Nitrogen economics of root foraging: Transitive closure of the nitrate–cytokinin relay and distinct systemic signaling for N supply vs. demand

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As sessile organisms, root plasticity enables plants to forage for and acquire nutrients in a fluctuating underground environment. Here, we use genetic and genomic approaches in a "split-root" framework—in which physically isolated root systems of the same plant are challenged with different nitrogen (N) environments—to investigate how systemic signaling affects genome-wide reprogramming and root development. The integration of transcriptome and root phenotypes enables us to identify distinct mechanisms underlying "N economy" (i.e., N supply and demand) of plants as a system. Under nitrate-limited conditions, plant roots adopt an "active-foraging strategy", characterized by lateral root outgrowth and a shared pattern of transcriptome reprogramming, in response to either local or distal nitrate deprivation. By contrast, in nitrate-replete conditions, plant roots adopt a "dormant strategy", characterized by a repression of lateral root outgrowth and a shared pattern of transcriptome reprogramming, in response to either local or distal nitrate supply. Sentinel genes responding to systemic N signaling identified by genome-wide comparisons of heterogeneous vs. homogeneous split-root N treatments were used to probe systemic N responses in *Arabidopsis* mutants impaired in nitrate reduction and hormone synthesis and also in decapitated plants. This combined analysis identified genetically distinct systemic signaling underlying plant N economy: (*i*) N supply, corresponding to a long-distance systemic signaling triggered by nitrate sensing; and (*ii*) N demand, experimental support for the transitive closure of a previously inferred nitrate–cytokinin shoot–root relay system that reports the nitrate demand of the whole plant, promoting a compensatory root growth in nitrate-rich patches of heterogeneous soil.

Systems analysis | root morphology | hormone

For all living organisms, the capacity to sense and adapt to environmental change is one of the foremost challenges for survival and propagation. The short-term physiological and morphological responses to fluctuations in the external nutrient environment are even more critical for sessile organisms like plants, giving a particular relevance to the network signaling involved in these adaptive mechanisms. Below ground, plant root plasticity to fluctuating environments is a primary mechanism for optimizing water and nutrient acquisition/use and depends on the integration of local and systemic signaling. Indeed, plant roots have the ability to sense their environment, enhance their uptake/assimilation systems, and proliferate specifically in nutrient-rich zones (local signaling). This phenomenon is enhanced when the internal nutrient availability is limited (systemic signaling) (1). This dual regulation by local and systemic signaling holds true for nutrients such as nitrate (NO_3^-), one of the most growth-limiting nutrients. The current model depicting this dual regulation proposes that root growth/development and NO_3^- transport are (*i*) regulated locally by NO_3^- itself and (*ii*) under a systemic feedback repression by reduced nitrogen (N) metabolites (2, 3). One major challenge is to identify the molecular components of these local and systemic N-signaling pathways and the mechanism for their integration that enables plant roots to properly respond to the varying environmental nutrient scenarios they encounter in the soil.

Recently, it has been demonstrated that root NO_3^- sensing originates from the functional activity of the NO_3^- transporter/sensor (transceptor) NRT1.1 (4–6). In particular, root proliferation in NO_3^--rich zones relies on the dual NO_3^-/auxin transport activity of this NO_3^- transceptor (NRT1.1) (7), which illustrates at a mechanistic level the intricate relationship between nutrients, hormones, and growth (8). Other key regulatory components of the NO_3^- perception and signaling pathway have also been identified, such as transcription factors (ANR1, NLP7, and SPL9) and kinases (CIPK8 and CIPK23). These regulators control root developmental and metabolic activity (e.g., lateral root growth and NO_3^- transport/assimilation) (5, 9–13). Thus, deciphering the signaling pathways that perceive and integrate external and internal N status will improve our understanding of how plants coordinate the different N-signaling mechanisms to respond and grow in heterogeneous soil habitats.

Despite progress in understanding the nature of local nutrient signaling, the signaling mechanisms and the long-distance (systemic) signals through which a plant regulates root activity according to its nutrient status remain largely unknown (3, 14–16). The accumulation of N assimilation products (e.g., amino acids) as a negative feedback signal to mediate root activity (e.g., in particular NO_3^- uptake), has been proposed (17). Physiological evidence has highlighted their unequivocal role (18) and it has been shown that Glu/Gln signaling is involved in the N repression of lateral root outgrowth, involving mir167 and the auxin response factor, ARF8 (19). However, the direct involvement of N assimilation products in systemic N signaling has not been demonstrated (20).

Other putative systemic signals of nutrient status are hormones that have been shown to play an important role in nutrient signaling (8, 20, 21), especially in the case of nitrogen (22). Importantly, a specific role for cytokinin (CK) as part of systemic N signaling has been proposed because (*i*) NO_3^- supply induces an increase in CK content in the xylem of roots and shoots, due to the specific induction of *IPT3*, which encodes an adenosine phosphate-isopentenyltransferase (the first enzyme involved in CK biosynthesis) (23–25); (*ii*) CK regulates the expression of N uptake- and assimilation-related genes (8, 21, 22), as well as root architecture (26–29); and (*iii*) CK may function as a "root-to-shoot" long-distance signal related to NO_3^- supply (23, 25). However, an essential experimental validation is missing, because to date, no evidence supports the role of CK as a systemic

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relay that integrates N status and the regulation of root activity (N transport and architecture) accordingly (22).

In this paper, we establish that CK is a crucial component of a root–shoot–root signaling/relay mechanism involved in conveying the NO_3^- status of the plant as a "system", thus enabling a compensatory increase of lateral root growth in NO_3^--rich zones of a root system foraging for N resources in a heterogeneous N environment. In addition, our results led us to extend the current model saying that root architecture is under the control of a dual signaling pathway (one local and one systemic) by proposing the existence of at least two genetically independent systemic signaling mechanisms reporting the N supply and demand of a plant. This study has led to our coining of the term and discovery of the systemic signaling mechanisms controlling "plant nitrogen economics".

Results and Discussion

Split-Root System: A Framework to Study the Nitrogen-Signaling Network in *Arabidopsis*. To study N-related systemic signaling controlling root development, we used the split-root system, in which a single plant is manipulated to create two physically separated root systems that can be supplied with different nutrient media to mimic a heterogeneous soil environment (2, 4, 30–32). Because NO_3^- is an essential, growth-limiting nutrient and a key signal for gene expression, metabolism, growth, and development (3, 33–36), we focused on the different responses of *Arabidopsis* when the NO_3^- concentration was varied between physically isolated root systems (Fig. 1*A*). We quantified root architecture in three different NO_3^- environments: (*i*) a homogeneous N-replete environment (C.NO3: both compartments have 5 mM KNO3), (*ii*) a homogeneous N-deprived environment (C.KCl: both compartments have 5 mM KCl), and (*iii*) a heterogeneous split environment (Sp.NO3/Sp.KCl: one compartment has 5 mM KNO3, and the other has 5 mM KCl), from 2 to 4 d after transfer to these conditions. There were no significant differences in the main root [that we considered hereafter as a primary root (PR)] length in any of the conditions, showing that root plasticity largely targeted lateral roots (LR) under these growth conditions (Fig. S1).

Overall, roots adopted either an "active-foraging strategy", characterized by outgrowth of LRs in the disparate Sp.NO3 and C.KCl conditions, or a "dormant foraging strategy" characterized by a repression of LR outgrowth in the disparate Sp.KCl and C.NO3 conditions. (Note that a thorough analysis of root development under these diverse conditions is provided in *SI Results*). It is noteworthy that the plant as an integrated system maintained a constant level of root proliferation within the compartments that contained NO_3^-, 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 combined [(2.29 ± 0.21 cm LR·PR^{-1}) vs. (2.15 cm LR.PR^{-1} = 1.07 ± 0.15 cm LR·PR^{-1} × 2 root parts); Fig. 1*B*]. Altogether, the LR responses in this split-root system seem to display a logical overall adaptive strategy that plants use to optimize nutrient acquisition in different environments. These responses fit with the current model of dual regulation by local NO_3^- and systemic feedback repression (2, 3): i.e., (*i*) C.NO3 roots are under a systemic feedback repression in response to high NO_3^- supply. (*ii*) In split-root plants that are exposed to NO_3^- only on one-half of their root system, the level of this systemic repression is lower—compared with plants exposed to a homogeneous N supply. In the split-root case, the combination of a low systemic repression and local NO_3^- availability leads to LR outgrowth in Sp.NO3 conditions, whereas Sp.KCl roots are subjected only to the systemic repression. (iii) In C.KCl plants, the systemic repression of LR growth is totally absent, leading to root proliferation. The findings support the basis for the former model for the control of root development by local and systemic signals (2).

To further our understanding of the molecular underpinnings of the integrated root growth responses, we performed analysis of genome-wide transcriptional changes occurring in short- and long-term exposure to N supply and deprivation in the split-root experimental framework.

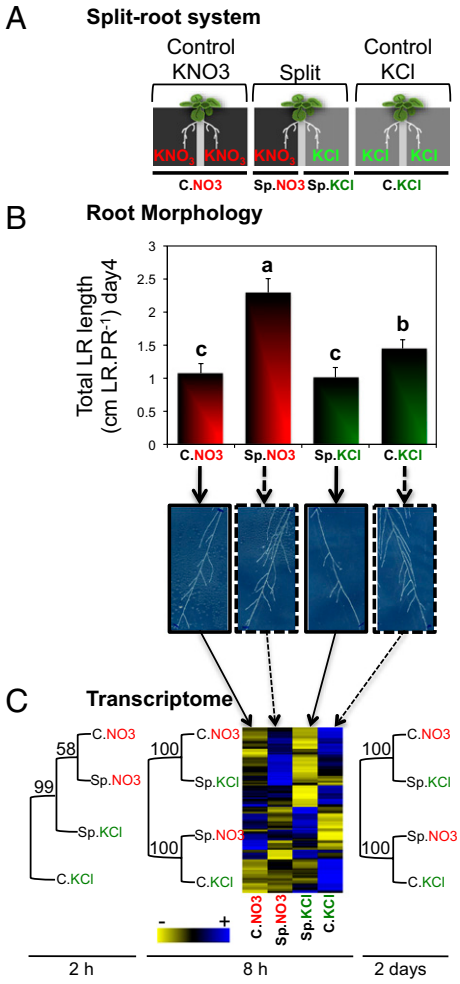

Fig. 1. *Arabidopsis* roots display a coordinated morphological and molecular strategy in response to a heterogeneous NO_3^- environment. (*A*) Diagram shows the physical split-root experimental setup used to detect N-related systemic signaling. Such roots are subjected to three different treatments: "Control KNO3" plants received KNO3 on both sides of the root system (C.NO3), "Control KCl" plants received KCl on both sides (C.KCl), and "Split" plants received KNO3 (Sp.NO3) on one side and KCl (Sp.KCl) on the other. The gray line in each setup represents a physical 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. (*Upper*) Bar graph depicts the total LR length (cm) normalized by the length of the primary root (PR) (cm) as cm LR·PR^{-1}. For C.NO3 and C.KCl, measurements made on both halves of the root systems were pooled and averaged. Each bar graph represents the mean of at least 10 roots. The different letters on top of the bars indicate statistically significant differences ($P \leq 0.05$; *t* test). Error bars equal SE. At the bottom, one representative set of LRs illustrating the trends in LR length in the different treatments is shown. (*C*) Genes whose NO_3^- response was altered in the split-root experiments showed a similar pattern of change to that of LRs. The heat map depicts the expression pattern of 123 genes that showed an interaction between NO_3^- availability and split conditions in ANOVA. The same set of genes was used to generate dendrograms to cluster experiments at the different time points. The numbers at each node in the dendrogram represent bootstrap values from permutation tests.

Genome-Wide Reprogramming in Response to Local vs. Systemic Nitrogen Environments. To understand the molecular basis of the integration of local vs. systemic signals, we undertook a transcriptomic approach across the panel of split-root conditions presented above. RNA from roots of plants exposed to a distinct

combination of local and distal N signals (C.NO3, Sp.NO3, Sp. KCl, and C.KCl) was extracted at early time points (2 h and 8 h) and at a later time point (2 d), after the beginning of the –N or +N treatment. These early time points were selected in an effort to sample early responses and the dynamics of regulatory change before any significant changes in root morphology. A three-way analysis of variance (ANOVA) was performed to statistically analyze these data as a whole (e.g., nitrogen effect, split-root effect, and time effect) (*SI Materials and Methods*). From this ANOVA analysis, we identified a set of genes whose N responses were altered by the split-root conditions, i.e., genes that showed a significant interaction between NO_3^- availability (i.e., presence or absence) and split-root conditions (i.e., homogeneous or heterogeneous): "N-interaction set" of 123 genes (Q value <0.2 and P value <0.001; Dataset S1).

These 123 genes whose expression is significantly affected by a systemic N signal (e.g., of N supply or demand) were used to cluster the corresponding split-root experiments on a dendrogram, to probe dominant trends in gene expression. At the earliest time point (2 h), this experimentwise clustering paired the two NO_3^- treatments together (C.NO3 and Sp.NO3), showing that genes responded at first to the local NO_3^- environment (Fig. 1*C*). However, by the later time points (8 h and 2 d), large-scale changes in genome-wide expression among the 123 genes rearranged the dendrogram of experimental treatment by unexpectedly pairing the Sp.NO3 with the C.KCl treatments and the C.NO3 with the Sp.KCl treatments (Fig. 1*C*). This unexpected genome-wide resemblance of disparate conditions closely parallels that observed with LR architecture after 4 d in the same treatments (compare Fig. 1*B* and 1*C*). Thus, the genes affected by the interaction between NO_3^- availability and split conditions initially respond to the local root N environment, but are later controlled by systemic regulatory signals that integrate information about N status from other parts of the plant. The overall effect is to orchestrate a revised and apparently more effective genome-wide strategy in which a set of molecular changes precedes change in LR architecture in response to system-wide integration of N-systemic signal.

These molecular responses observed at the level of the transcriptome correspond to the morphological responses of the root and appear to represent a coordinated strategy to anticipate assimilation of newly foraged N. Indeed, despite the different local NO_3^- conditions, the N-foraging roots (Sp.NO3 and C.KCl) both showed an induction of genes involved in N uptake and assimilation, such as *AtNRT3.1* (*NAR2.1/WR3*) and *NIR1* (*Nitrite Reductase 1*), and genes involved in metabolism providing reducing equivalents for N assimilation, such as *G6PD3* (*Glucose-6-phosphate Dehydrogenase 3*) or *FNR2* [*Root Ferredoxin: NAPD(H) oxidoreductase 2*] (Dataset S1). Previously, split-root experiments using *Medicago* (4 d posttreatment) identified these same sentinel genes, among others, as responding to an N-related systemic signaling in addition to a local NO_3^- signal (31). Interestingly, in the present study, we show that these genes are rapidly regulated by the split-root N-treatment conditions (within 8 h), suggesting that their regulation is likely among the first targets of systemic N signaling and not a long-term consequence of root adaptation to physiological modifications triggered by the split-root treatment. Overall, these results indicate that systemic signals rapidly (within hours) communicate the NO_3^- status of the whole root system to alter the expression of a subset of genes mainly involved in N metabolism and that later changes in genome expression ultimately result in alterations in root architecture.

Surprisingly, very few genes known to be directly involved in LR development or growth were found among this set of systemically regulated genes (discussed in *SI Results*). However, we do not rule out the possibility that genes categorized in "N-metabolism function" have a direct role in LR architecture response. For example, the NO_3^- transporter *AtNRT2.1* has a role in LR development independently of its NO_3^- uptake function (37, 38) and has been previously identified as a main target of N-related systemic signaling (18, 39). Indeed, we confirmed the early transcriptional regulation of this gene by the systemic signals using q-PCR assays (Fig. S2) and revealed an expression pattern of *AtNRT2.1* that is similar to that of its functional partner *AtNRT3.1* (40).

Coordinated Molecular and Morphological Responses Triggered by Split-Root Conditions Are Driven by NO_3^- Itself and a Shoot-Integrated Systemic Signal. A central question is to determine which signals mediate the root growth adaptations to the different levels of NO_3^- supply in the environment, with respect to gene expression and LR architecture. To efficiently monitor the N × split-root systemic interaction response in a number of different conditions (e.g., mutants and treatments), we identified a set of 8 sentinel genes that responded robustly and showed the same pattern as the dominant trend of the N-interaction set of the 123 genes (identified above from the ANOVA analysis), as well as the LR responses in the four types of compartments (i.e., genes up-regulated in Sp.NO3 and C.KCl compared with C.NO3 and Sp.KCl compartments; *SI Results* and Fig. S2). Interestingly, the genes whose expression best correlates with LR architecture largely belong to the NO_3^- uptake/assimilation functions (*SI Results*).

Because both NO_3^- and its downstream assimilates have been implicated in mediating morphological and molecular responses (2, 18, 31, 41, 42), we tested their distinct roles in the split-root responses using an *Arabidopsis* double mutant in which Nitrate Reductase (*NIA1* and *NIA2* genes) activity is abolished (41). Interestingly, the *NR-null* mutant still exhibited the usual N-regulated response of the eight sentinel genes at the 8-h time point (Fig. 2 *A* and *B*). This result shows that NO_3^- itself, rather than the NO_3^- assimilates, is sufficient in our conditions to mediate the complete set of early transcriptional N-regulated reprogramming. Therefore, our results demonstrate again that these growth changes are supported by dedicated signaling pathways anticipating (and thus independent of) any change in the nutritional status of the plant (8).

To our knowledge, definitive evidence for the role of the shoots themselves in this long-distance root–shoot–root N signaling was still missing (31, 32) (direct root-to-root could technically have been invoked). We determined that the roots of decapitated plants indeed completely lost the response to the N-systemic signaling, but still responded to local NO_3^- conditions (Fig. 2 *A* and *C*). Taken together, these investigations imply that root foraging responses rely on the perception of the systemic NO_3^- imbalance/absence of the whole plant and are mediated through a verified root–shoot–root signaling mechanism.

Cytokinin Biosynthesis Is Essential for Root–Shoot–Root Signaling Triggering the Compensatory Root Responses to Partial NO_3^- Limitation. How does NO_3^- as a signal of N supply (presence) or demand (absence) mediate/amplify a system-wide plant growth response? To date, there have been two types of data linking CK as a second messenger of NO_3^- signaling. First, CK has been shown to be a root-to-shoot NO_3^--derived messenger that modulates shoot growth (22–24, 43). Second, CK has been shown to control several aspects of N nutrition, including NO_3^- -transport and -assimilation steps (thoroughly reviewed and commented on in refs. 8, 21, and 22). Thus, it was tempting to speculate—by transitive closure—that NO_3^- controls CK content that in turns feeds back on N nutrition (8, 22). However, experimental evidence showing a defect in N signaling itself in response to a mutation in the CK signaling pathway remains to be demonstrated. Because our experimental split-root framework allows us to uncover systemic N signaling, it represents an ideal experimental design to address the question of the role of CKs in systemic NO_3^- root–shoot–root signaling.

To test the connection between CK and the N-systemic responses in our experimental system, we repeated the split-root treatments in an *Arabidopsis* triple mutant for ATP/ADP isopentenyltransferases (*ipt3,5,7*), which has severely reduced CK biosynthesis (28). We first tested the impact of the CK synthesis mutations on the response of the eight sentinel genes regulated at early time points in response to systemic signaling triggered by NO_3^-. Strikingly, we observed that the *ipt3,5,7* mutant was

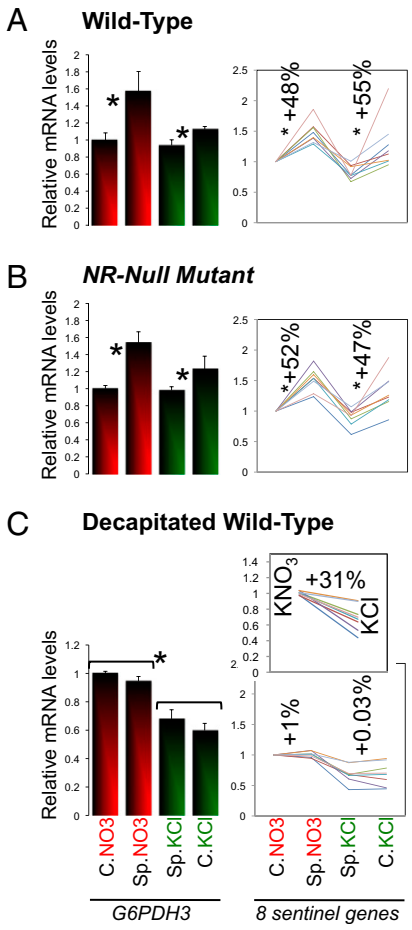

Fig. 2. The coordinated response of roots in these disparate N environments requires sensing of NO_3^- itself and is mediated through the shoot. (*A–C*) Bar graphs represent the relative mRNA levels of the *Glucose-6-Phosphate Dehydrogenase 3* (*G6PDH3*) gene, and the line graphs represent the relative mRNA accumulation of the eight genes used to monitor N and split-root interaction effects (as described in the text). The asterisks in between the two bars indicate significant differences between the corresponding two compartments. The numbers on the line graph are the average percentage of relative mRNA accumulation increase for the eight genes, either between Sp.NO3 and C.NO3, or between Sp.KCl and C.KCl, or between total NO_3^- (C.NO3 + Sp.NO3) and total KCl (C.KCl + Sp.KCl). Trends are shown for (*A*) the wild-type background (plants were grown in the conditions used for the NR-null mutant), (*B*) the *NR-null* mutant (41) in which Nitrate Reductase activity is abolished, and (*C*) wild-type roots of plants decapitated at the time they were transferred to the split or homogeneous treatments. All roots were harvested for RNA expression analysis 8 h after treatment.

impaired *only* in the differential response between C.NO3 and Sp.NO3, but not in the differential response between C.KCl and Sp.KCl (Fig. 3 *A* and *B*). Given that this result favored a specific role for CK in the systemic integration of the NO_3^- status available to the whole root system, our reasoning was that the experimental application of CK specifically to the NO_3^- compartments (both root compartments of the C.KNO3 plants and only the Sp.NO3 compartment for the split plants) would mimic the NO_3^- imbalance in the CK biosynthesis triple mutant. Indeed, the induction of the eight sentinel genes was restored in Sp.NO3 roots, when CK was supplied to the NO_3^--containing compartments (Fig. 3 *A–C*). This induction was also observed when CK was supplied only to the Sp.KCl roots (Fig. S3). This shows that (*i*) shoots are definitively essential for the compensatory response in the Sp.NO3 compartment and (ii) local NO_3^- supply is mandatory for the complementation by cytokinins, but the supply does not have to be in the same compartment. In other words, genes respond to a combination between a cytokinin-derived systemic signaling and a NO_3^- local signal.

Development-wise, we observed that the total LR lengths in C.NO3, Sp.KCl, and C.KCl were unchanged between the wild type and the *ipt* triple mutant in CK synthesis, ruling out the possibility that the mutant caused a general root growth defect (Fig. 3*D*). However, as found at the transcriptome level, LR growth stimulation was lost in the Sp.NO3 compartment, compared with C.NO3 (1.46 ± 0.18 cm LR·PR^{-1} vs. 1.28 ± 0.12 cm LR·PR^{-1}, not significant) in the *ipt3,5,7* mutant compared with wild type, but the stimulation in LR growth in the C.KCl compared with the Sp.KCl was maintained (2.06 ± 0.17 cm LR·PR^{-1} vs. 0.75 ± 0.06 cm LR·PR^{-1}; P value $= 4 \times 10^{-6}$) (Fig. 3*D*).

These results led to two conclusions. First, root responses to systemic N status can no longer be explained by the existence of only one systemic signaling as previously proposed (2), but require the existence of at least two genetically independent systemic signaling pathways. From the evidence above, we propose that the differential response between C.NO3 and Sp.NO3 relies on a systemic N-demand signaling (–N) whereas the differential response between C.KCl and Sp.KCl relies on a systemic N supply (+N). Second, we identified an essential component of the systemic N signaling, by demonstrating that the N-demand signaling depends on CK biosynthesis. In the N-economics model described below, we develop and discuss the role of –N and +N systemic signaling and their interplay in mediating the response of the plant as an integrated system.

Plant Nitrogen Economics: A Model for Systemic Signaling of Nitrogen Supply and Demand. In this study, we integrated the split-root experimental framework with genomic and genetic approaches, to decipher N-related systemic signaling controlling root architecture. Overall, the dissection of systemic signaling supports the existence of distinct systemic signaling controlling plant N economics, in which plants balance and respond to N supply (+N) and N demand (–N) to efficiently control root growth and the expression of N-uptake/assimilation genes, as depicted in Fig. 4. Our results build a model for plant N economics that proposes the coexistence of systemic signaling for both N supply and N demand. Our data provided the following main components in support of this plant nitrogen economics model:

i) *Systemic signaling for N supply and demand*: The LR growth differences observed between root compartments exposed to distinct N-supply/demand environments highlighted the occurrence of several types of N-related systemic signaling, for which the genetic independence has been proved by using the *ipt3,5,7 Arabidopsis* mutant in CK synthesis (Fig. 4). Specifically, the differential response between C.NO3 and Sp.NO3 highlighted a CK-dependent systemic N demand (–N signaling), whereas the differential response between C.KCl and Sp.KCl highlighted the existence of a previously unknown systemic N supply (+N signaling) (Fig. 1*B*).

ii) *NO_3^- supply is the signal for N supply and demand*: Using an *Arabidopsis* NR-null mutant (41) in split-root experiments (Fig. 2*B*), we showed that both local and distal NO_3^- signaling responses are preserved, indicating that NO_3^- is the signal for both sides of the N-economics equation in plants.

iii) *N-supply and -demand signaling involves a root–shoot–root relay*: Decapitation experiments showed that whereas local NO_3^- responses are preserved in shootless plants, the systemic signaling for distal N supply or distal N demand is lost, invoking a root–shoot–root relay for each (Fig. 2*C*).

The –N systemic signaling for N demand of our N-economics model (C.NO3 vs. Sp.NO3) has been highlighted in previous studies where root morphological responses were measured (2, 4, 30–32). In our new study, the ability to monitor both root and

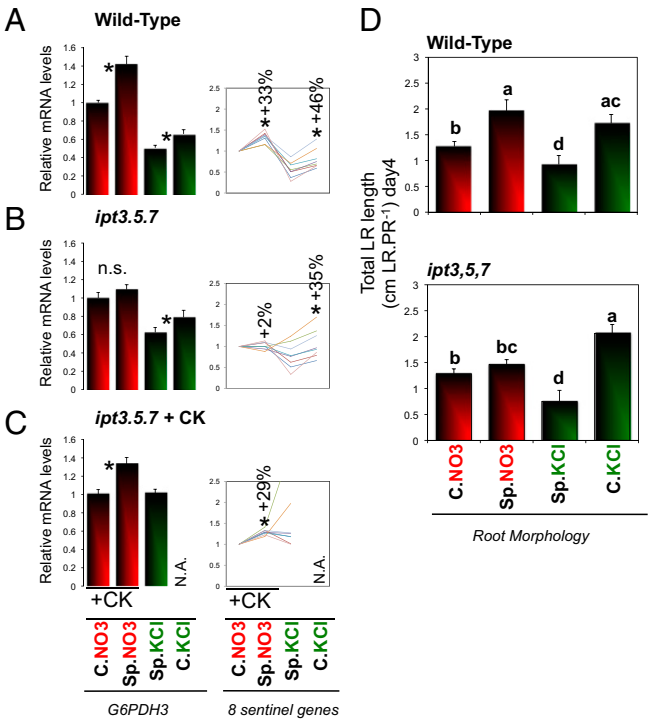

Fig. 3. CK mediates coordination of root responses in a heterogeneous environment by allowing a compensatory LR growth in the NO_3^- rich area. Expression of *G6PDH3* and the eight sentinel genes was assayed by q-PCR in the standard set of treatments used in (*A*) the wild type, (*B*) the CK synthesis mutant *ipt3,5,7* background (28), and (*C*) the *ipt3,5,7* mutant in which CK was added back to the roots in the NO_3^- compartments. The asterisks indicate the significant differences between two compartments. The numbers on the line graphs are the average percentage of relative mRNA accumulation increase for the eight sentinel genes, either between Sp.NO3 and C.NO3 or between C.KCl and Sp.KCl. N.A., nonapplicable. (*D*) Total LR length (cm LR·PR^{-1}) is shown in WT compared with the *ipt3,5,7* mutant. The different letters on top of the bars indicate statistically significant differences ($P \leq 0.05$; *t* test). Error bars equal SE.

transcriptome responses enabled us to identify a genetically independent +N-systemic signaling for N supply that accounts for the differences between C.KCl and Sp.KCl conditions, the latter of which is exposed to a distal N supply. Moreover, these differences also imply that a different –N signaling (local or systemic) occurs in response to total N deprivation (in C.KCl), which is distinct from the –N signaling perceived in Sp.NO3, because it is not dependent on CK. In the Sp.KCl scenario, this N-deprivation signaling system is repressed by the +N-systemic signaling (from Sp.NO3) and accounts for the regulation of expression of N-deprivation sentinels such as *AtNRT2.5* (44) (Fig. S4). As such, our study, which combines split-root conditions, root morphology, and genome-wide transcriptome analysis, enabled us to discover distinct signaling for N supply and demand and to refine previous hypotheses.

Finally, our studies provide experimental evidence to support a "transitive closure" for the role of CK in mediating the shoot–root systemic N signal controlling N uptake/assimilation and LR growth. In previous studies, NO_3^- (A) was shown to induce CK (B) synthesis, providing evidence for the relationship A → B (22). Previous studies also showed that CK (B) supplied to plants could regulate (C) the expression of genes involved in N uptake/assimilation and root development, providing experimental evidence for the relationship B → C (22). The transitive closure of A → B and B → C suggests (by transitive closure) a relationship of A → B → C, but this important relationship in the NO_3^-/CK relay was not experimentally validated. The inference of this relationship is noted by the dashed lines in the model in figure 4 of Kiba et al. that postulates a nitrate–cytokinin relay (22). In our study, by combining the split-root system, NO_3^- treatment, and a CK biosynthesis mutant, we provide experimental evidence that supports the transitive closure of NO_3^- → CK (systemic signal) → root–shoot–root signaling → activation of root responses, including N uptake/assimilation and LR development.

CK and its antagonistic partner, auxin, are well known to act in concert to tune plant development (45). Thus, it will be of interest to integrate the recent findings on the role of auxin signaling in the control of LR development by local NO_3^- availability with the work on CK and systemic N signaling presented herein. On one hand, NO_3^- promotes (through the action of NRT1.1) auxin accumulation in lateral roots, which promotes its elongation (7). On the other hand, CK synthesis is necessary to induce LR development in response to a systemic –N signaling (Fig. 3). According to our results, the role of CKs in a root–shoot–root communication is clear, but several scenarios can be hypothesized for the exact location of their actions. Because we show that systemic N-demand signaling is lost in decapitated plants, it is very likely that CKs act in shoots. Given that NO_3^- provision induces CK production and translocation toward the shoots (23–25), we believe that CKs play an important role as an integrator of the NO_3^- status in shoots. However, whether the CKs themselves function in the shoot-to-root relay—or as yet unknown downstream signals of CK—remains to be experimentally explored. Preliminary results tend to indicate that it is likely that another signal downstream of CK plays a role in this shoot–root N-demand signal. Indeed, by examining the *type-A ARR* genes, which constitute a family of primary CK-response genes (46), we observed that these genes are globally regulated by the local NO_3^- presence in roots (up-regulation in C.NO3-Sp.NO3 vs. Sp.KCl-C.KCl; microarray data), whereas *ARR* expression is up-regulated in proportion to global NO_3^- levels in shoots (up-regulation in Control.NO3 vs. Split vs. Control.KCl; q-PCR assays) (Fig. S5). As auxin transport from shoot to root is believed to be a reporter of N status of the plant (20), and CK may control auxin transport and synthesis (47–49), it is tempting to hypothesize that auxin may be part of the long-distance signal informing the roots of the integrated N status of the shoot. Furthermore, because auxin is taken up by NRT1.1 locally to stimulate root development, according to the local NO_3^- environment, the combination of the auxin and CK models would provide a large panel of developmental programs of N economics of root development described herein.

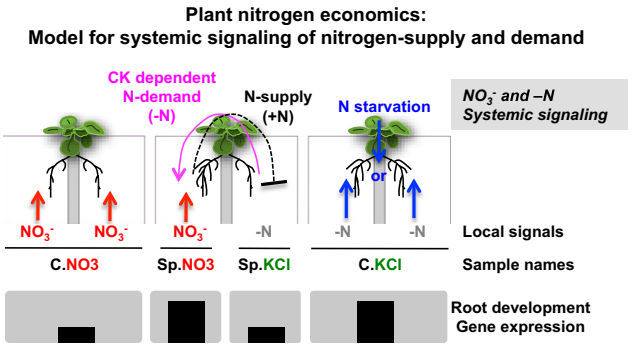

Fig. 4. A model for systemic signaling involved in plant nitrogen economics: Systemic signaling for N supply and N demand that control NO_3^- metabolism genes and LR development in plants exposed to heterogeneous nitrogen environments. We propose the existence of several systemic signalings to account for a systems-wide integration of nitrogen economics coordinating the root responses in heterogeneous N environments: (*i*) inductor CK-dependent N-demand (–N) signaling (in pink), (*ii*) repressive N-supply (+N) signaling in split-root plants (in black), and (*iii*) N- starvation signaling that is either local or systemic in C.KCl conditions and that is CK independent (in blue). These systemic signalings of N supply and N demand act likely in combination with NO_3^- local signal (in red), to control root molecular and developmental phenotypes and coordinate a plant system-wide response to its perceived nitrogen economics.

Materials and Methods

Plant Materials. All *Arabidopsis thaliana* plants were in Columbia background, the wild type used in this study. The *NR-null* mutant in nitrate reductase (*chl3-5/nia1-2*) and the *ipt3,5,7* triple mutant in CK biosynthesis were respectively obtained from Nigel Crawford (University of California at San Diego, La Jolla, CA) (41) and Sabrina Sabatini (University "La Sapienza," Rome) (28).

Split-Root System and Treatments. Split-root conditions applied to *Arabidopsis* were adapted from ref. 4. Details of the procedures used are given in *SI Materials and Methods*.

Analysis of Root Growth. Two, 3, and 4 d after the transfer of the split-root plants to selected treatment media, a minimum of 10 plates for each condition were scanned at 400 dpi (Epson Perfection V350 Photo). Root growth was analyzed as previously described (4). Statistical comparisons of means between treatments and/or genotype were performed using Student's *t* test. Each experiment including wild type and/or mutants was performed twice and displayed the same result and one experiment was shown.

Analysis of Genome-Wide Expression. Genome-wide expression was performed using Affymetrix (ATH1) and for selected examples by q-PCR. Statistical analysis was performed according to ref. 13. Details of the procedures are given in *SI Materials and Methods*.

The Affymetrix Microarrays data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus in compliance with Minimum Information About a Microarray Experiment standards (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession no. GSE22966.

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1. Schachtman DP, Shin R (2007) Nutrient sensing and signaling: NPKS. *Annu Rev Plant Biol* 58:47–69.
2. Zhang H, Jennings A, Barlow PW, Forde BG (1999) Dual pathways for regulation of root branching by nitrate. *Proc Natl Acad Sci USA* 96:6529–6534.
3. Gojon A, Nacry P, Davidian JC (2009) Root uptake regulation: A central process for NPS homeostasis in plants. *Curr Opin Plant Biol* 12:328–338.
4. Remans T, et al. (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proc Natl Acad Sci USA* 103:19206–19211.
5. Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. *Cell* 138:1184–1194.
6. Gojon A, Krouk G, Perrine-Walker F, Laugier E (2011) Nitrate transceptor(s) in plants. *J Exp Bot* 62:2299–2308.
7. Krouk G, et al. (2010) Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Dev Cell* 18:927–937.
8. Krouk G, et al. (2011) A framework integrating plant growth with hormones and nutrients. *Trends Plant Sci* 16:178–182.
9. Castaings L, et al. (2009) The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. *Plant J* 57:426–435.
10. Hu HC, Wang YY, Tsay YF (2009) AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. *Plant J* 57:264–278.
11. Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR (2009) Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. *Plant Cell* 21:3567–3584.
12. Zhang H, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. *Science* 279:407–409.
13. Krouk G, Mirowski P, LeCun Y, Shasha DE, Coruzzi GM (2010) Predictive network modeling of the high-resolution dynamic plant transcriptome in response to nitrate. *Genome Biol* 11:R123.
14. Liu TY, Chang CY, Chiou TJ (2009) The long-distance signaling of mineral macronutrients. *Curr Opin Plant Biol* 12:312–319.
15. Hermans C, Hammond JP, White PJ, Verbruggen N (2006) How do plants respond to nutrient shortage by biomass allocation? *Trends Plant Sci* 11:610–617.
16. Desnos T (2008) Root branching responses to phosphate and nitrate. *Curr Opin Plant Biol* 11:82–87.
17. Imsande J, Touraine B (1994) N demand and the regulation of nitrate uptake. *Plant Physiol* 105:3–7.
18. Lejay L, et al. (1999) Molecular and functional regulation of two NO3- uptake systems by N- and C-status of Arabidopsis plants. *Plant J* 18:509–519.
19. Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. *Proc Natl Acad Sci USA* 105: 803–808.
20. Forde BG (2002) Local and long-range signaling pathways regulating plant responses to nitrate. *Annu Rev Plant Biol* 53:203–224.
21. Rubio V, et al. (2009) Plant hormones and nutrient signaling. *Plant Mol Biol* 69: 361–373.
22. Kiba T, Kudo T, Kojima M, Sakakibara H (2011) Hormonal control of nitrogen acquisition: Roles of auxin, abscisic acid, and cytokinin. *J Exp Bot* 62:1399–1409.
23. Sakakibara H, Takei K, Hirose N (2006) Interactions between nitrogen and cytokinin in the regulation of metabolism and development. *Trends Plant Sci* 11:440–448.
24. Takei K, Sakakibara H, Taniguchi M, Sugiyama T (2001) Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: Implication of cytokinin species that induces gene expression of maize response regulator. *Plant Cell Physiol* 42:85–93.
25. Takei K, et al. (2004) AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant Cell Physiol* 45:1053–1062.
26. Higuchi M, et al. (2004) In planta functions of the Arabidopsis cytokinin receptor family. *Proc Natl Acad Sci USA* 101:8821–8826.
27. Laplaze L, et al. (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19:3889–3900.
28. Miyawaki K, et al. (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc Natl Acad Sci USA* 103: 16598–16603.
29. Werner T, et al. (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15:2532–2550.
30. Jeudy C, et al. (2010) Adaptation of Medicago truncatula to nitrogen limitation is modulated via local and systemic nodule developmental responses. *New Phytol* 185: 817–828.
31. Ruffel S, et al. (2008) Systemic signaling of the plant nitrogen status triggers specific transcriptome responses depending on the nitrogen source in Medicago truncatula. *Plant Physiol* 146:2020–2035.
32. Gansel X, Muños S, Tillard P, Gojon A (2001) Differential regulation of the NO_3^- and NH_4^+ transporter genes *AtNrt2.1* and *AtAmt1.1* in Arabidopsis: Relation with long-distance and local controls by N status of the plant. *Plant J* 26:143–155.
33. Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010) Nitrate signaling: Adaptation to fluctuating environments. *Curr Opin Plant Biol* 13:266–273.
34. Vidal EA, Gutiérrez RA (2008) A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. *Curr Opin Plant Biol* 11:521–529.
35. Zhang H, Rong H, Pilbeam D (2007) Signalling mechanisms underlying the morphological responses of the root system to nitrogen in Arabidopsis thaliana. *J Exp Bot* 58: 2329–2338.
36. Scheible W-R, et al. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. *Plant Physiol* 136:2483–2499.
37. Little DY, et al. (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *Proc Natl Acad Sci USA* 102: 13693–13698.
38. Remans T, et al. (2006) A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. *Plant Physiol* 140:909–921.
39. Girin T, et al. (2007) Identification of a 150 bp cis-acting element of the AtNRT2.1 promoter involved in the regulation of gene expression by the N and C status of the plant. *Plant Cell Environ* 30:1366–1380.
40. Krouk G, Tillard P, Gojon A (2006) Regulation of the high-affinity NO3- uptake system by NRT1.1-mediated NO3- demand signaling in Arabidopsis. *Plant Physiol* 142: 1075–1086.
41. Wang R, et al. (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. *Plant Physiol* 136:2512–2522.
42. Zhang H, Forde BG (2000) Regulation of Arabidopsis root development by nitrate availability. *J Exp Bot* 51:51–59.
43. Rahayu YS, et al. (2005) Root-derived cytokinins as long-distance signals for NO_3–induced stimulation of leaf growth. *J Exp Bot* 56:1143–1152.
44. Okamoto M, Vidmar JJ, Glass AD (2003) Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: Responses to nitrate provision. *Plant Cell Physiol* 44:304–317.
45. Moubayidin L, Di Mambro R, Sabatini S (2009) Cytokinin-auxin crosstalk. *Trends Plant Sci* 14:557–562.
46. D'Agostino IB, Deruère J, Kieber JJ (2000) Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol* 124: 1706–1717.
47. Dello Ioio R, et al. (2008) A genetic framework for the control of cell division and differentiation in the root meristem. *Science* 322:1380–1384.
48. Jones B, et al. (2010) Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *Plant Cell* 22:2956–2969.
49. Ruzicka K, et al. (2009) Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proc Natl Acad Sci USA* 106:4284–4289.