Supporting Online Material

Materials and Methods

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SOM Methods

**Plant material**

Arabidopsis thaliana plants were of the Columbia ecotype (wild-type). The CycB1::GUS line used to measure the lateral roots (LR) initiation events was obtained from Philippe Nacry (Biochemistry and Plant Molecular Physiology at Montpellier, France). The *NR-null* mutant (chl3-5/nia1-2) was obtained from Nigel Crawford (University of California at San Diego, USA). The *ipt3,5,7* triple mutant was obtained from Sabrina Sabatini (University ‘La Sapienza’ at Rome, Italy).

**Split-root system and treatments**

Plants were grown at 22 ºC with a 16 hr/8 hr light/dark cycle and a light intensity of 50 µmol.m-2.s-1. Surface sterilized seeds were sown in 10x10 cm plates on 40 ml of solid medium (1% type A agar) containing custom-made Murashige and Skoog basal medium (*i.e*., without nitrogen, sucrose and glycine; GIBCO Invitrogen). The medium is supplemented with 0.5mM NH4+-succinate and 0.1mM KNO3 as the nitrogen source and 0.3mM sucrose as a carbon source, except for the experiments testing the *NR-null* mutant where the nitrogen source was 0.5mM NH4+-succinate. After 8-10 days, the primary root was cut below the first two LRs and roots still grown on these plates for 4 more days. Then, each plant with two main roots was transferred on individual 10x10 cm plates containing the 40 ml solid medium supplemented with 0.5mM NH4+-succinate as the nitrogen source. The solid medium was divided in two compartments by a trench to allow the physical separation of the two roots. After 4 days, we selected the plants with balanced roots on both sides and they were transferred on 12x12cm segmented agar plates containing either 5mM KNO3 or 5mM KCl on both sides for the controls or 5mM KNO3 on one side and 5mM KCl on the other side for the split. These plates contained 60 ml of solid basal MS medium supplemented with 0.3mM sucrose. The KNO3 or KCl compartments were obtained by spreading concentrated KNO3 and KCl solution on the solid medium 24 hr before the experiments to allow diffusion of the solutions, as previously described (*1*) (Fig. S2A). Concentrated cytokinins solution has been added to the KNO3 solution before spreading. The final concentration was 1 nm trans-zeatin cytokinin (Sigma).

**Analysis of root growth**

Two, three and four days after the transfer of the plants, a minimum of 10 plates for each condition were scanned at 400 dpi (Epson Perfection V350 Photo). Root growth parameters were analyzed by using the Optimas image analysis software (MediaCybernetics; AES). Histochemical analysis of the GUS reporter enzyme activity for the CycB1::GUS line has been done as previously described (*2*). Three main variables were measured at each time point: the primary root length, the LRs length and the number of emerged LRs. In addition, the number of initiated LRs was measured for the CycB1::GUS (at days 2 and 4). As only LRs longer than 1mm are visible on the scans, each root was examined under a dissecting microscope (Bausch & Lomb) to correct the number of emerged LRs. The main parameter presented in this study is the total LR length corresponding to the sum of the LRs length normalized by the primary root length. For the dissection of the LRs responses, we used three parameters i) mean LRs length = total LRs length / number of emerged LRs, ii) mean LRs density = number of emerged LRs / length of the primary root and, iii) mean initiation density = number of initiated LRs / length of the primary root. Each parameter is the mean of at least 10 roots. Statistical comparisons of means between treatments and/or genotype were performed using the student’s t test. Each of the root growth experiment using Columbia, CycB1::GUS or the *ipt3,5,7* mutant was performed twice. The same results were obtained and only one experiment was shown.

**Analysis of genes expression**

For the microarrays and q-PCR experiments, we performed three independent experiments. RNA extractions were carried out on roots collected at 2 hours, 8 hours and 2 days after the beginning of the split-root treatment with TRIzol (Invitrogen). Standard Affymetrix protocols were then used for amplifying, labeling and hybridizing RNA samples (1μg) to the ATH1 GeneChip (Affymetrix). Raw data were processed with MASv5.0 software. All the data analysis was performed using custom-made R functions. The reproducibility of replicates was analyzed using the correlation coefficient and r2 value of replicate pairs. r2 values were in the range of 0.97 to 0.99. The normalized data were analyzed using a three-way ANOVA that was modeled as follows: Y = μ + αnitrate + αsplit + αtime + αnitrate\*split + αnitrate\*time + αsplit\*time + αnitrate + αnitrate\*split\*time + ε, where Y is the normalized expression signal of a gene; μ is the global mean; the α coefficients correspond to the effects of NO3- (roots in presence or absence of NO3-), of the split treatment (roots coming from a ‘split’ or a ‘control’ plant), of the time (2hrs/8hrs/2days), the interaction between nitrate, split and time, and ε represents unexplained variance. The measures of the significance of each probe were done by the Q-value method (q < 0.2 corresponding to pANOVA < 0.001) (*3*). Then, in order to extract probes differentially regulated between Sp.NO3 and C.NO3, and between Sp.KCl and C.KCl, the post-hoc tukey-test (ptukey < 0.05) was used on the probes that passed the q-value for the interaction nitrate\*split and nitrate\*split\*time. Arabidopsis Gene Identifier and annotation of the genes corresponding to each probe were obtained from the TAIR9 annotation. The clustering analysis was performed using MultiExperiment Viewer v4.4 (MeV) (*4*). The determination of the functions overrepresented in the gene lists was performed using the Biomaps and the significance of the overlap between two genes lists was obtained using Genesect from VirtualPlant software (*5*). For q-PCR tests, double-stranded cDNA was synthesized by using the Invitrogen RT-PCR system according to manufacturer’s instructions. The PCRs were done using the LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche), according to the manufacturer’s instructions. The relative concentration of each gene has been normalized using three genes: Clathrin (at4g24550), and two genes extracted from our normalized microarrays data which displayed the lower covariance across the experiments: SIP1A (at3g04090) and ATJ3 (at3g44110). The primers used are listed in Table S7.

The Affymetrix Microarrays data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through accession number GSE22966.

SOM Text

**1) Lateral roots development and growth responses to the split-root treatment were dissected.**

In this study, we examined the total lateral roots (LR) length response of the whole roots system. However, this measurement depends on two main visible parameters that are LRs elongation and emergence rates; the former partly depending on the LRs initiation rates (*6*).Our aim was to determine whether the regulation of LRs growth was due to the same or different LR parameters. Moreover, as the root system was well developed at the day of the transfer in the split-root treatment plates (d=0) (Fig. S2A), our reasoning was that the strategy to adapt the morphology to the environment was different along the root depending on its status at d=0. Therefore, each root has been virtually divided in 3 parts: part 1 (P1) which already displayed visible LRs at d=0, part 2 (P2) which displayed none visible LR at d=0 and part 3 (P3) which is the newly developed root part (Fig. S2A). Then, we examined the LRs length and density in these 3 virtual parts and on days 2 to 4 (Fig. S2B).

We observed that the stimulation of the LRs growth in the Sp.NO3 and C.KCl compartments were explained by the stimulation of the same visible parameters along the primary root. Indeed, in the older part of the root, the plant reacts by increasing the LR elongation (P1-Mean LR length; Fig. S2B) and in the middle part, an increase of the emerged LR number (P2-Mean LR density; Fig. S2B). In the new part, the increase of the emerged LR number is observed only in the Sp.NO3 compartment (P3-Mean LR density; Fig. S2B). However, the rapidity of LR emergence in the C.KCl compartment between days 3 and 4 suggests that this parameter is also stimulated in this compartment and will likely become significant later (Fig. S2B).

In order to determine whether the regulation of LR density in P2 is due to a modification of the level of LR initiation or emergence *per se*, we measured the LRs initiation/pre-emerged density (LRs from stage I to VII according to (*7*)) on days 2 and 4, using the CYCB1::GUS line (Fig. S2B). First, we checked that the transgenic line displayed the stimulation of LR density in the Sp.NO3 and C.KCl compartments as we previously observed in wild-type (Fig. S2B).

On day 2, we observed that the LR initiation density was lower in the C.NO3 compartment than in other compartments (Fig. S2B). The repression of LR density at day 4 seemed partly due to a repression of the LRs initiation rate in this compartment whereas the repression of LRs density for Sp.KCl roots was likely the result of the repression of the emergence rate. This result suggests a specificity of the signaling beyond the C.NO3 and Sp.KCl repression.

On day 4, we observed that the initiation density was higher in the Sp.NO3 compared to the C.KCl compartment (Fig. S2B). This result indicates that the LRs proliferation in the Sp.NO3 is also followed by a higher stimulation of the LR initiation rates whereas in the C.KCl compartment this parameter is probably not stimulated due to a limitation of energy. The allocation of the resources to the roots appeared to be dedicated to the emergence and elongation of LR in the newer part of the primary root exploring for new area (P3- Fig. S2B). This behavior would be another illustration of the plant’s strategies to optimize soil exploration and then nutrients uptake.

Overall, the dissection of the LR responses to the split-root treatment highlights that plants have different strategies to adapt the root system architecture to various nitrogen constraints (retard or proliferate the LR growth depending on the local environment and internal needs).

It’s noteworthy that using totally different conditions, we were able to make a parallel between our results and a previous study focusing on the responses of the root system architecture to nitrogen limitation (*8*). The authors have shown that a moderate NO3- limitation (0.5-1mM) has an impact on the density of LRs resulting from the stimulation of LR initiation, as we observed for the NO3--supply roots of the split plants. Conversely, they have shown that LR emergence and elongation are stimulated during a strong NO3- limitation (0.1-0.05mM). In that study, they demonstrated the specific action of the NO3- transporter NRT2.1 on LR initiation under moderate nitrogen-limited conditions (*8*). Interestingly, this NO3- transporter would have a role in coordinating LR development with NO3- availability, independently from its NO3- transport activity. Therefore, in our following transcriptomic approach the regulation of this gene has been carefully looked at. [My overall comment: this is all way to speculative sounding for a top notch journal like Science. Also, when LR is used as an adjective there should not be an s]

**2) A transcriptomic approach was used to decipher the molecular basis of the decision-making process for morphological root adaptation.**

In order to explore the root molecular changes prior to the visible root morphological adaptation, the transcriptome of the C.NO3, Sp.NO3, Sp.KCl and C.KCl roots have been determined at 2 hours, 8 hours and 2 days after the transfer of the plants in the split-root treatment. (Recall that Sp.NO3 means high NO3 in a split root and C.NO3 means high NO3 in a homogeneous environment.) Our aim was mainly to identify the genes acting upstream of the LR growth stimulation in the Sp.NO3 and C.KCl as potential early molecular players in this decision-making process.

A three-way ANOVA followed by a post-hoc Tukey-test were used to analyze the normalized data (see Methods). We focused our interest on the 123 genes regulated by the interaction between NO3- availability and split conditions for all pooled time points (Table S1). In order to extract the genes with a relevant expression pattern regarding the LR responses, we identified genes differentially regulated between roots growing in the same local environment. To do so, the Tukey post-hoc testing identified genes i) differentially regulated between C.NO3 and Sp.NO3 roots (41 genes; p-val<0.05; Table S2) and, ii) differentially regulated between Sp.KCl and C.KCl roots (94 genes; p-val<0.05; Table S3). The differential regulation of genes between Sp.NO3 *versus* C.NO3 or between Sp.KCl *versus* C.KCl roots can only be the response to a signal related to the heterogeneity of the nutrient environment for the split plants. Because these roots [which roots?] were in the same environment, we eliminated any confusion that could be made with a response related to the local environment. For instance, the comparison between the C.NO3 and C.KCl roots encompasses both the response to the internal nutrient status and also to the external NO3-. In addition, 1267 genes responding to the presence or absence of NO3- (KNO3 versus KCl roots) were also identified from this analysis (Table S4).

In Arabidopsis, the transcriptome reprogramming to the NO3- supply has been previously documented (*9-11*). We first checked whether our split-root system triggers the previously known NO3- responses. We compared the 1267 genes with the 595 NO3- responsive genes identified in a previous study using a nitrate reductase (NR) null-mutant (*11*). Thirty six percent of the known NO3- responsive genes were found within the 1267 genes (Table S5-a). [This is rather low] The significance of the overlap has been tested by a non-parametric randomization test (Gensect) and the p-value is below 0.001 (see Methods). Those genes displayed the over-representation of the following functional categories: Nitrate transport and assimilation (including ammonia assimilation and amino acid metabolism), Carbon metabolism, Pentose phosphate pathway and Sulfur metabolism(Table S5-b), which are representative of the context-independent NO3- response (*12*). This result showed that the roots respond properly to the NO3- signal in the split-root system.

We also observed that genes involved in nitrogen-related functions are over-represented within the 41 and 94 genes [which genes are those?] and are generally induced in Sp.NO3 and C.KCl more than in Sp.KCl and C.NO3 roots, respectively (Table S6). Overall, these results showed, at a global level, an interconnection of the different transcriptional reprogramming based on the fact that functions related to the nitrogen metabolism are found regulated in the three gene lists defined by the analysis. [I don’t understand this last sentence]

**3) A set of reporter genes for the root decision was identified.**

First, based on the clustering of the 123 genes, we have shown that these genes display, by 8 hrs, an expression pattern which matches with the LR growth response in the split-root treatment (See main text). Our aim was to identify from these genes a set of genes as early reporter sof the morphological root decision.

To do so, we extracted the 14 genes overlapping between the two lists of genes differentially regulated between C.NO3 *versus* Sp.NO3 and C.KCl *versus* Sp.KCl (Fig. S3A). From them, we selected a cluster of 6 genes based on two main properties. 1- Their expression pattern follows the dominant trend highlighted by the clustering approach, meaning that at 2 hrs, the presence of NO3- drives the expression pattern and by 8 hrs, the level of expression is similar between Sp.NO3 and C.KCl and conversely between C.NO3 and Sp.KCl. 2- By 8 hrs, the expression level of these genes in the Sp.NO3 and C.KCl compartments are higher than in the C.NO3 and Sp.KCl compartments, respectively, matching with the LR pattern at day 4 (Fig. S3A and Fig. 1B). For instance, the expression pattern of the *NiR1* gene coding for the Nitrite Reductase enzyme follows these two properties (Fig. S3A).

In addition, we examined the expression pattern of the NO3- transporters *NRT2.1* that constitutes a main component of the transport system for root uptake and LRs development (*8, 13, 14*), and its partner *NRT3.1* (*15*) (Fig. S3B). It is also known that the expression of these genes are induced by NO3- but also regulated by a nitrogen demand related to the availability of the downstream NO3- products at the whole plant level (*16*). Interestingly, these two genes display the two properties defined above for the set of indicators genes and so have been added to our set (Fig. S3B).

Altogether, our approach allowed the identification of a set of 8 reporter genes for the root decision. Unexpectedly, almost all these genes belong to the NO3- metabolism functions (Fig. S3C), suggesting an early stimulation of this metabolic function in the Sp.NO3 and C.KCl, as a compensatory response to the nitrogen deprivation (*17*). However, it was surprising to observe the stimulation of this function in the completely NO3- deprived roots. Indeed, the expression level of genes like the *NiR* gene for instance is usually observed down regulated during a nitrogen limitation treatment because of the lack of NO3- (*18, 19*). One possibility would be that the NO3- stored in vacuoles during the first growth days could be remobilized, leading to the stimulation of the NO3- metabolism function for its assimilation (*20*). Finally, the clustering of these 8 genes suggests that the transferase and the unknown function genes could be also involved in the NO3- metabolism function. [Again, very speculative]

**4) The cytokinin biosynthesis is specifically involved into the decision to stimulate the lateral roots emergence in the Sp.NO3 compartment.**

First, we have shown that the early alteration of the cytokinin biosynthesis in the *ipt3,5,7* mutant specifically affects the up-regulation of the 8 transcriptional indicators in the Sp.NO3 (by 8 hrs). Later, this specificity was confirmed by the observation that in the *ipt3,5,7* mutant the decision to make more LRs to overcome the nitrogen limitation was affected only in the Sp.NO3 compartment. These results validated that the 8 genes are appropriate early reporters for the LR responses in our system. They also led us to point to the molecular specificity of the signaling pathway triggered in the Sp.NO3 and C.KCl compartments. Indeed, we did not observe any effect of the cytokinin biosynthesis alteration on the stimulation of LR growth in the C.KCl compartment (Fig. 3B), confirming that the cytokinin related signal relies on the perception of the NO3- availability.

Second, to determine which LR parameters are affected by the cytokinin biosynthesis alteration in the Sp.NO3 compartment, we analyzed the LR responses of the triple mutant, as described above for the wild-type (SOM Text-1). We observed that the mutations affected specifically the stimulation of LR emergence in P2 and P3, and not the elongation in the Sp.NO3 compartment (Fig. S4). The effect of cytokinins on LR formation rather than on elongation is consistent with what has been previously described (*21*). However, exogeneous cytokinin treatment or over-expression of *IPT* genes have usually a negative and direct effect on LR formation (*21, 22*), suggesting that a removal of the cytokinin-repression could be expected in the triple mutant. Instead, we observed an opposite effect since the LR emergence was repressed in the Sp.NO3 compartment. Overall, our results suggest that it is not the cytokinin that acts directly on roots development but rather a cytokinin-shoot-derived signal.

**5) The type-A ARRs genes expression in the split-root system supports the hypothesis of an integrated cytokinin signal in the shoots.**

In order to go further into the cytokinin-derived signal, we looked at the expression of the type-A ARRs genes, which are a family of primary cytokinin response genes (*23*). The family contains 10 members: *ARR3, 4, 5, 6, 7, 8, 9, 15, 16, 17* (*24*). We focused only on *ARR5, 6, 7, 8, 9* since *ARR3, 4* expression were not regulated at 8 hrs and *ARR15, 16, 17* were expressed at a low level. The expression regulation of these 5 *ARRs* genes in roots suggests that cytokinins do not have a direct role in roots. If a lack of cytokinin itself acted on LRs stimulation in the Sp.NO3 compartment, we would expect a down-regulation of these marker genes in the Sp.NO3 compared to the C.NO3 compartment. However, our microarray data indicated that the global expression pattern of *ARR5 to 9* corresponds to an up-regulation of these genes due to the NO3- presence only (Fig. S5A).

We tested the expression of these 5 genes in shoots by qPCR experiments. Interestingly, in the shoot parts, almost all these ARRs genes display an expression pattern suggesting a gradual accumulation of cytokinins from the control KCl (low), split (medium) to the control KNO3 (high) shoots (Fig. S5B). This would tend to show that the quantity of NO3- available for the roots is integrated into the shoots through the cytokinins (*25*). According to these results, our hypothesis is that cytokinins would act in shoots to modulate the expression of the basipetal signal involved into the stimulation of LRs growth in the Sp.NO3 compartment. [this sounds very speculative]

SOM Figures legend

**Fig. S1. Roots growth responses in the split-root system across the time.**

(**A**) The bar graph depicts the primary root length (cm) in the four root compartments from days 2 to 4. This graph shows that the split-root treatment had no effect on the rate of the primary root across the time. n.s. = no significant.

(**B**) The bar graphs depict the total LR length normalized by the length of the primary root (PR) as cm LR/cm PR at days 2 and 3. They show that the effect of the split-root treatment on the total LRs length was not visible on day 2 and turned to be slightly significant on day 3. The numbers above the 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): any two bars or any numbers above bars with a different letter showed a significant difference between them. Error bars=standard error.

**Fig. S2. Dissection of the lateral roots development and growth responses to the split-root treatment.**

**(A)** Scans of Arabidopsis plants in the split-root system at day 0 and day 4. At day 0, the primary root parts displaying visible LRs are named P1, and non-visible LRs, named P2, are marked on the plates. P3 is the newly grown root part during the split-root treatment.

(**B**) The bar graphs depict either the mean LR density (number of emerged LRs / length of the primary root part), the mean LR length (total LRs length of the part / length of the primary root part) or the mean initiation density (number of initiated LRs / length of the primary root) at days 2, 3 and 4 and, in the P1, P2 and P3. 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. They are indicated only for the measures on day 4, except for the mean initiation density graph where the statistically significant differences are indicated on days 2 and 4. Error bars=standard error. At bottom, the table displays all the lateral roots measurements made on day 4 for the wild-type to facilitate the graph reading.

**Fig. S3. Identification of a set of 8 reporter genes for the root decision.**

(**A**) Identification of 6 genes as reporters of the LR responses. First, we selected the 14 common genes to the lists of 41 (C.NO3 vs Sp.NO3) and 94 (Sp.KCl vs C.KCl) genes. The clustering of these 14 genes allowed the identification of 6 genes displaying two main properties: i) their expression pattern follows the dominant trend highlighted by the clustering approach and, ii) by 8 hrs, the expression level of these genes in the Sp.NO3 and C.KCl compartments are higher than in the C.NO3 and Sp.KCl compartments, respectively, matching with the LR pattern at day 4. The bar graph displays the normalized expression value of the *Nitrite reductase 1* (*NiR1*) gene in the four compartments, over time, as an example of the expression pattern of the 6 genes. The table displays the AGI and annotation of the 6 genes.

(**B**) Normalized expression value of the two NO3- transporters *NRT3.1* and *NRT2.1* genes showing that they are reporter genes of the LRs responses.

(**C**) Scheme summarizing the role of these reporter genes in the NO3- assimilation pathway, except for the transferase and the unknown genes. These genes are indicated in black boxes or circles.

**Fig. S4. Dissection of the LRs development and growth in wild-type and *ipt3,5,7* mutant.**

The bar graphs depict either the mean LR density or the mean LR length, on day 4 in the P1, P2 and P3. The asterisks indicate the statistically significant differences between C.NO3 and Sp.NO3 compared with Sp.KCl and C.KCl. The graphs in the grey box indicate the main differences between the wild-type and the triple mutant. Indeed, in P2 and P3, the triple mutant is specifically altered in the stimulation of LRs emergence in the Sp.NO3 compartment.

**Fig. S5. The expression pattern of type-A ARRs in the roots and shoots of plants in the split-root treatment.**

(**A**) Normalized expression value of ARR5 to 9 genes in the roots extracted from the microarrays data.

(**B**) Relative mRNA accumulation of ARR5 to 9 genes in the shoots of the split-root plants, at 8 hrs. Relative mRNA accumulation has been determined by qPCR experiments.

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