Plant decision-making coordinating root responses to nitrogen cues involves cytokinin

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Plants are non-motile but explore their surroundings through post-embryonic growth, navigating a heterogeneous environment. Here, we investigate the logic of nitrate foraging strategies in *Arabidopsis thaliana*. By using a framework in which physically isolated root systems of the same plant can be challenged with different environments, we highlight that plants are able to integrate information from isolated appendages and make decisions resulting in remarkably flexible behaviors. In particular, we show that roots in a nitrate-rich compartment alter their molecular (genes expression) and morphological (lateral root architecture) programs in response to the nitrogen deprivation of the other root part like the roots do in a homogeneous nitrogen-deprived environment, presumably to optimize the acquisition of nitrate. Shoot decapitation and cytokinin synthesis mutants do abolish this type of conditional behavior, leading 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-shoot-root communication.

For all living organisms, the capacity to sense and adapt 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*). Belowground, 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 (*6-8*). 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 manipulated to create two independent root systems that can be supplied with different media to mimic a heterogeneous environment (*9-13*). Because NO3- is an essential, limiting nutrient and a key signal for gene expression, metabolism, growth and development (*14-19*), we focused on the different responses of *Arabidopsis* when nitrate (NO3-) concentrations varied between physically isolated root systems (Fig. 1A). First, we quantified root architecture in three different environments: (1) a homogenous nitrogen-rich environment (C.NO3: both compartments have 5 mM NO3), (2) a homogenous nitrogen-deprived environment (C.KCl: both compartments have 5 mM KCl and no nitrate), and (3) a heterogeneous split environment (Sp.NO3/Sp.KCL: one compartment has 5 mM NO3 and the other 5 mM KCl, respectively), from 2 to 4 days after transfer to these conditions. Across this panel of conditions, we also sampled the early global transcriptional status of roots to determine the coordination between 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 (*20*). This is reflected in our split root system where the total length of the lateral root (LR) system averaged 2.15±0.32 cm (cm LR/cm PR=Primary Root) in the C.NO3 environment against 2.90±0.23 cm in the C.KCl environment 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 is not limited in the experimental conditions by a lack of nitrogen.

Plants can also exhibit compensatory behavior in which morphological or transcriptional responses in constant local conditions are altered when conditions in the other part of the root system are changed (*9, 11, 13, 21*). We were particularly 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 homogeneous and heterogeneous environments. We found that the plant completely reversed its growth strategy in the heterogeneous split environment, with LR growth increased in the nitrogen-rich compartment (Sp.NO3, 2.29±0.21 cm) compared to roots in a nitrogen-rich homogenous environment (C.NO3, 1.07±0.15 cm; p-val=0.0002) (Fig. 1B). Conversely, roots in the nitrogen-deprived half of the heterogeneous environment decreased growth (Sp.KCl, 1.01±0.15 cm)compared to the nitrogen-deprived homogenous environment (C.KCl, 1.45±0.13 cm; p-val=0.02) (Fig. 1B). Overall, LR length in Sp.KCl environment resembled roots in C.NO3 environment, and, similarly, we observed root proliferation in Sp.NO3 and C.KCl environments. These similarities extended to most metrics of LR architecture, as they showed highly similar trends in LR 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 LRs (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 LR growth in the nitrogen deprived environment in what would appear to be logical overall strategy to optimize nutrient acquisition in different environments. Moreover, the plant maintained a constant root volume 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. 2.15±0.32 cm; Fig. 1B). This shows how the plant balances overall nitrogen needs with the most effective strategy to acquire this growth-limiting nutrient. This complex behavior can be assimilated as a decision-making process involving communication between the two roots systems confronted to different environments.

To understand the molecular basis of this remote sensing 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). An analysis of variance first identified genes responding to an interaction between NO3- availability (presence or absence) and split conditions for all pooled time points: “interaction set” of 123 genes (q-val<0.2 and p-val< 0.001; Table S1).

These 123 genes were used to cluster experiments on a dendrogram to probe dominant trends in gene expression. At 2 hrs, the dendogram paired the two nitrate treatments together, showing these genes responded first to local nitrate concentration (Fig. 1C). However, by 8 hrs and 2 days, large-scale changes in expression among the 123 genes re-arranged the dendrogram by pairing the Sp.NO3 with the C.KCl treatments and the C.NO3 with the Sp.KCl treatments (Fig. 1C). This surprising resemblance of disparate conditions closely parallels that observed with LR architecture after four days in the same treatments. Thus, the genes affected by the interaction between NO3- availability and split conditions first respond to the local root environment but are then controlled by regulatory signals that integrate information from other parts of the plant. The effect is to orchestrate a revised and apparently more effective strategy in which a set of molecular change precedes change in LR 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 in 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 the marker line *CYCB1::GUS* for which the GUS activity is associated with early divisions of the “transient” stem cells that form LRs within pericycle cells and consequently identified the earliest stages of LR initiation (*22, 23*). By 2 days, we observed an increase in the number of LR initiation events in pericycle founder cells in Sp.NO3 roots compared to C.NO3 roots (One tailed t-test, 1.87±0.36 versus 1,18±0.2, p-val=0.06; SOM text-1; Fig. S2B). This increased LR initiation was consistent with increases observed in LR density in the nitrogen-foraging roots by day 4.

Overall, these results suggest that early cues rapidly communicate the global environment of the plant to alter the expression of a subset of genes and ultimately reshape the plant body.

A central question is to determine which signals mediate the conditional decision-making process with respect to gene expression and LR architecture. To efficiently monitor the interaction response in a number of conditions, we identified a set of 8 genes that robustly reported the dominant trend of the “interaction set” of genes (SOM Text-3). In the first step of nitrogen perception (8 hrs), we determined that NO3- itself rather than its assimilates [you should name them], both being involved in morphological and molecular reprogramming (*11, 12, 24-26*), was the critical signal. Specifically, mutants in which Nitrate Reductase (*Nia1* and *Nia2 genes*) activity were severely reduced (*25*) still exhibited the usual response of the 8 reporter genes (Fig. 2a-b). 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, in our framework, the root decision-making 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 (*27, 28*). 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 (*29*). 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 the NO3- deprivation of the other root part (Fig. 3A-B and SOM Text-4). However, the conditional response that repressed LRs in Sp.KCl compared to C.KCl remained intact, showing that the *ipt3,5,7* triple mutant did not cause 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 wild-type and the mutant, ruling out an effect of the mutation on general root growth (Fig. 3B). Consistently, the induction of the 8 reporter genes was restored in Sp.NO3 roots when cytokinin was added back to the NO3- compartments (Fig. 3A). Thus, the result demonstrates that cytokinin is an essential component of the signaling system from root to root in the heterogeneous environment.

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 (*30*), to monitor cytokinin signaling in the root and shoot at 8 hrs 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-5). Thus, cytokinin activity in the shoot appears to be 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 (*27, 31*). 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 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). 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 (LR) responses in the split-root treatments showing the total LR proliferation in each of the four distinct conditions. At top, the bar graph depicts the total 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 the bar graph are the total average LR 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 or numbers above the bar with a different letter showed a significant difference between them. Error bars=standard error. At bottom, one representative set of LRs illustrating the trends in LR length in the different treatments is shown. (**C**) Genes whose NO3- response was altered in the split-root experiments showed a similar pattern of change as LRs. The heat map depicts the expression pattern of 123 genes that showed an interaction between NO3- availability and split conditions 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 and 2 days, 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) the wild-type (WT) background (plants were grown in the conditions used for the NR-null mutant; see Methods), (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 homogeneous treatments. All roots were harvested for RNA expression analysis 8 hrs 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 the WT (top), the *ipt3,5,7* background (middle), and the *ipt3,5,7* in which cytokinin was added back to the roots in the NO3- compartments (bottom), showing the rescue of gene induction in the Sp.NO3 compartment. The add-back treatment used 1nM trans-zeatin cytokinin, which is known to move from the root to the shoot (*32*). 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 or between C.KCl and Sp.KCl. N.A.=Non Applicable. (**B**) Total LR length (cm LR/ cm PR) is shown in WT 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 LR 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 LRs 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. [This model cannot be enough I think. Here is why: look at the world from the perspective of the Sp.KCL environment. Either it hears that there is enough NO3 or not. Let’s say it hears that there is not enough. Then it would grow lateral roots regardless of knowing that the other side was rich in NO3. Let’s say it hears that there is enough then neither it nor the SP.NO3 side would grow lateral roots. So the mechanism must be more sophisticated, e.g. if cytokinin levels indicate more than would be expected from what my part of the root sends up, then another part of the root must be happier and so I will not grow more LRs; conversely, if cytokinin levels indicate less than would be expected from what my part of the root sends up, then I must be doing everything and so I should keep sending out LRs.]

**References and Notes**

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