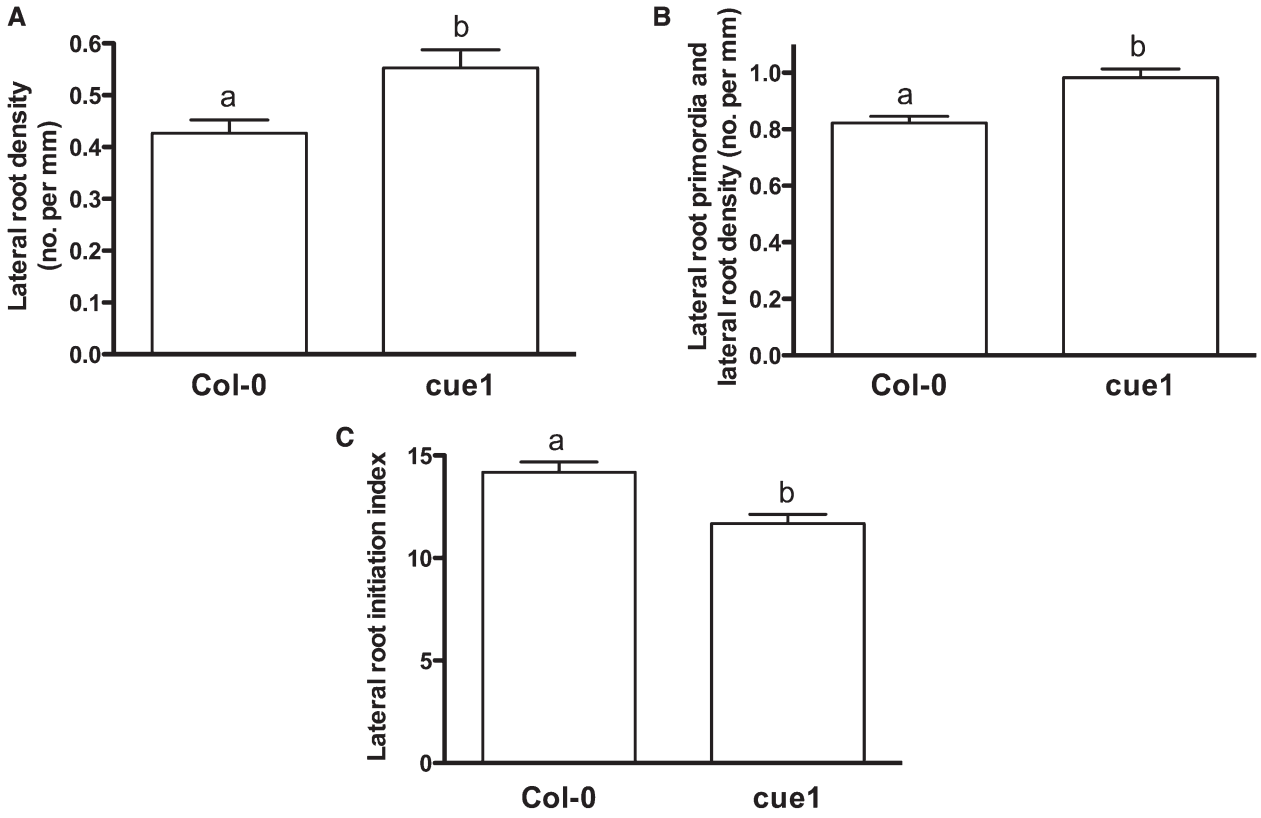

Fig. 4. Lateral root density and lateral root initiation index of *Arabidopsis thaliana* wild type (Col-0) and the mutant *cue1/nox1* that accumulates more nitric oxide than the wild type. Ten-day-old plants were analyzed. (A) Lateral root density estimated in the root branching zone (from the root base to the distalmost emerged lateral root). (B) Density of all initiation events, expressed as the number of both lateral roots and primordia, per mm of the primary root between the root base and the distalmost lateral root primordium. (C) Lateral root initiation index estimated as the number of all initiation events per 100 cortical cells along the root. Combined data of three independent experiments (n = 23–47) are shown (mean ± SE). Letters above graph bars indicate statistical differences among treatments at $P < 0.05$ in Tukey's multiple comparison test.

affected by a treatment or a genotype. As we saw, density measurements do not provide sufficient information to evaluate quantitatively the LRI process even if LRP are taken into account. Furthermore, if the analysis of this study is entirely based on density values, one can conclude that LRI is promoted. However, when the more developmentally relevant LRI index is applied, one can find that LRI is not promoted, but significantly inhibited (because the treatment produces a reduction in cell length). Therefore, a heuristic aspect of the LRI index parameter is that it reveals otherwise-hidden developmental changes in the LRI. The example shown here demonstrated a strong inhibition of LRI in the root portion formed during the treatment. Therefore, despite the generally accepted view that NO has a promoting effect on LR formation (see the Introduction section), our analysis permitted the identification of a strong inhibitory component of NO action on the LRI process. Because the primary root growth is affected significantly by NO, not only in *A. thaliana* (this study; Méndez-Bravo et al., 2010; Fernández-Marcos et al., 2011; Bai et al., 2012) but also in other studied species (Correa-Aragunde et al., 2004; Xiong et al., 2009), the overall inhibitory effect on the root system formation appears to be a general phenomenon. Our data indicate that NO has a dual action on LRI, with only a slight induction in the root portion formed before the treatment and a strong inhibition in the root portion formed during the treatment, indicating that NO action depends on the tissue differentiation state. This dual action resembles that of auxin (Ivanchenko et al., 2010) and can be an important mechanism involved in the control of the root architecture.

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