An *Arabidopsis* MADS Box Gene That Controls Nutrient-Induced Changes in Root Architecture

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The development of plant root systems is sensitive to the availability and distribution of nutrients within the soil. For example, lateral roots proliferate preferentially within nitrate (NO_3^-)-rich soil patches. A NO_3^--inducible *Arabidopsis* gene (*ANR1*), was identified that encodes a member of the MADS box family of transcription factors. Transgenic plants in which *ANR1* was repressed had an altered sensitivity to NO_3^- and no longer responded to NO_3^--rich zones by lateral root proliferation, indicating that *ANR1* is a key determinant of developmental plasticity in *Arabidopsis* roots.

The structure of a root system is determined by an interplay between the intrinsic developmental program and external biotic and abiotic stimuli (*1*). One important role of such plasticity is to enable the plant to forage for mineral nutrients (*2*), which are usually distributed unevenly within the soil. Nitrate is the major source of the mineral N for many higher plant species, and its supply often limits plant growth and crop yields. Plants respond to its presence in a variety of ways that enhance their ability to absorb and metabolize it (*3*). Most dramatic of these is the stimulation of lateral root development that occurs specifically within NO_3^--enriched patches of soil (*4*).

In the course of screening *Arabidopsis* roots for NO_3^--inducible genes we identified a cDNA clone, pANR1, that has homology to the MADS (MCM1, AGAMOUS, DEFICIENS, and serum response factor) box family of transcription factors (*5*). Products of MADS box genes, found in a variety of eukaryotes, share a conserved motif within the DNA binding domain (*6*). In plants, most of the MADS box genes so far identified (more than 24 in *Arabidopsis* alone) are expressed in flowers; many of these affect floral organ identity (*7*). The *ANR1* gene belongs to the same subfamily (*6*) as two MADS box genes of unknown function: *AGL17*, an *Arabidopsis* root-specific gene (55% amino acid identity) (*8*), and *DEFH125*, an *Antirrhinum* pollen-specific gene (59% amino acid identity) (*9*). Hybridization of pANR1 to Southern blots of *Arabidopsis* genomic DNA showed that no other members of the MADS box family are sufficiently similar to cross-hybridize with it at high stringency (*10*).

Northern (RNA) blots showed that *ANR1* expression, undetectable in N-starved roots, is rapidly induced when NO_3^- is supplied (Fig. 1A) (*11*). We were unable to detect *ANR1* mRNA in stems or leaves of mature plants (Fig. 1B), suggesting that *ANR1*, like *AGL17* (*8*), is expressed preferentially or specifically in roots. We investigated whether *ANR1* is induced as part of a general response to nutrient starvation and resupply (Fig. 1C) and found that expression of the gene was maintained in NO_3^--grown roots regardless of changes in the supply of either K^+ or inorganic PO_4^{3-}.

To investigate the function of *ANR1*, we generated transgenic *Arabidopsis* lines in which expression of *ANR1* was down-regulated to various degrees by antisense or cosuppression effects (*12*) (Fig. 2A). The response of the transgenic root systems to either ubiquitous or locally concentrated supplies of NO_3^- was then analyzed.

First we examined the effect on root growth of a range of external NO_3^- concentrations (Fig. 2B) (*13*). Previous studies have demonstrated that lateral root development is sensitive to changes in the NO_3^- supply (*4*, *14*). In the parental line (C24) and a control transgenic line (43-3), increasing the concentration of NO_3^- ($[NO_3^-]$) from 10 μM to 1 mM had no significant effect on the growth of lateral roots, whereas at 10 mM and particularly at 50 mM their development was inhibited (Fig. 2B). When we compared the numbers of emerged and unemerged laterals in roots grown on 1 mM and 50 mM KNO_3 we found little difference (*15*), showing that the suppression of lateral root development that occurs at concentrations ≥10 mM is due to an effect on lateral root elongation rather than on initiation. As has been found for other species (*4*), primary root elongation was insensitive to $[NO_3^-]$ (Fig. 2B), demonstrating that the inhibition of lateral root growth by $[NO_3^-]$ ≥10 mM is not part of a general inhibitory effect on plant growth.

The sensitivity of lateral root elongation to $[NO_3^-]$ was altered in the *ANR1*-repressed lines (Fig. 2B). At 10 μM KNO_3, lateral root growth in the antisense lines did not differ from that in the control lines. However, concentrations of 100 μM or 1 mM, which had no effect on the control lines, significantly inhibited lateral root growth in A1 and A13, the most strongly down-regulated lines (Fig. 2B). S10, a line carrying the *ANR1* sense construct (*12*), had the lowest amounts of *ANR1* mRNA (Fig. 2A), probably because of cosuppression (*16*), and this line had a phenotype similar to A1 and A13 (*10*). Two lines with intermediate amounts of *ANR1* mRNA (A11 and A15) showed an intermediate sensitivity to NO_3^- inhibition (Fig. 2B). As in the control lines, primary root growth in the *ANR1*-repressed lines was unaffected by $[NO_3^-]$ (Fig. 2B), showing that the consequences of down-regulating *ANR1* are specific to lateral roots.

To investigate further the phenotype of the *ANR1*-repressed lines, we tested the effect of locally concentrated supplies of NO_3^- on root development (Fig. 3) (*17*). In the control lines (C24 and 43-3), lateral root growth in the NO_3^--enriched segment was stimulated two- to threefold compared with KCl-treated controls, whereas growth of the lateral roots in the top (low $[NO_3^-]$) segment was slightly (30%) inhibited (Fig. 3B). The localized NO_3^- treatment had no significant effect on the number of lateral roots in C24 (*18*), and more detailed analysis has shown that the increased proliferation of lateral

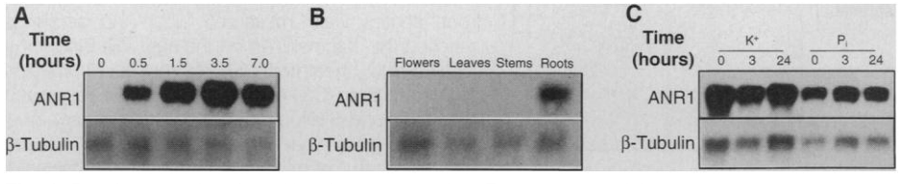

Fig. 1. Developmental and nutritional regulation of *ANR1* expression. (**A**) *ANR1* is rapidly induced by NO_3^-. Nitrogen-starved seedlings were treated with KNO_3 at time 0. Lanes contain total RNA from roots harvested at intervals after NO_3^- treatment, and the blot was hybridized with an *ANR1* cDNA probe (*11*). A β-tubulin cDNA was hybridized to the same filter as a loading control. (**B**) *ANR1* is expressed mainly or exclusively in roots. Lanes contain total RNA extracted from the aerial parts of greenhouse-grown plants and from roots of seedlings grown in liquid culture (*11*). (**C**) *ANR1* is not under general nutritional control. Seedlings were starved of K^+ or inorganic PO_4^{3-} (P_i) and at time 0 were resupplied with the appropriate nutrient (*11*). Lanes contain total RNA from roots harvested at intervals after nutrient resupply.

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roots is due to a twofold increase in the average rate of lateral root elongation in the treated zone (*10*).

The localized NO_3^- treatment was also applied to an *Arabidopsis* mutant (*nia1 nia2*) that has just 0.5% of wild-type nitrate reductase (NR) activity and is therefore ineffective at using NO_3^- as a source of N (*19*). This mutant showed a similar response to the localized NO_3^- treatment as the control lines (Fig. 3B). This result argues against previous hypotheses that it is the assimilation of NO_3^- locally at its site of uptake, and the consequent increased flux of photosynthate to that region of the root, that leads to localized lateral root proliferation (*20*) and supports an earlier suggestion (*21*) that the effect is due to the signaling properties of the NO_3^- ion itself.

When the localized NO_3^- treatment was applied to the three strongly *ANR1*-repressed lines, there was no significant effect on lateral root growth in the treated zone (shown for A13 and S10 in Fig. 3); the two intermediately repressed lines (A11 and A15) did show some response, but one which was diminished by about 70% compared with the response by C24 (*10*). Thus, the stimulation of lateral root elongation by localized applications of NO_3^- is dependent on expression of the *ANR1* gene (*22*).

To explain the manner in which down-regulation of *ANR1* alters the root's responses to localized and ubiquitous supplies of NO_3^-, we suggest a model in which the NO_3^- supply has two opposing effects on lateral root elongation: a localized stimulatory effect that requires *ANR1* expression and depends on the external [NO_3^-] at the lateral root tip, and a systemic inhibitory effect that results from its influence on the N status of the shoot, which in turn depends on how much NO_3^- is absorbed by the whole root system. The latter effect would not involve *ANR1* and would require some shoot-derived signal that suppresses lateral root elongation (*23*). This model can explain how a localized NO_3^- treatment stimulates lateral root elongation specifically within the treated segment. In this situation, the inhibitory effect should be felt by all the lateral roots, both outside and inside the NO_3^--rich zone, whereas the stimulatory effect should be sensed only by those lateral roots present within the NO_3^--rich zone.

The model is also consistent with both aspects of the phenotype of the *ANR1*-repressed lines. The absence of a positive response to the localized NO_3^- treatment (Fig. 3) would be due to the impairment of the signal transduction pathway that normally mediates the stimulatory effect of external NO_3^-. The increased sensitivity of the lateral roots to NO_3^- inhibition when NO_3^- is ubiquitously supplied (Fig. 2) would be the phenotype expected if the stimulatory effect of NO_3^- had been blocked, but the inhibitory effect had not.

What role might *ANR1* play in converting a NO_3^- stimulus at the lateral root tip into an increased rate of elongation? One possibility is suggested by analogy with another MADS box transcription factor, the human serum response factor. This protein is responsible for the rapid and coordinate activation of a set of "immediate-early" genes when quiescent human cell lines receive an extracellular mitogenic stimulus (*24*). In a similar way the *ANR1* gene product might be the transcriptional regulator of a set of genes that modulate the rate of lateral root elongation.

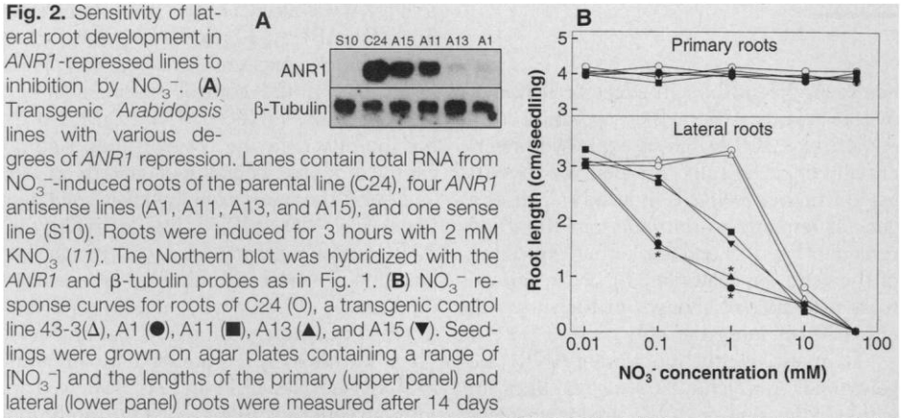

Fig. 2. Sensitivity of lateral root development in *ANR1*-repressed lines to inhibition by NO_3^-. (**A**) Transgenic *Arabidopsis* lines with various degrees of *ANR1* repression. Lanes contain total RNA from NO_3^--induced roots of the parental line (C24), four *ANR1* antisense lines (A1, A11, A13, and A15), and one sense line (S10). Roots were induced for 3 hours with 2 mM KNO_3 (*11*). The Northern blot was hybridized with the *ANR1* and β-tubulin probes as in Fig. 1. (**B**) NO_3^- response curves for roots of C24 (O), a transgenic control line 43-3(Δ), A1 (●), A11 (■), A13 (▲), and A15 (▼). Seedlings were grown on agar plates containing a range of [NO_3^-] and the lengths of the primary (upper panel) and lateral (lower panel) roots were measured after 14 days (*13*). Each point represents the mean of data from four to eight seedlings; those marked with an asterisk differed significantly from the 43-3 and C24 controls ($P < 0.05$ in a t test).

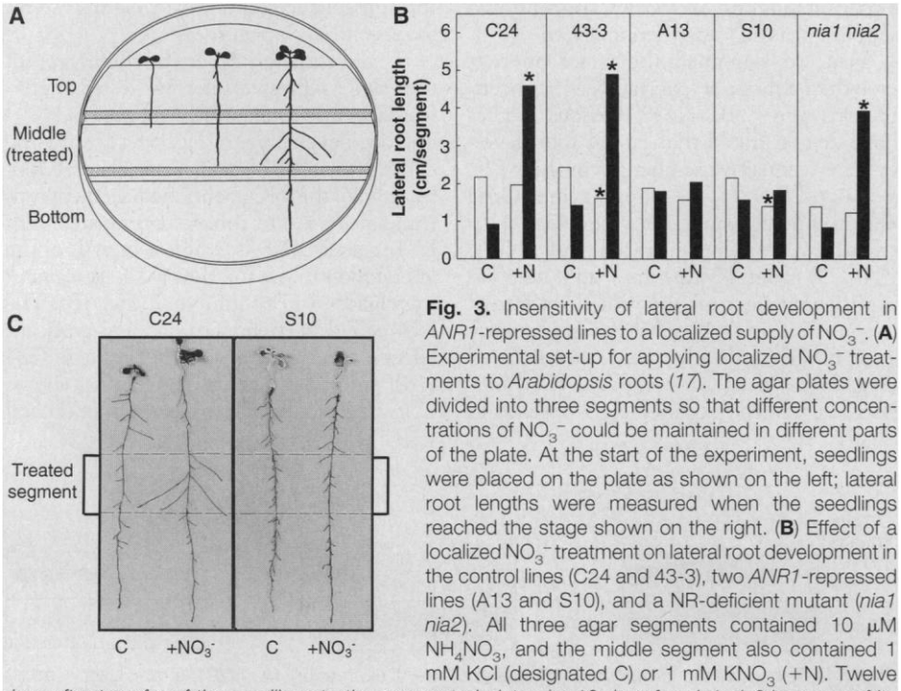

Fig. 3. Insensitivity of lateral root development in *ANR1*-repressed lines to a localized supply of NO_3^-. (**A**) Experimental set-up for applying localized NO_3^- treatments to *Arabidopsis* roots (*17*). The agar plates were divided into three segments so that different concentrations of NO_3^- could be maintained in different parts of the plate. At the start of the experiment, seedlings were placed on the plate as shown on the left; lateral root lengths were measured when the seedlings reached the stage shown on the right. (**B**) Effect of a localized NO_3^- treatment on lateral root development in the control lines (C24 and 43-3), two *ANR1*-repressed lines (A13 and S10), and a NR-deficient mutant (*nia1 nia2*). All three agar segments contained 10 μM NH_4NO_3, and the middle segment also contained 1 mM KCl (designated C) or 1 mM KNO_3 (+N). Twelve days after transfer of the seedlings to the segmented plates (or 13 days for *nia1 nia2* because of its slower growth rate) lateral root lengths were measured in the top (open bars) and middle segments (closed bars) (*17*). (The bottom segment was not included in the analysis because it did not yet contain many laterals). Each bar represents the mean of data from 8 to 12 seedlings; those marked with an asterisk differ significantly ($P < 0.05$ in a t test) from the control treatment to the same line. (**C**) Root morphology of seedlings of C24 and S10 that received (+NO_3^-) or did not receive (C) a localized NO_3^- treatment. Representative seedlings from the experiment in (B) were stained with toluidine blue and photographed.

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11. Seedlings of *Arabidopsis thaliana* ecotype Columbia were grown aseptically in liquid culture at 25°C in B5 medium [O. L. Gamborg, R. A. Miller, K. Ojima, *Exp. Cell. Res.* **50**, 151 (1968)] containing 2% glucose and 23 mM MES, pH 5.7. The seedlings were grown for 4 days in the absence of N (starved) in B5-N medium [B5 in which the KNO_3 and $(NH_4)_2SO_4$ were replaced by 1 mM K_2SO_4] and then induced with NO_3^- by treating them with 2 mM KNO_3. Starvation and resupply of K^+ or PO_4^{3-} were performed in the same way except that B5-N was substituted by B5 lacking either K^+ or PO_4^{3-}, and the roots were resupplied with either 2 mM KCl or 2 mM sodium phosphate buffer (pH 5.7) as appropriate. Standard techniques were used to extract total root RNA [L. J. Trueman, A. Richardson, B. G. Forde, *Gene* **175**, 223 (1996)] and for Northern blots [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)]. After hybridization to a ^{32}P-labeled cDNA probe, the membrane was washed at 65°C in 0.1× standard saline citrate (SSC) and autoradiographed.
12. *Arabidopsis thaliana* ecotype C24 was transformed with *Agrobacterium tumefaciens* strains carrying sense and antisense *ANR1* constructs. The sense construct was generated by fusing the pANR1 cDNA in the sense orientation to a duplicated cauliflower mosaic virus 35*S* promoter [F. Guerineau, A. Lucy, P. Mullineaux, *Plant Mol. Biol.* **18**, 815 (1992)]. For the antisense construct, a polymerase chain reaction fragment corresponding to bases 262 to 969 of the pANR1 sequence (which excludes the conserved MADS-box domain) was fused in the antisense orientation to the same promoter. Both constructs were transferred to pBIN19 and introduced into *A. tumefaciens* by electroporation. Root explants were transformed as described [D. Valvekens, M. Van Montagu, M. Van Lijsebettens, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536 (1988)].
13. Seeds of the parental and transgenic lines were surface-sterilized and sown in 9-cm petri dishes on medium containing 23 mM MES (pH 5.7), 0.5% (w/v) sucrose, 1% agar-agar (Fisons), and B5 salts (1:50 final dilution) in which KNO_3 and $(NH_4)_2SO_4$ were replaced with 1 mM KCl and 10 μM NH_4NO_3. The agar plates were placed vertically in continuous light at 25°C so that the roots grew over the agar surface. Once roots had developed (3 to 4 days), seedlings of similar size were transferred to fresh plates (three per plate) containing the same medium except that the 10 μM NH_4NO_3 was replaced by a range of KNO_3 concentrations.
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15. Seedlings were grown on vertical agar plates containing 1 mM or 50 mM KNO_3 (*13*), and the roots, still on the agar, were bathed in water and examined at ×100 magnification. The total numbers of emerged and unemerged lateral roots per centimeter of primary root were 1.9 ± 0.3 (1 mM KNO_3) and 1.5 ± 0.4 (50 mM KNO_3).
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17. Young seedlings with primary roots ~20 mm long were transferred to nutrient agar plates (*13*) that had been divided into three segments as shown in Fig. 3A. The top and middle segments were each 15 mm wide, with gaps of 3 to 4 mm between each segment. The middle segment had been adjusted to 1 mM KCl or 1 mM KNO_3 by spreading 50 μl of a 100 mM solution over its surface and leaving it overnight to diffuse.
18. The numbers of lateral roots in KCl-treated C24 were 3.7 ± 0.8 (top segment) and 3.9 ± 1.1 (middle); for NO_3^--treated C24, the numbers were 3.7 ± 1.1 (top) and 4.4 ± 0.8 (middle).
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22. We have found no change in the NO_3^--inducibility of NR (*3*) in the *ANR1*-repressed lines, indicating that this aspect of the NO_3^- response is not mediated by *ANR1* (*10*).
23. The growth rate of the root relative to the shoot is inversely correlated with the plant's total N content [G. I. Ågren and T. Ingestad, *Plant Cell Environ.* **10**, 579 (1987)], and recent experiments indicate that the inhibitory effect on root growth arises from the accumulation in the shoot of NO_3^- itself as well as organic N compounds [W.-R. Scheible *et al.*, *Plant J.* **11**, 671 (1997)].
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25. We thank N. Crawford for the *nia1 nia2* mutant, D. Marks for the β-tubulin cDNA, A. Williams for assistance with some experiments, and D. Clarkson for critical reading of the manuscript. IACR-Rothamsted is grant-aided by the Biotechnology and Biological Sciences Research Council.

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