Dual pathways for regulation of root branching by nitrate

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ABSTRACT Root development is extremely sensitive to variations in nutrient supply, but the mechanisms are poorly understood. We have investigated the processes by which nitrate (NO_3^-), depending on its availability and distribution, can have both positive and negative effects on the development and growth of lateral roots. When *Arabidopsis* roots were exposed to a locally concentrated supply of NO_3^- there was no increase in lateral root numbers within the NO_3^--rich zone, but there was a localized 2-fold increase in the mean rate of lateral root elongation, which was attributable to a corresponding increase in the rate of cell production in the lateral root meristem. Localized applications of other N sources did not stimulate lateral root elongation, consistent with previous evidence that the NO_3^- ion is acting as a signal rather than a nutrient. The *axr4* auxin-resistant mutant was insensitive to the stimulatory effect of NO_3^-, suggesting an overlap between the NO_3^- and auxin response pathways. High rates of NO_3^- supply to the roots had a systemic inhibitory effect on lateral root development that acted specifically at the stage when the laterals had just emerged from the primary root, apparently delaying final activation of the lateral root meristem. A nitrate reductase-deficient mutant showed increased sensitivity to this systemic inhibitory effect, suggesting that tissue NO_3^- levels may play a role in generating the inhibitory signal. We present a model in which root branching is modulated by opposing signals from the plant's internal N status and the external supply of NO_3^-.

A plant's ability to explore the soil and to compete effectively for soil resources is critically dependent on the architecture of its root system (1). Root architecture is determined by the pattern of root branching and by the rate and trajectory of growth of individual roots. These properties of a root system are not only under direct genetic control but are also highly plastic, being influenced by a wide range of physical, chemical, and biological factors (2, 3).

A striking example of plasticity in root development is seen in the way many plant species respond to an uneven distribution of nutrients (NO_3^-, NH_4^+ or inorganic phosphate) by proliferating their lateral roots preferentially within nutrient-rich zones (3, 4). This ability to "forage" for localized supplies of nutrients is believed to be important in determining a plant's ability to compete for limiting resources (5). In cereals, the increased proliferation of laterals in the nutrient-rich zone is caused by an increase in both their numbers and their elongation rates (6–8). These localized responses have generally been explained in terms of either a direct or an indirect nutritional effect. Thus, it has been suggested that the roots directly exposed to a localized source of NO_3^- are stimulated because they benefit most from the increased N supply (6), or, alternatively, that increased metabolic activity in those same

roots leads to a growth-stimulating influx of carbohydrates and auxin (7–10).

In contrast to the stimulatory effect of a localized NO_3^- supply, a high rate of N supply to the root system as a whole usually is associated with a reduced allocation of resources to root growth (i.e., decreased root/shoot ratios) (11). Similar apparently contradictory effects of NO_3^- on root growth were observed in *Arabidopsis* (12), which led to a model in which it was proposed that the NO_3^- supply modulates lateral root (LR) development in two distinct ways: through a systemic inhibitory effect that results from the accumulation of NO_3^- (and/or its metabolites) in the shoot and through a localized stimulatory effect that depends on the local concentration of NO_3^- at the LR tip. An analogous "feedback–feedforward" model for NO_3^- regulation of shoot–root allocation in tobacco has been postulated by Stitt and colleagues (13, 14).

The finding that LR proliferation in the *nia1nia2* mutant of *Arabidopsis* [which is nitrate reductase (NR)-deficient and has a low capacity for NO_3^- assimilation] responds as strongly as the wild-type to a localized NO_3^- treatment (12), led to the suggestion that the stimulatory effect of the NO_3^- on LR proliferation is triggered by a signal from the NO_3^- ion itself rather than by its nutritional properties. The *Arabidopsis ANR1* gene, which encodes a NO_3^--inducible and root-specific member of the MADS-box family of transcription factors, was identified as a key component of the signal transduction pathway by which NO_3^- stimulates LR proliferation (12).

Arabidopsis offers a number of advantages for the study of the nutritional control of root development, not least its small size (which allows root growth studies to be done in standard Petri dishes) and the availability of a wide range of nutritional and hormonal mutants to aid in the dissection of signal transduction pathways. In the present study we have examined in detail the effects of NO_3^- on root branching in *Arabidopsis* and present a model for how NO_3^- modulates LR growth and development via two distinct pathways.

MATERIALS AND METHODS

Plant Material. Seed of *Arabidopsis thaliana* ecotype Columbia (Col) was from Lehle Seeds, (Round Rock, TX, catalogue no. WT-1A). The *axr2–1* and *axr4–2* mutants were kindly provided by O. Leyser (University of York, United Kingdom), the *aux1–7* mutant by M. Bennett (University of Warwick, United Kingdom), END199 by P. Benfey (New York University), and the *nia1nia2* mutant G′4-3 by N. Crawford (University of California, San Diego).

Growth of Seedlings. The use of segmented agar plates to make localized applications of nutrients to *Arabidopsis* roots under aseptic conditions has been described (12). Unless otherwise stated, all plates contained 0.5% sucrose and 0.01 mM NH_4NO_3 and were incubated at 24–26°C under a 16:8 h

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light/dark regime. Seedlings were transferred to the segmented plates when their primary roots were long enough to extend into the middle segment ($\approx$2 cm). For the uniform nutrient treatments, seedlings were germinated for 3 d and subsequently transferred to agar plates containing the appropriate nutrients. Lengths of individual primary and lateral roots were measured with a ruler directly or from images captured by using an Eagle Eye II Still Video System (Stratagene).

Cytology and Histochemistry. Epidermal cells of LRs that had been fixed in 4% (vol/vol) formaldehyde were observed with differential interference contrast microscopy in either a Zeiss Photomicroscope III or a Leica DMRB microscope by using a $\times$40 objective. Mean mature cell lengths were determined from measurements on 7–10 cells per root in the zone where root hairs had recently emerged. β-glucuronidase (GUS) activity in roots of the GUS marker line END199 was detected histochemically by using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate (15).

RESULTS

Even Very Low NO$_3^-$ Concentrations Applied Locally Can Stimulate LR Proliferation. By using a technique in which *Arabidopsis* seedlings are grown aseptically on segmented vertical agar plates, we previously showed that a localized supply of 1 mM KNO$_3$ to the primary roots of *Arabidopsis* seedlings stimulated LR proliferation specifically in the zone of treatment (12). To investigate the concentration dependence of this effect, a range of KNO$_3$ concentrations from 0.05 to 50 mM was supplied to the middle segment of the segmented agar plates, the top and bottom segments receiving only the basal level of N (0.01 mM NH$_4$NO$_3$).

Surprisingly, even 0.05 mM NO$_3^-$ was sufficient to produce a localized 50% increase in LR length in the zone of treatment (Fig. 1*A*). However, the strongest responses (2.5- to 3-fold stimulation) were seen at concentrations from 0.1 to 10 mM (Fig. 1*A*). At 50 mM NO$_3^-$, LR growth in the treated segment was inhibited by 50% compared with the control (which received 1 mM KCl). This effect, which is investigated in more detail below, is consistent with previous evidence that high concentrations of NO$_3^-$ can inhibit LR development in *Arabidopsis* (12). In all cases, the effect of the localized NO$_3^-$ supply was specific to the root segment receiving the treatment, LR lengths in the top segment not being significantly affected. (Over the short time span of this and the other experiments reported here, there was insufficient LR growth in the bottom segment of the plate for meaningful measurements to be made.)

A Localized NO$_3^-$ Supply Specifically Stimulates LR Elongation Rates Without Affecting LR Initiation. In previous studies, mainly with monocots, localized NO$_3^-$ treatments were found to stimulate both initiation and elongation of LRs (6–8). To investigate the nature of the response in *Arabidopsis,* roots were exposed to a localized supply of 1 mM KNO$_3$ or 1 mM KCl, and the LR elongation rates were monitored. Although there was a wide variation in the growth rates of individual LRs within each treatment (Fig. 1*B*), the mean rate of LR elongation in the localized NO$_3^-$ treatment was twice that in the controls. On the other hand, the numbers of emerged LRs in the treated segment did not differ significantly (3.1 $\pm$ 0.5 in the control and 2.8 $\pm$ 0.4 in the localized NO$_3^-$ treatment). Thus, in *Arabidopsis*, a localized supply of NO$_3^-$ specifically stimulates LR elongation.

The mean final cell length was determined for 29 individual lateral roots elongating at different rates either with or without the localized NO$_3^-$ treatment (Fig. 1*C*). Only relatively small differences in the lengths of the mature cells were seen despite a wide variation in elongation rates, and there was no significant difference between the data for the NO$_3^-$-treated LRs and the controls. Thus, we can conclude that the stimulation
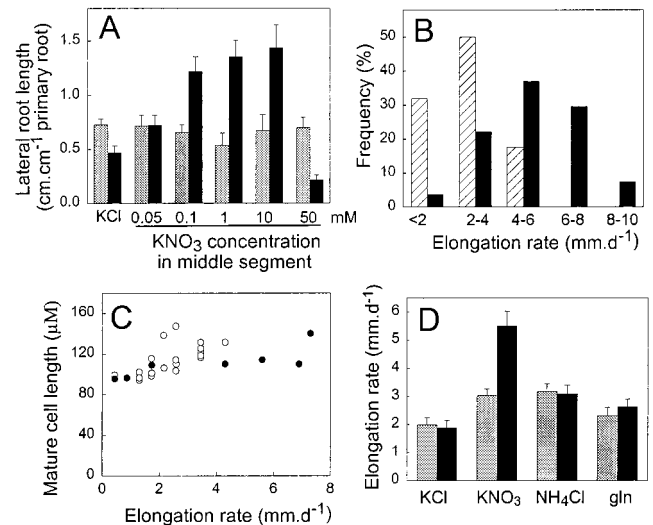

FIG. 1. Effects of localized supplies of different N sources on LR growth. (*A*) Effect of different KNO$_3$ concentrations in the "NO$_3^-$-rich" zone. *Arabidopsis* seedlings were grown on vertical agar plates that had been divided horizontally into three segments to allow different nutrient treatments to be applied to the basal, middle, and apical zones of the primary root (12). The top and bottom segments of the agar plate contained 0.01 mM NH$_4$NO$_3$ as sole N source, and the middle segment contained in addition the indicated concentrations of KNO$_3$. The controls received 1 mM KCl in the middle segment (in preliminary experiments, the 1 mM KCl treatment was found to have no effect on LR growth compared with a water control). Nine days after transfer, LR lengths in the top (shaded bars) and middle (filled bars) segments were measured for each seedling (n = 23–33). (*B*) Effect of a localized supply of KNO$_3$ on LR elongation rates in the NO$_3^-$-rich zone. Seedlings (12 per treatment) were grown at 25°C and under continuous light on segmented agar plates containing 0.01 mM NH$_4$NO$_3$ that were supplemented in the middle segment with either 1 mM KCl or 1 mM KNO$_3$. The elongation rates of individual LRs in the middle segment were estimated by measuring their lengths on days 8 and 9 after transfer, and the frequency distribution of different elongation rates was plotted for the KCl (hatched bars) and KNO$_3$ (filled bars) treatments. The mean rate of LR elongation in the KCl controls was 2.7 $\pm$ 0.26 and in the KNO$_3$ treatment was 5.4 $\pm$ 0.38 mm·day^{-1}. (*C*) Relationship between the elongation rate of a LR and the length of its mature cells. The elongation rates of individual LRs growing in a localized supply of 1 mM KCl or 1 mM KNO$_3$ as described for *B* were determined for the 24-h period before they were excised and fixed for cytological examination. Mean mature cell lengths for the KCl ($\bigcirc$) and KNO$_3$ ($\bullet$) treatments were estimated as described in *Materials and Methods*. (*D*) Effect of localized supplies of NH$_4^+$ and glutamine on LR elongation rates. Seedlings (11–13 per treatment) were grown on segmented agar plates containing 0.01 mM NH$_4$NO$_3$ and supplied in the middle segment with KCl, KNO$_3$, NH$_4$Cl, or glutamine (each at 0.1 mM). LR elongation rates in the top (shaded bars) and middle (filled bars) segments were measured between days 9 and 10 after transfer.

of LR elongation by a localized NO$_3^-$ treatment is primarily caused by a higher rate of cell production in the LR meristem rather than an increase in mature cell length.

Localized Supplies of Other N Sources Fail to Stimulate LR Elongation. Ammonium and glutamine can each serve as alternative N sources for *Arabidopsis*, although at high concentrations ($\geq$1 mM), they can inhibit growth (unpublished results). In preliminary experiments, we found that localized treatments with either 1 mM NH$_4$Cl or 1 mM glutamine did not stimulate localized LR growth (data not shown). Because these results may have been influenced by the inhibitory effects of these relatively high concentrations, we repeated the experiment, using just 0.1 mM of each N source. The results (Fig. 1*D*) demonstrate that whereas this concentration of KNO$_3$ stimulated LR elongation in the middle (treated) segment by almost 2-fold compared with the top segment, the

same concentration of either NH_4^+ or glutamine produced no localized increase in LR elongation rates.

Evidence for an Overlap Between the Signal Transduction Pathways for NO_3^- and Auxin. Elongation of both primary and lateral roots is highly sensitive to auxin, with either stimulatory or inhibitory effects being seen, depending on the auxin concentration (16). To look for evidence of interactions between the signal transduction pathways for NO_3^- and auxin, we tested the sensitivity of three auxin-resistant mutants, *aux1* (17), *axr2* (18), and *axr4* (19), to a localized supply of 1 mM NO_3^-.

As shown in Fig. 2, LR growth in the *aux1* and *axr2* mutants responded to the NO_3^- treatment in a similar way as the wild type. On the other hand, LR growth in the *axr4* mutant was not stimulated by the localized supply of NO_3^-. For *axr4*, as for the wild type, the localized NO_3^- treatment did not affect the numbers of emerged LRs (data not shown), indicating that the lack of response in the *axr4* mutant was caused by a failure to stimulate LR elongation.

High Rates of NO_3^- Supply Inhibit LR Development at a Specific Stage. We previously reported that high NO_3^- concentrations ($\geq$10 mM), when applied uniformly to the whole of the primary root, had a strong inhibitory effect on LR production without affecting the numbers of LRs initiated or the rate of primary root growth (12). Fig. 3*A* compares the root morphology of seedlings grown on 1 mM and 50 mM KNO_3 illustrating the apparent absence of LRs at the higher NO_3^- concentration. However, closer examination of these seedlings showed the presence of many short but otherwise normal-looking LRs (Fig. 3*B*).

We used the GUS marker line END199 to identify the stage(s) of LR development that are blocked or delayed by high NO_3^- concentrations. Seedlings grown on 1 mM KNO_3 or 50 mM KNO_3 were stained for GUS activity and the emerged and unemerged LRs were classified into four developmental stages (see legend to Fig. 3). The mean number of LRs (emerged and unemerged) per seedling at 1 mM and 50 mM KNO_3 was similar (33.2 and 30.6, respectively), but the mean length of LRs in the seedlings grown on 1 mM KNO_3 was 4.5 cm per seedling, whereas on 50 mM it was only 0.3 cm, confirming that this line was as sensitive to the inhibitory effect of NO_3^- as the C24 line studied previously (12).

Fig. 3*C* shows the proportion of LRs at each stage that were found in successive 1 cm segments from the apex to the base
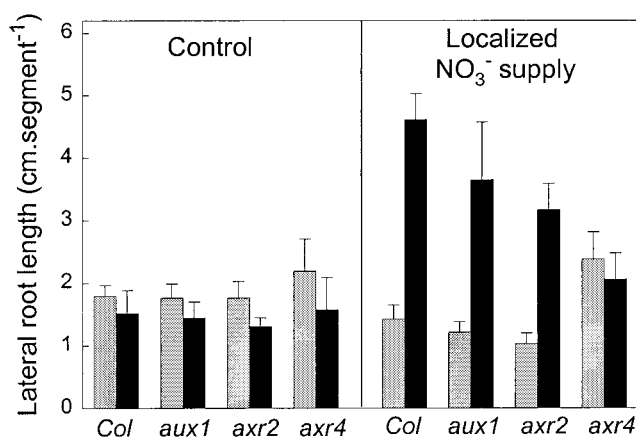

FIG. 3. Effect of a high rate of NO_3^- supply on LR development. (*A*) Photograph showing the suppression of LR development in seedlings grown for 7 days on 50 mM KNO_3 compared with those grown on 1 mM KNO_3. (*B*) Close-up of a typical stunted LR from a seedling grown on 50 mM KNO_3. The primary root is approximately 0.2 mm in diameter. (*C*) LR development is specifically inhibited at a stage just after LR emergence. Seedlings of the END199 GUS marker line (30) were grown on 1 mM KNO_3 (shaded bars) or 50 mM KNO_3 (filled bars) for 7 days and then stained for GUS activity (9–15 seedlings per treatment). Each LR or LR primordium was classified according to its stage of development and its distance from the primary root tip. The relative frequency of each of the four developmental stages within each 1-cm segment of the primary root has been plotted. Stage A, up to 3 cell layers; Stage B, unemerged, >3 cell layers; Stage C, LR emerged, <0.5 mm long; Stage D, ≥0.5 mm long.

of the primary root. The distribution along the primary root of unemerged LRs (stages A and B) was very similar in the two NO_3^- treatments, with the frequency of both stages generally declining with increasing distance from the root tip as more and more of the LR primordia emerged through the epidermis. There was, however, a marked difference between the two treatments in the distribution of stage C and stage D laterals: whereas most of the LRs growing on the lower NO_3^- concentration quickly progressed through stage C to stage D, LRs growing on 50 mM NO_3^- accumulated at stage C, with very few progressing to stage D by the day of measurement.

To investigate whether mature LRs are sensitive to 50 mM KNO_3, seedlings were grown initially on 1 mM KNO_3 and subsequently shifted to 50 mM KNO_3. It was found that LRs that had already emerged at the time of the shift were insensitive to the high NO_3^- concentration and grew at the same average rate as LRs that were kept on 1 mM KNO_3 throughout (data not shown). Thus, LRs appear to be sensitive to the inhibitory effect of a high rate of NO_3^- supply only during a very specific stage of their development, just after emergence and before maturation.

The phenotype of the *alf3* mutant of *Arabidopsis*, which forms a primary root covered with stunted LRs (20), is superficially similar to that of wild-type seedlings growing on 50 mM KNO_3 (Fig. 3). However, whereas the arrested *alf3* LRs

FIG. 2. The responses of three auxin-resistant mutants to a localized supply of KNO_3. The auxin-resistant mutants (*aux1–7, axr2–1*, and *aux4–2*) and the wild type (Col) were grown under standard low-N conditions (see Fig. 1) on segmented agar plates, and the middle segment was supplied with either 1 mM KCl (control) or 1 mM KNO_3 (localized NO_3^- supply). Nine days after transfer, LR lengths were measured in the top (shaded bars) and middle (filled bars) segments (13 seedlings of each line per treatment).
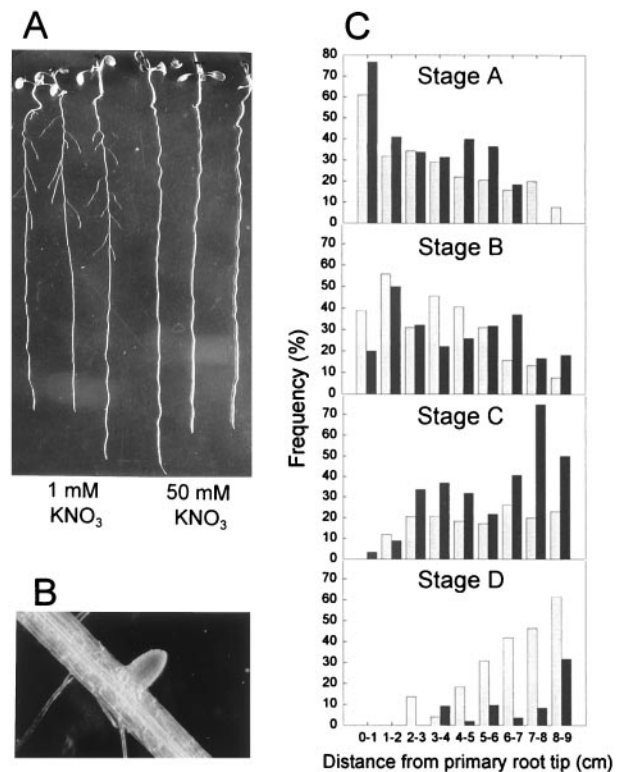
are dead and cannot be rescued once formed (20), many of the stunted LRs developing on 50 mM KNO$_3$ will eventually grow out normally if left for long enough and will recover immediately if the seedlings are transferred to 1 mM NO$_3^-$ (data not shown).

The Inhibitory Effect of NO$_3^-$ Is Systemic and Is Not Alleviated in an NR-Deficient Mutant. To investigate whether a high NO$_3^-$ concentration has a localized or a systemic effect on LR development, we grew seedlings on segmented agar plates which contained 50 mM KNO$_3$ in both the top and bottom segments and a much lower NO$_3^-$ concentration in the middle segment (0.01 mM NH$_4$NO$_3$ plus either 1 mM KCl or 1 mM KNO$_3$). Compared with the controls (which received only the basal level of N in the top and bottom segments), LR development in the middle segment was inhibited by about 70% in each case, despite the LRs not being directly exposed to the 50 mM KNO$_3$ (Fig. 4*A*). Thus, in contrast to its stimulatory effect, the inhibitory effect of NO$_3^-$ on LR development is systemic. When a similar experiment was performed by using 1 mM glutamine in the top and bottom segments, LR growth in the middle segment was inhibited by about 60% (data not shown), indicating that N sources other than NO$_3^-$ are capable of producing a systemic inhibitory effect.

In experiments with detached leaves, it was shown that feedback inhibitory effects of downstream metabolites (such as glutamine) on expression of the genes for NR and nitrite reductase can be counteracted by supplying a C source (21). To investigate whether a similar response occurred in the case of the inhibitory effect of NO$_3^-$ on LR development, we measured the growth of LRs of seedlings grown on a range of NO$_3^-$

concentrations and either the standard sucrose concentration (0.5%) or a higher concentration of sucrose (2%). Fig. 4*B* shows that the strong inhibitory effect of 10 mM and 50 mM KNO$_3$ seen at 0.5% sucrose was markedly alleviated at 2% sucrose.

These experiments suggested that the inhibitory effect of 50 mM KNO$_3$ was due to its influence on the N status of the plant. To determine the importance of NO$_3^-$ assimilation in the process, we investigated the sensitivity of the NR-deficient *nia1nia2* mutant (22) to high rates of NO$_3^-$ supply. Seedlings of the wild type (Col) and the *nia1nia2* mutant were grown on plates containing a range of NO$_3^-$ concentrations from 1 to 10 mM, and the numbers of stage C and stage D laterals were determined. As is evident from the data in Fig. 3*C*, the relative numbers of stage C/stage D laterals provides a sensitive indicator of the specific inhibitory effect of NO$_3^-$ on stage C laterals. As expected, the ratio of stage C/stage D laterals in both the wild-type and mutant lines increased as the NO$_3^-$ concentration was increased. However, whereas at 1 mM KNO$_3$, the proportion of stage C and stage D laterals in the mutant and wild-type seedlings was similar, higher concentrations of NO$_3^-$ led to a greater accumulation of stage C laterals in *nia1nia2* than in Col, with the differences between the lines becoming increasingly pronounced with increasing NO$_3^-$ concentrations. Thus, the NR-deficient mutant has enhanced sensitivity to the inhibitory effect of high rates of NO$_3^-$ supply on LR development.

DISCUSSION

The evidence reported here and elsewhere (12), demonstrates that the availability and distribution of the NO$_3^-$ supply have very marked effects on LR growth and development in *Arabidopsis*. Most of these effects are consistent with a model in which NO$_3^-$ has both positive and negative effects on LR proliferation (12). In the present study, we show that these opposing effects of NO$_3^-$ not only operate by different pathways but also act at different stages of LR development.

Nitrate concentrations as low as 0.05 mM, if applied to just one zone of the primary root, were found to be able to stimulate LR proliferation within that zone, and this was because of an increased rate of cell production in the LR meristem (Fig. 1). In contrast to similar studies in other species (6–8), the localized supply of NO$_3^-$ had no effect on LR initiation. Furthermore, whereas a localized supply of NH$_4^+$ is reported to stimulate LR initiation and elongation in barley (23), we found no effect of localized supplies of either NH$_4^+$ or glutamine on *Arabidopsis*.

We previously showed that an NR-deficient *Arabidopsis* mutant, with a much reduced capacity to assimilate NO$_3^-$, is able to respond normally to a localized NO$_3^-$ supply (12). This result, together with the present finding that other N sources fail to stimulate LR elongation, does not support models in which localized LR proliferation is attributed either directly or indirectly to the nutritional role of NO$_3^-$ (6–8, 10). Our data are more consistent with the previous suggestion that NO$_3^-$ is acting as a signal rather than as a nutrient (12), i.e., that meristematic activity in the LR tip is responding directly to the external NO$_3^-$ concentration. A similar signaling role for NO$_3^-$ in stimulating root growth in tobacco was proposed on the basis of split-root experiments with an NR-deficient mutant (24).

The product of the NO$_3^-$-inducible *ANR1* gene, a putative transcription factor of the MADS-box family, was recently identified as a likely component of the signal transduction pathway linking external NO$_3^-$ to increased LR proliferation (12). Additional insight into this pathway comes from the finding that LR elongation in the *axr4* mutant failed to respond to a localized NO$_3^-$ supply, whereas two other auxin-resistant mutants resembled the wild type (Fig. 2). The *axr4* mutant is
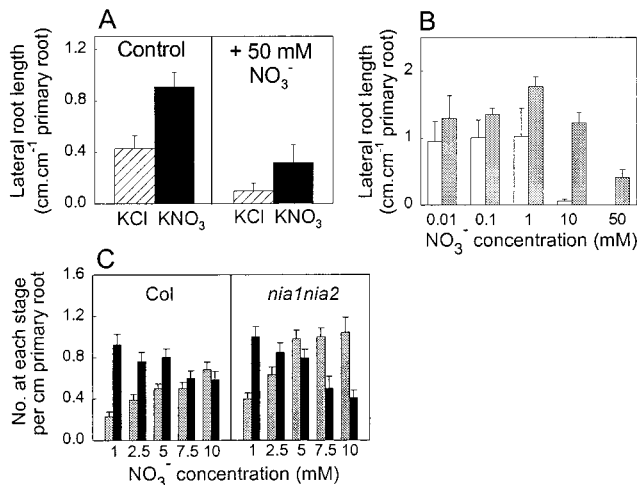

FIG. 4. Characterization of the inhibitory effect of NO$_3^-$ in wild-type and an NR-deficient line. (*A*) The inhibitory effect of a high NO$_3^-$ concentration is systemic. Seedlings were grown on segmented agar plates in which the top and bottom segments contained either 0.01 mM NH$_4$NO$_3$ (control) or 50 mM KNO$_3$, whereas the middle segments received either 1 mM KCl (hatched bars) or 1 mM KNO$_3$ (filled bars). LR lengths in the middle segment were measured 8 days after transfer (11–17 seedlings per treatment). (*B*) Increasing the sucrose concentration in the medium partially relieves the inhibitory effect of high NO$_3^-$ concentrations. Seedlings were grown for 7 days on unsegmented agar plates containing a range of KNO$_3$ concentrations and either 0.5% (open bars) or 2% (shaded bars) sucrose. Note that there was no significant LR growth in the 50 mM NO$_3^-$ treatment at 0.5% sucrose. (*C*) An NR-deficient mutant is more sensitive than the wild type to the inhibitory effect of high NO$_3^-$ concentrations. Seedlings of the wild type (Col) and of the *nia1nia2* mutant (22) (12–18 per treatment) were grown for 7 days on agar plates containing a range of KNO$_3$ concentrations, and the numbers of LRs at stages C (shaded bars) and D (filled bars) (see Fig. 3) were scored by bathing the roots in water and examining them at ×100 magnification with an inverted microscope.

unusual among auxin-resistant mutants, in that its sensitivity to other hormones such as ethylene and cytokinins is unaffected (19, 25), so its lack of responsiveness to the stimulatory effect of NO_3^- provides clear evidence for an overlap between the auxin and NO_3^- response pathways.

The inhibitory effect of NO_3^- on LR development differs in two important respects from its stimulatory effect. First, whereas the stimulatory effect acts on the mature LR, the inhibitory effect acts specifically on immature LRs, just after their emergence from the primary root (Fig. 3). Second, whereas the stimulatory effect is localized to the LRs directly exposed to the NO_3^-, the inhibitory effect is systemic (Fig. 4*A*).

The finding that the inhibitory effect of a treatment with 50 mM KNO_3 is much diminished when it is applied to just one part of the root system (Figs. 1*A* and 4*A*) indicates that this effect depends not on its local concentration but on the total amount of NO_3^- taken up by the plant. The further finding that LR development in the NR-deficient *nia1nia2* mutant is more sensitive rather than less sensitive to the inhibitory effects of high NO_3^- concentrations (Fig. 4*C*) suggests that the accumulation of NO_3^- itself within the plant is capable of generating the inhibitory effect. It is well established that NR-deficient mutants growing on NO_3^--containing media accumulate high concentrations of NO_3^-, particularly in their leaves (26–28). By using tobacco lines with different degrees of NR deficiency, it has previously been found that there is a strong positive correlation between the leaf NO_3^- content and the shoot/root ratio (13), leading to the conclusion that NO_3^- levels in the shoot act as a signal to regulate the allocation of resources between shoots and roots. Root growth in a NR-deficient line growing on 12 mM NO_3^- was inhibited 2- to 3-fold compared with controls growing on 0.2 mM NO_3^-, and the resultant root system was shorter and less "bushy" than the controls (13). There therefore appear to be strong parallels between the inhibitory effects we observe in *Arabidopsis* and those seen in tobacco, but whether LR development is affected in the same stage-specific way in tobacco as in *Arabidopsis* has yet to be established.

Although the evidence supports a role for tissue NO_3^- in generating the systemic inhibitory signal, we cannot rule out a possible additional contribution by downstream metabolites of NO_3^-. When 1 mM glutamine was included in the medium, it too had a systemic inhibitory effect on LR development (data not shown), and in tobacco it was similarly found that shoot growth was stimulated and root growth was inhibited when the medium was supplemented with either NH_4^+ or glutamine (13). These results suggest that products of NO_3^- and NH_4^+ assimilation (such as glutamine or other amino acids) may serve as additional indicators of the plant's internal N status, so that in plants that are not NR-deficient, the pools of these other N compounds may act in concert with the NO_3^- pool to determine the intensity of the inhibitory signal.

Previous work on LR development in *Arabidopsis* has identified the stage just after emergence of the LR primordium as a critical step (29). Evidence from cytological studies (30) and from the phenotype of the *rml1* (root meristemless) mutant (31) indicates that although the structure of the LR meristem is already fully formed before emergence, it is only after emergence that the meristem is activated to allow continued growth of the mature LR. It appears that the stage between differentiation of the meristem and its activation is a time when the LR primordium becomes susceptible to the postulated systemic inhibitory signal. As a result, the duration of this developmental stage is extremely flexible, to the degree that under very high rates of NO_3^- supply, it can be extended by several days or more. Because not all LRs are delayed equally at any given NO_3^- concentration (see Fig. 3*C*) it may be that individual LRs differ in their sensitivity to the inhibitory signal. Studies on LR development in *Vicia faba* identified two

periods of temporary mitotic quiescence or dormancy during early LR development, the second of which occurred just before emergence of the LR primordium (32).

Our current model for the dual pathways by which NO_3^- regulates root branching in *Arabidopsis* is summarized in Fig. 5. The nature of the proposed systemic inhibitory signal is unknown, but it seems likely that it emanates from the shoot. Split-root experiments with tobacco indicate that shoot-derived signals are responsible for regulating shoot/root partitioning in tobacco in response to tissue NO_3^- levels (13). Similarly, the processes of NO_3^- uptake and symbiotic N_2 fixation in plant roots are subject to feedback repression from regulatory signals that originate in the shoot (33–35).

The pool of amino acids that cycles between the shoot and the root is considered to be one possible means of transmitting information about the N requirements of the shoot (33, 35, 36). In tobacco, the high levels of leaf NO_3^- that were associated with an inhibition of root growth were correlated with a lower rate of sucrose export from the shoot and a lower sugar content in the root (24), leading to the suggestion that the reduced C allocation to the root may be responsible for the effects on root growth. Our finding that increasing the sucrose concentration in the medium from 0.5% to 2% partially relieved the inhibitory effect of 50 mM KNO_3 (Fig. 4*B*) would appear to be consistent with this hypothesis. However, the highly specific way in which LR development in *Arabidopsis* is inhibited by a high NO_3^- supply argues against the notion that the roots are simply C starved. It seems more likely that the stimulatory effect of sucrose is due to its effect on the plant's N status or its N/C ratio: there is evidence for regulatory mechanisms that monitor the balance between C and N metabolism in plants (21, 37), as there are in prokaryotes (38).

Although our observations have been made with *Arabidopsis*, an annual weed from disturbed habitats, the model we propose is consistent with the manner in which root architecture in species as diverse as cereals and trees is affected by the availability and distribution of NO_3^- (3). A key feature of the model is that it indicates how a plant could modify its pattern of root development in a way that integrates information about the spatial distribution of NO_3^- in the soil and the demand for N in the shoot. Whereas the localized stimulatory effect of NO_3^- allows for autonomous responses by individual lateral roots, the systemic inhibitory effect ensures that these re-
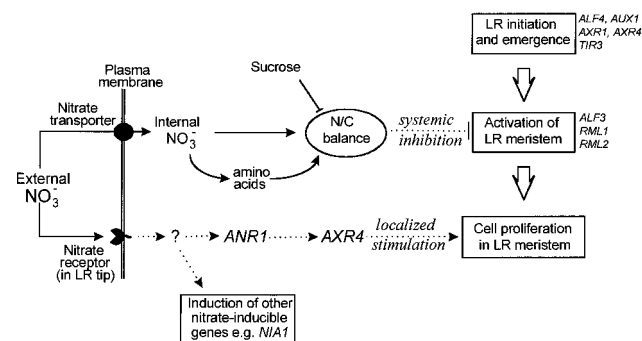

FIG. 5. Dual-pathway model for regulation of LR growth and development by NO_3^-. Because the *ANR1* gene is rapidly induced by NO_3^- (12), the putative NO_3^- receptor and the mechanism for transcriptional activation of *ANR1* are likely to be shared with other NO_3^--inducible genes such as the *NIA1* genes encoding NR (39, 40). We have tentatively placed *ANR1* upstream of *AXR4* in the signal transduction pathway. This arrangement makes a number of predictions that can be tested experimentally by using *axr4* mutants (19) and *ANR1* antisense lines (12). Other genes implicated in controlling particular stages in LR initiation or development (29) are shown on the right. Broken arrows indicate signaling steps, solid arrows indicate transport or metabolic steps, and large open arrows indicate developmental steps.

sponses are modulated according to the plant's nutritional needs, thus optimizing resource allocation within the plant as a whole.

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