A framework for complex decisions in a plant nutrient foraging strategy [Dennis thinks a better title might be: The Molecular Basis for a Nutrient Foraging Strategy in Plant Roots]

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Abstract:

Plants are non-motile but they still explore their surroundings through post-embryonic growth, navigating a heterogeneous environment. Here, we investigate the logic of nitrate foraging strategies in *Arabidopsis thaliana* using the split root system, in which isolated root systems of the same plant can be challenged with different environments. We show that plants integrate information from isolated appendages to mount remarkably flexible behaviors. Roots in a rich nitrogen environment alter their molecular and morphological program to resemble roots in a nitrogen-deprived environment under certain conditions. This strategy appears to optimize the acquisition of nitrogen in a heterogeneous environment, with the disparate conditions sharing up-regulation of nitrate assimilation genes, activation of lateral root stem cells, and ultimately similar overall root architectures. Shoot decapitation and cytokinin synthesis mutants do not affect local responses to nitrate but do abolish one type of conditional behavior. The results lead to a model in which cytokinin signaling in the shoot acts as a reservoir to integrate nitrate status from all root systems, forming one critical component of root-to-root communication. These results show how the plant coordinates molecular and morphological programs by processing information on conditions throughout the plant body with plausibly simple signaling systems and without a central nervous system.

For all living organisms, the capacity to respond to environmental change is one of the foremost challenges for survival and propagation. Despite the lack of any central nervous system, plants are able to display a repertoire of behaviors in response to their unpredictable environments (1). Unlike animals, the basis of the behavior does not rely on long distance movement but rather on phenotypic plasticity (2, 3). Underground foraging for nutrients and water in a heterogeneous environment drives much of root plasticity (4). For instance, it is well established that roots have the ability to sense and proliferate in nutrient-rich zones and decide to invest more of these resources in roots when the internal nutrient availability is limited (5). Some mechanisms of nutrient sensing are starting to be understood (6). However, little is known about the basis of plant decision-making processes and the signaling mechanisms that permit complex behaviors in plants.

To study conditional decision making in the plant, we utilized the split-root system in which a single plant is pruned to create two independent root systems that can be supplied with different media to mimic a heterogeneous environment (7-10). We focused on the different responses of *Arabidopsis* when nitrate (NO3-) concentrations varied between isolated root systems (Fig. 1A). As in all plants, NO3- is an essential, limiting nutrient and a key signal for gene expression, metabolism, growth and development in *Arabidopsis* (11-15). First, lateral root architecture was quantified in a homogenous nitrogen-rich environment (5mM NO3-; C.NO3), a homogenous nitrogen-deprived environment (5mM KCl for osmotic control; C.KCl,), and a heterogeneous environment (Sp.NO3/Sp.KCL) from 2 to 4 days after transfer to these conditions (Fig. 1A-B and SOM Fig. S1). Across this panel of conditions, we also sampled the early global transcriptional status of roots to quantify the coordination of morphological and molecular responses.

To establish the plant’s strategy when faced with a simple environment, we first examined the homogenous control conditions. For example, root systems proliferate when plants encounter nutrient deprivation in an apparent strategy to forage for the resources in short supply (16). This is reflected in our split root system where the total length of the lateral root system averaged 2.15±0.32 cm (cm LR/cm PR) in the nitrogen rich environment (C.NO3) and 2.90±0.23 cm in the nitrogen-deprived environment (C.KCl) after 4 days (p-val=0.05) (Fig. 1B). This showed that, in our system, roots exhibit a growth-in-deprivation response in the homogeneous environments and that growth is not limited in the experimental conditions by a lack of nitrogen.

[Dennis thinks this paragraph is unnecessary as you’ve already made this point.] Plants can also exhibit compensatory behavior in which morphological or transcriptional responses in constant local conditions are altered when conditions in the isolated root system are changed (7, 9, 17). But how do such compensatory responses represent a change in strategy tailored to a new environment? In particular, we were interested in changes in the plant’s strategy when confronted with an environment that challenged the logic of the growth-in-deprivation response. Thus, we compared root growth in both similar and disparate nitrate conditions in homogeneous and heterogeneous environments.

Theplant completely reversed its growth strategy in the heterogeneous environment, with lateral root growth increased in the nitrogen-rich compartment compared to roots in a nitrogen-rich homogenous environment (2.29±0.21 cmvs. 1.07±0.15 cm, p-val=0.0002) (Fig. 1B). [Sandrine, will everyone know the difference between lateral root growth and lateral root length?] Conversely, roots in the nitrogen-deprived half of the heterogeneous environment decreased growth compared to the nitrogen-deprived homogenous environment (1.01±0.15 cmvs. 1.45±0.13 cm, p-val=0.02) (Fig. 1B). Overall, lateral root length in nitrogen-rich media in the heterogeneous environment (Sp.NO3) resembled root architecture in a nitrogen-deprived media in a homogeneous environment (C.KCl), and, similarly, roots [Sandrine, do you mean root length? If not, what about the roots? Physical appearance?] in the nitrogen-deprived heterogeneous environment (Sp.KCl) resembled roots in the nitrogen-rich homogeneous environment (C.NO3). The similarities of the disparate conditions extended to most metrics of lateral root architecture, as they showed highly similar trends in lateral root emergence and elongation in different regions of the root (SOM Text-1). There were no significant differences in primary root length in any of the conditions, showing that plasticity largely targeted lateral roots (SOM Fig. S1A).

Thus, the plant reverses its growth-in-deprivation strategy to instead forage in the nitrogen rich half of its environment and retard lateral root growth in the nitrogen deprived environment in what would appear to be logical overall strategy to optimize nutrient uptake in different environments. Within the nitrogen acquisition strategy, the plant maintained a constant root-shoot ratio [Sandrine: what was that ratio and was there no variance?] in environments where nitrogen could be harvested, as the total LR length in the Sp.NO3 compartment was virtually the same as the total LR length in both compartments of the C.NO3 roots (2.29±0.21 cm vs. 2.14±0.32 cm; Fig. 1B). This demonstrates how the plant balances overall nitrogen needs with the most effective strategy to acquire this growth-limiting nutrient.

To understand the molecular basis of this complex behavior, we undertook a transcriptomic approach. RNA from C.NO3, Sp.NO3, Sp.KCl and C.KCl roots was extracted at 2 hrs, 8 hrs and 2 days after the beginning of the treatment in an effort to sample early responses and the dynamics of regulatory change (SOM Text-2). ANOVA first identified genes for which expression was affected by the interaction between NO3- presence and imbalance of nitrogen environmental conditions for all pooled time points, allowing dominant patterns to emerge but also permitting some dynamic patterns over time (“interaction set,” 123 genes; q-val<0.2 and p-val< 0.001; Table S1).

First, the 123 genes were used to cluster experiments on a dendrogram to probe global trends in gene expression in this interaction set. The ANOVA identified genes whose nitrate response was affected by heterogeneous environment, but it did not rule out any of the 15 possible clustering patterns of the four experiments on a dendrogram (SOM Fig. S3). At 2 hours, the experiments paired the two nitrate treatments together [Sandrine: please state explicitly which two treatments], showing that genes first respond to local nitrate concentration (Fig. 1C). However, by 8 hours and 2 days, large-scale changes in expression among the 123 genes [Sandrine: which 123 genes? The 123 genes first responding?] re-arranged the dendrogram by pairing the Sp.NO3 (the nitrogen-rich portion of the split root system) with the C.KCl (homogeneous KCL) treatments and the C.NO3 (homogeneous nitrogen) treatment with the Sp.KCl (split-root KCL treatment), precisely the reverse pattern created by similarities in total lateral root length after four days in the same treatments (Fig. 1C). Thus, the genes affected by nitrogen in the split root conditions first respond to local signals but are then controlled by regulatory signals that integrate information from other parts of the plant. [Sandrine: the following sentence and the first one of the next paragraph should be unified or this one should be dropped]The effect is to orchestrate a revised and apparently more effective strategy in which a set of molecular changes precede changes in lateral root architecture

The molecular and morphological responses appeared to represent a coordinated strategy to anticipate assimilation of newly foraged nitrogen or absorb stored nitrogen. For example, despite the different local nitrate conditions, the nitrogen-foraging roots (Sp.NO3 and C.KCl) showed an induction of genes involved nitrogen uptake and assimilation, such as *AtNRT3.1* and *NIR1* (SOM Text-2).

To determine when the earliest signs of developmental responses occurred, we used a molecular marker that reports the highly localized activity of lateral root founder cells within pericycle cells, which identify the earliest stages of lateral root initiation (18, 19). By two days, we observed a significant increase in the *CYCB1*::GUS reporter line in pericycle founder cells in Sp.NO3 roots compared to C.NO3 roots. (One tailed t-test, LR initiation density is 1.87±0.36 versus 1,18±0.2, p-val=0.06). This reporter activity is associated with early divisions of the “transient” stem cells that form lateral roots (18, 19). The increased lateral root initiation observed with the marker at day 2 was consistent with increases observed in lateral root density in the nitrogen-foraging roots by day 4. Overall, these results suggest that early cues [Sandrine: what constitutes the cues here?] rapidly communicate the global environment of the plant to alter the expression of a subset of genes and ultimately reshape the plant body. [Dennis finds this last sentence troublesome, because it appears to claim a lot but it’s not clear how the claim is tied to evidence]

A central question is to determine which signals mediate the conditional decision-making process with respect to gene expression and lateral root architecture? To efficiently monitor the interaction response in a number of conditions, we identified a set of 8 genes that robustly reported the interaction set of genes that showed the reverse response [Sandrine: which reverse response?] at eight hours (SOM Text-3). In the first step of nitrogen perception, we determined that NO3- itself was the critical signal rather than the assimilates of NO3-. Mutants, in which *Nitrate Reductase* (*Nia1*) gene expression and the accumulation of nitrate assimilates were severely reduced, still exhibited all nitrate responses (Fig. 2b). We also determined that conditional root-to-root responses required signaling to the shoot, as the roots of decapitated plants still responded to local nitrate conditions but completely lost conditional responses in the split root system (Fig. 2c). Altogether, these results show that the optimal foraging strategy of the plant rely on the perception of the NO3- imbalance through root-shoot-root signaling.

The phytohormone cytokinin has been shown to be a root-to-shoot NO3--derived messenger that modulates shoot growth (23, 24). However, it has not been implicated as a signal that can mediate NO3- status from one root system to another in the same plant. To test the connection between cytokinin and the conditional responses of the split root system, we repeated the split root treatments in a triple mutant for ATP/ADP isopentenyltransferases (*ipt3,5,7*), which has severely reduced cytokinin biosynthesis (25). The *ipt3,5,7* mutant was not impaired in local nitrate responses but the mutant lost part of the conditional response; that is, roots in the Sp.NO3 environment lost the ability to respond to conditions in the Sp.KCl environment of the same root (Fig. 3A-B). However, the conditional response that repressed lateral roots in Sp.KCl compared to C.KCl remained intact, showing that the *ipt3,5,7* triple mutant did not just cause general defects that mimicked a loss of all shoot signaling (Fig. 3B). In addition, the total LR length in C.NO3, Sp.KCl and C.KCl was unchanged between the control [Sandrine: do you mean wildtype?] and the mutant, ruling out an effect of the mutation on the general root growth (Fig. 3B). Consistently, the induction of the eight reporter genes was restored in Sp.NO3 roots when cytokinin was added back to the same compartment (Fig. 3A). Thus, the result demonstrates that cytokinin is an essential signaling component for the conditional Sp.NO3 response that requires information flow from one root system to another.

To reveal the spatial integration of signaling, we used the type-A Arabidopsis Response Regulators (ARRs), which are a family of primary cytokinin response genes (26), to monitor cytokinin signaling in the root and shoot at eight hours after treatment. In the root, the ARRs were up-regulated in proportion to local nitrate concentration while, in the shoot, the ARRs were upregulated in proportion to global nitrate levels, as reflected by an average nitrate concentration in both compartments (SOM Text-4). Thus, cytokinin activity in the shoot appears to be [Sandrine: “appears to be” is too weak for Science] correlated with a summation of nitrogen concentration and cytokinin activity in all root systems.

These results suggest a model in which the local nitrate supply induces cytokinin biosynthesis in roots and leads to cytokinin accumulation in the shoot, likely by direct movement through xylem (23, 27). Translocation of cytokinin in the shoot would act as a global integrator of the nitrate status from all root systems of the plant, solving the problem of requiring distinct signals from all isolated roots. The model also predicts the existence of a second modifying descending signal to instruct the root system to proliferate. The shoot-derived cytokinin signal could either act in combination with a local NO3--derived signal or be guided directionally into a specific root system driven by the NO3- supply (Fig. 3C). [Sandrine: It’s a pretty model. Is there any way to radioactively trace this behavior?]

Overall, the flexible strategies of root nitrate foraging can be viewed as a complex decision-making process, such as a decision tree from the perspective of a given isolated root system (Fig. 4). Root behavior is influenced by multiple inputs and their spatial origin, which can be modeled as different levels of the tree. For example, roots may shut down foraging when a root system and its isolated counterparts are in a nitrogen rich environment (Fig. 4, top leaf) but override this program when another root system of the plant is starved for nitrogen (Fig. 4, second from top leaf). Crop improvement and domestication has frequently targeted the plant’s intrinsic programs to balance its modular growth, such as the ratio between grain and total biomass. The results show how canonical signaling pathways are used by the plant to coordinate a complex strategy that can be altered through potentially simple signaling cues.

**Fig. 1.** *Arabidopsis* roots display a coordinated morphological and molecular strategy in response to a heterogeneous NO3- environment. (**A**) Diagram shows the physical split-root experimental set up to detect long-range sensing between plant roots and conditional responses. All plants are grown in an identical manner that creates two separate root systems joined by a short segment of the primary root. Such roots are subjected to three different treatments: Control KNO3 (C.NO3) plants received KNO3 on both sides of the root system, Control KCl (C.KCl) plants received KCl on both sides, and Split plants received KNO3 (Sp.NO3) on one side and KCl (Sp.KCl). The gray line in each set up represents a gap between the media in the two compartments that keeps conditions on the two sides isolated. (**B**) Lateral root responses in the split-root treatments showing the total lateral root (LR) proliferation in each of the four distinct conditions. At top, the bar graph depicts the total lateral root (LR) length normalized by the length of the primary root (PR) as (cm LR/cm PR). In C.NO3 and C.KCl, measurements on both root systems were pooled and averaged. The numbers above bar graph are the total average LRs length of the whole root system per plant in each of the conditions. Each bar graph represents the mean of at least 10 roots. The different letters on top of the bars indicate statistically significant differences (p≤0.05; t-test), such that any two bars with a different letter showed a significant difference between them. Error bars=standard error. At bottom, one representative set of lateral roots illustrating the trends in lateral root length in the different treatments is shown. (**C**) Genes whose nitrate response was altered in the split-root experiments showed a similar pattern of change as lateral roots. The heat map depicts the expression pattern of 123 genes that showed an interaction between nutrient treatment and environmental heterogeneity in ANOVA. The same set of genes was used to generate dendrograms to cluster experiments at the different time points (see Methods). At 2 hrs, roots in the presence of NO3- cluster together. At 8 hrs, roots in C.NO3 and Sp.KCl cluster together and Sp.NO3 and C.KCl roots cluster together. The numbers at each node in the dendrogram represent bootstrap values from permutation tests.

**Fig. 2.** The coordinated response of roots in a heterogeneous environment requires sensing of NO3- itself and is mediated through the shoot. For each 3 panels, the bar graph represents the relative mRNA accumulation of the *Glucose-6-Phosphate Dehydrogenase 3* (*G6PDH3*) gene and the line graph represents the relative mRNA accumulation of the 8 genes used to monitor interaction effects (as described in text). The asterisks indicate significant differences between two compartments. The numbers on the line graph are the average percentage of relative mRNA accumulation increase for the 8 genes, either between Sp.NO3 and C.NO3, C.KCl and Sp.KCl, or NO3- and KCl. Trends are shown for (a) an untreated wildtype (WT) background, (b) the NR-null mutant in which nitrate reductase activity is dramatically reduced and, (c) WT roots decapitated at the time they were transferred to the split or control treatments. All roots were harvested for RNA expression analysis 8 hours after treatment.

**Fig. 3.** Cytokinin mediates coordination of root responses in a heterogeneous environment. (**A**) Expression of G6PDH3 and the 8 reporters of the conditional response were assayed by qPCR in the standard set of treatments used in a WT background (top), the *ipt3,5,7* background (middle), and the *ipt3,5,7* in which cytokinin was added back to the roots in the Sp.NO3 compartment (bottom), showing the rescue of gene induction in that compartment. The add-back treatment used 1nM trans-zeatin cytokinin, which is known to move from the root to the shoot (28). The asterisks indicate the significant differences between two compartments. The numbers on the line graph are the average percentage of relative mRNA accumulation increase for the 8 genes, either between Sp.NO3 and C.NO3, C.KCl and Sp.KCl. N.A.=Non Applicable. (**B**) Total LR length (cm LR/ cm PR) is shown in WT control compared to the *ipt3,5,7* mutant, which shows a loss of the Sp.NO3 response, similar to the genes in middle panel of (A). (**C**) A model of cytokinin as an integrator of nitrate conditions in different root compartments in which a reservoir of cytokinin activity in the shoot integrates nitrate readouts from all root systems (left). In a second stage of the model, the cytokinin-activity reservoir communicates system-wide nitrogen status to all roots (signal A). The spatial specificity of the system requires that signal A interact with at least one other signal that provides information on a particular root system’s nitrogen status (signal B, right). The modifying signal B may act in combination with signal A or signal B may provide directional information for signal A that induce gene responses and lateral root architectural changes.

**Fig. 4.** The strategic behavior of the plant root in homogeneous and heterogeneous environments is represented by a decision tree. The decision tree has been built from the perspective of the root in environment A. The first branches of the tree define the status of the local nitrogen environment (Yes = NO3- rich environment and No = NO3- deprived environment). The second level similarly defines the status of the distant, isolated root nitrogen environment. The dark grey box indicates that cytokinin (CK) plays a role in overriding the suppression of lateral roots in a nitrogen rich environment (First Level=Yes) when the distant root system is deprived of nitrogen (Second Level=No) but also alters the expression responses of the genes preceding the morphological adaptation.

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