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Integration of local and systemic signaling pathways for plant N responses

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Nitrogen (N) is an essential macronutrient and a signal that has profound impacts on plant growth and development. In order to cope with changing N regimes in the soil, plants have developed complex regulatory mechanisms that involve short-range and long-range signaling pathways. These pathways act at the cellular and whole plant scale to coordinate plant N metabolism, growth and development according to external and internal N status. Although molecular components of local and systemic N signaling have been identified and characterized, an integrated view of how plants coordinate and organize the N response is still lacking. In this review, we discuss recent advances toward understanding the mechanisms of local and systemic N responses and provide an integrated model for how these responses are orchestrated.

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Introduction

The mineral nutrient required in the greatest amount by plants is nitrogen (N). Nitrogen comprises 1.5–2% of plant dry matter and approximately 16% of total plant protein [1]. Owing to its metabolic importance, N availability is one of the key factors limiting crop production [1].

N abundance in soils can vary several orders of magnitude (reviewed in [2]). As a consequence, plants have evolved sophisticated mechanisms for N acquisition as well as for coordinating plant growth and development based on N supply. The regulatory mechanisms that govern plant responses to N comprise both local signaling pathways acting at the cell level and systemic signaling pathways that communicate internal nutrient status across different tissues and plant organs. An illustrative example of both local and systemic regulatory pathways at work can be found in the nitrate modulation of root system architecture (RSA). A local supply of nitrate promotes lateral root elongation in the nitrate-rich patch, while a high nitrate supply to whole roots has an inhibitory effect on lateral root development [3]. When two separate parts of the root are exposed to different nitrate concentrations, systemic signals can communicate the N status of the plant and impact lateral root growth away from the site of nitrate perception. The long-distance systemic signaling is mediated by nitrate, but hormones such as cytokinins, can report the nitrate demand of the whole plant [4••]. Nitrate uptake systems are also under control by both local signaling and systemic signaling driven by the N status of the plant (reviewed in [5,6]). For instance, the expression of NRT2.1 that encodes a main component of the high-affinity transport system for root nitrate uptake [7] is induced by local nitrate supply [8] and repressed by systemic feedback signals exerted by high N status [9]. The interplay of local and systemic regulatory mechanisms for control of RSA allows plants to optimize nutrient acquisition in soils where N is heterogeneously distributed.

In this review, we discuss recent advances and perspectives on deciphering local and systemic signaling pathways regulating plant N responses and how these processes may be integrated in cells and organs to generate a coherent organismal response.

Molecular mechanisms of the local N response

In land plants, the initial site of nutrient perception and acquisition is typically the root organ. Since nutrient availability fluctuates in soils, plants must have efficient sensing systems to signal the external nutrients concentrations. Since nitrate represents the main N source for plants in most agricultural soils, most research efforts have centered in the identification of molecular components of the signaling pathways mediating plant responses to nitrate. Less is known about the local plant response to ammonium or other N nutrient/metabolites.

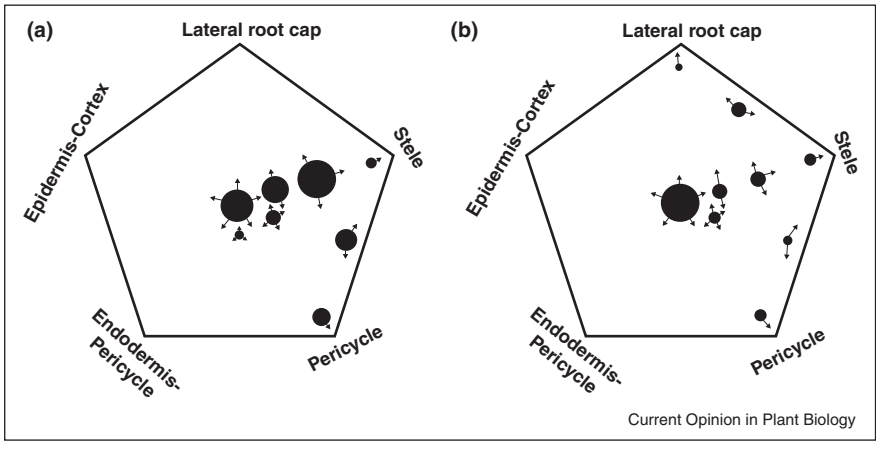
Several pieces of evidence indicate the nitrate transporter NRT1.1 as one of the nitrate sensors in *Arabidopsis thaliana* [10,11••,12•]. NRT1.1 is a dual affinity nitrate transporter that changes between low and high affinity based on the phosphorylation status of the threonine residue 101 [10,11••]. Low nitrate concentration triggers

phosphorylation of T101 by CIPK23 [11••]. This phosphorylation switches NRT1.1 to the high affinity function leading to a weak induction of *NRT2.1* gene expression [11••]. Conversely, in experiments with high nitrate concentration T101 is not phosphorylated, NRT1.1 switches to the low affinity function and NRT2.1 gene expression is strongly induced [11••]. Additional evidence of a function for the NRT1.1 protein in N responses come from studies on nascent primary and lateral roots [13], in stimulation of germination [14], in root colonization in nitrate rich patches [15] and in the antagonistic effect of nitrate on the response of the primary root to L-glutamate [16]. These studies however did not clearly distinguish between phenotypic effects due to a transport deficiency versus a signaling defect of the transporter. A more recent work directly addressed this issue by characterizing the EMS mutant *chl1-9*. The point mutation in *chl1-9* resulted in a change of the proline residue 492 to leucine. This amino acid change reduced NRT1.1 nitrate uptake in the low and high affinity range but did not affect the primary nitrate response of *NRT2.1* [11••]. These experiments showed that nitrate transport activity is not required for the sensing function of NRT1.1.

Transcriptome analysis with cell-type resolution have shown that nitrate elicits coordinated but distinct cellular responses in different cell types and that the nitrate response is highly cell-specific [17••]. Out of 6142 total nitrate regulated genes identified, only 771 of them respond across all cell types [17••]. Consistent with NRT1.1 expression in primary root tips [13,15] and in internal layers of the root [13], the most responsive cell types correspond to pericycle, stele and the lateral root cap (LRC) [17••]. A recent study investigated the early nitrate response in detailed time course experiments to identify the early events underlying the root nitrate response [18••]. This work identified genes whose expression changed shortly after exposure to nitrate, including more than 200 genes responding at 12 min or earlier after nitrate treatment [18••]. When comparing with the cell-specific data by Gifford *et al*. [17••] the majority of the very early nitrate responsive genes (3 and 6 min after treatment) have cell-specific regulation in LRC, pericycle and/or stele (Figure 1a). This simple comparison suggests that nitrate might be sensed in specific cells of the root organ, including sites of NRT1.1 expression (Figure 2), further supporting its role as a nitrate sensor. Interestingly, at later time points (9, 12, 15 and 20 min after treatment) a higher proportion of the genes is regulated in all cell types (Figure 1b), suggesting that the gene response is first initiated in LRC, pericycle and/or stele and is then propagated to the rest of the root (Figure 2). Additional cell specific experiments at early time points are needed to dissect the onset of the nitrate response and how it is propagated between cells in roots. It has been shown that NRT1.1 is required for the normal expression in response to nitrate of more than 100 genes in Arabidopsis roots [12•]. However, this result also points to an additional nitrate sensing mechanism besides NRT1.1, since not all nitrate responsive genes are affected by mutations in NRT1.1 [12•]. Additional putative sensors may include the NRT2.1 transporter [19,20].

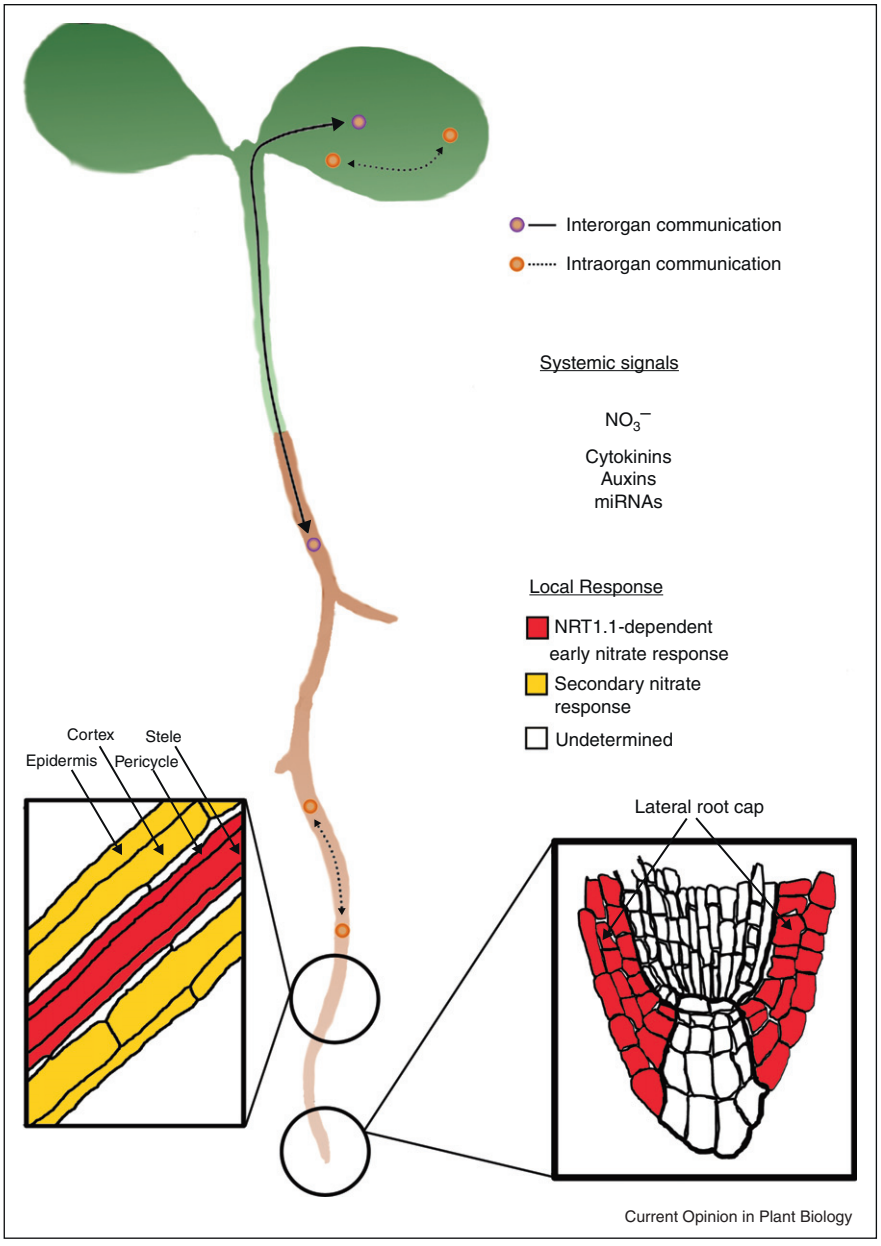
Although nitrate is the major source of N for plants growing in aerobic soils, other forms of N can contribute to plant nutrition as well as influence several aspects of

Figure 1



The nitrate response is initiated in specific cell types and is then propagated to the whole root. Nitrate-responsive gene list at the cell type resolution were obtained from Supplementary material by Gifford *et al*. [17••] and intersect with the list of very early regulated genes at 3, 6 min **(a)** and with the list of genes regulated at 9, 12, 15 and 20 min **(b)** that were obtained from the Supplementary material by Krouk *et al*., 2010 [18••]. For our analysis, we only considered genes that were regulated in both publications. The gene lists were used to perform a Sungear analysis [46,47] in the VirtualPlant webpage (www.virtualplant.org). Vertices in the polygon represent the different gene lists used in our analysis. The circles inside the polygon represent the number of genes shared among one or more sets of genes. Arrows in the circles indicate which sets the genes belong to. The size of the circles is proportional to the number of genes contained in each circle.

Figure 2



Hypothetical model of local and systemic N responses in *Arabidopsis thaliana*. N nutrients and metabolites are perceived locally in one or more cell types and local and systemic signaling pathways are activated to orchestrate the plant response. Local signaling is associated with sensing N availability in the root environment by NRT1.1. The expression of NRT1.1 in root tips and in internal layers of the root [13,48] coincides with pericycle, stele and lateral root cap being the earliest and most responsive tissues to nitrate [17••,18••]. NRT1.1 may control part of the early nitrate response in a tissue specific manner. Systemic signaling pathway informing the status of the whole plant involves long-distance signals that move between different organs.

plant growth and development. Ammonium is the second most abundant nutrient in most agricultural soils and the primary N source in anoxic soil conditions [21]. At least four ammonium transporters (AMT1.1, 1.2, 1.3, and 1.5) have been implicated in ammonium uptake from the soil [22]. A recent report showed that AMT1.3 is an important regulator of lateral root branching in response to ammonium. AMT1.3 mutants showed a 30% decrease in lateral root density, whereas accumulation of ammonium was similar as compared to wild-type plants [23]. These results suggest a transport-independent function of AMT1.3 in lateral root growth [23]. On the other hand, AMT1.1 is phosphorylated in a threonine residue in an ammonium concentration and time-dependent manner,

leading to allosteric inactivation of the transporter, down-regulating ammonium transport to prevent ammonium toxicity [24,25]. This mechanism is reminiscent of the nitrate concentration-dependent NRT1.1 regulation by phosphorylation. Changes on phosphorylation status of AMT1.1 suggest that AMT1.1 itself might act as a transceptor, sensing external ammonium concentration and adjusting ammonium acquisition.

Nitrate and other N metabolites are able to regulate a myriad of genes involved in many different processes. Gene responses to nitrate have been thoroughly described, mainly by transcriptomics analyses using microarrays and different experimental conditions (reviewed in [26–28]). Signaling components of the nitrate response have been shown to include calcium-related kinases CIPK8 and CIPK23, transcription factors NLP7, ANR1, LBD37/38/39, SPL9 as well as components of hormone signaling pathways (reviewed in [27–29]).

The nitrate primary response is characterized by rapid changes in the expression of many genes independent of protein synthesis [30]. Primary response genes (PRGs) include genes involved in nitrate transport, reduction and assimilation such as *NRT1.1*, *NRT2.1*, *NIR* and *NIA1* [31]. The expression of these genes is significantly increased shortly after nitrate treatment [18••,31]. In an attempt to identify regulatory factors involved in the nitrate response of PRGs, Krouk *et al.* monitored genome-wide responses at early time points after exposure to 1 mM nitrate. Plants were treated for 3, 6, 9, 12, 15 and 20 min (PRGs typically begin to respond at 12–20 min of nitrate exposure) and gene expression was monitored using Affymetrix ATH1 gene chips [18••]. The results of these experiments showed that the initial nitrate response include genes involved in translation, suggesting that nitrate initially triggers a reprogramming of the transcriptome to ensure production of the necessary proteins for nitrate acquisition [18••]. Genes involved in N metabolism were regulated after 9 min of nitrate treatment and genes responding at later time points (after 12 min) include components of hormone signaling pathways. The sequential modulation of protein synthesis, metabolism and hormonal pathways suggest a model for how the nitrate signal is translated to developmental outcomes. Using a machine learning approach, the authors described a gene network involving transcription factors and PRGs [18••]. Analysis of a predicted hub of this network, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9), showed that this early nitrate responsive transcription factor regulates the expression of *CIPK23*, *NRT1.1*, *NIR* and *NIA2* [18••]. SPL genes have been implicated in promoting vegetative and floral phase transition [32] but their role in roots and more specifically, in the nitrate response has not been described before this study [18••].

Molecular mechanisms of systemic N response

Besides sensing the local availability of N, plants must integrate internal systemic signals that inform the N status of organs in order to finely adjust N transport, metabolism and plant growth and development. In contrast to local signaling events, much less is known about molecular components of systemic signaling. Analysis of root nitrate uptake regulation and recent microarray data had shed some light on the underlying molecular mechanisms integrating local and systemic responses to N.

Systemic regulation of nitrate uptake has been extensively studied as a model to systemic N responses. Analysis of systemic responses to nutrients has been conducted by 'split-root' experiments, in which the plant root grows on a vertical agar plate that is divided in the middle, physically separating two sectors of the agar. Using this experimental system, the root can be simultaneously exposed to two different nutritional conditions [9,33]. Experiments with the split-root design showed that increased N supply on one side of the root system results in a compensatory downregulation of root nitrate uptake in the untreated part of the root system [9,33]. This systemic control is thought to involve specific repression of root nitrate uptake systems by long-distance signals triggered by high N status of the plant [6,34]. *NRT2.1* gene is a major target of this N signaling mechanism [7]. *NRT2.1* expression is repressed in plants under high N supply and at least part of this mechanism is due to transcriptional control [8,9]. A recent forward genetic screen using a transgenic line expressing *pNRT2.1:LUC* construct as a reporter gene to isolate mutants defective in systemic N signaling showed that systemic feedback repression of *pNRT2.1* under high N conditions is impaired in the *hni9-1* mutant [35]. HNI9 is allelic to Arabidopsis INTERACT WITH SPT6 (IWS1), and codes for a component of the RNA polymerase II complex [36•]. HNI9 acts by increasing the accumulation of H3K27me3 at the *NRT2.1* locus in response to high N, leading to a down-regulation of the *NRT2.1* transcript [36•]. This work also shows that the deposition of H3K4me3 and H3K36me3 on *NRT2.1* chromatin in response to N supply does not depend on HNI9/AtIWS1, suggesting an additional pathway controlling chromatin modifications involved in systemic N signaling.

Phenotypic analysis of nitrate reductase-null (NR-null) mutants has suggested that nitrate is one of the signals that communicate shoot and root N status. NR-null mutants are more sensitive to systemic high nitrate inhibition of lateral root development than wild-type plants [3]. Using the NR-null mutant in the split-root experimental system, Ruffel *et al.* (2011) showed that nitrate can mediate early transcriptional N-regulated reprogramming, that precede changes in root architecture. It was also determined that the roots of decapitated plants,

completely lost the response to N systemic signaling. These experiments showed that whereas local nitrate response is preserved in shootless plants, the systemic signaling is lost, requiring a root–shoot–root communication for the integration of the N signal [4••] (Figure 2). In addition to nitrate, other N nutrient/metabolites such as internal amino acid pools may function as systemic signals to control N responses in plants [6].

More recently, microRNA-mediated regulation of auxin components has been shown to depend on metabolites generated downstream of nitrate reduction and assimilation [37•]. microRNAs are good candidates for systemic regulation since they are found in the vascular system [38,39]. For example, phosphate starvation induces miR399 in shoots and translocates through the phloem to repress its target *PHO2* in roots [40,41]. MiR395 and miR398 are also expressed in vascular tissue and control sulphur and copper homeostasis, respectively [42,43]. Using the glutamine synthetase inhibitor MSX, Gifford *et al*., identified an organic N responsive microRNA167/ARF8 regulatory module that controls a connected network of 128 potential ARF8 targets [17••]. miR167/ARF8 was shown to regulate the ratio of lateral root initiation and elongation, coordinating rooting to organic N availability [17••]. The miR393/AFB3 module was shown to regulate primary and lateral root growth in response to N [37•]. This module represents a type I incoherent feed-forward loop in which *AFB3* is induced by the nitrate signal and is downregulated by the organic N-responsive miR393 [37•]. Although there is no direct evidence showing that miR167 or miR393 is able to translocate from shoots to roots in response to N to regulate RSA, these and other microRNAs regulated in response to changes in N availability are promising candidates and should be evaluated for their role in systemic N responses.

Other putative systemic signals include phytohormones such as cytokinins (CK) and auxin. Auxin role as a mobile signal originating in shoots and systemically controlling lateral root development has been described before [44] (Figure 2). The role of CK as a systemic signal communicating roots and shoots has only recently described. Using the split-root system it was shown that the response of systemic N-responsive genes is severely affected in a triple mutant for the CK biosynthesis enzymes isopentenyl transferases (ipt3,5,7) [45], indicating that CKs are necessary to integrate the N status of the plant [4••] (Figure 2). In order to uncover genes whose expression is affected by the N systemic signal, Ruffel *et al*. (2011) performed time course transcriptomic analysis [4••]. Clustering analysis of the data showed that plants initially respond to the local nitrate environment, but at later points, systemic signals integrating the N status of the plant control gene expression [4••]. Rapidly regulated genes include *NRT3.1*, *NIR*, *Glucose-6-phosphate Dehydrogenase 3* (*G6PD3*) and root *ferredoxin NADP(H) oxidoreductase* (*FNR2*), suggesting that the first target of systemic signals is N uptake and metabolism [4••]. These results are similar in Medicago, where systemic N signals are also able to regulate important components of N transport and metabolism [33], indicating that systemic N signaling could be conserved between plant species. Since this early reprogramming of the root transcriptome is still present in nitrate reductase-null plants, it is concluded that nitrate, rather than other downstream N metabolites is the signaling molecule, at least under these experimental conditions (Figure 2). Moreover, authors describe the existence of two genetically systemic signaling pathways, one dependent on CK, that would control N demand signals and one independent of CK that would control N supply signals [4••].

Final remarks

Over the past few years several regulatory factors have been shown to participate in N local signaling pathways including receptors, putative components of the signal transduction pathway and effectors such as transcription factors that trigger N responses [26–28]. Models of local N regulatory networks are emerging. However, we still do not know how these components are interconnected to effect local N responses. Concerning nitrate, NRT1.1 appears to be responsible for only a fraction of the nitrate regulated genes. Hence, additional nitrate sensing and signal transduction mechanisms may exist. Local response mechanisms that sense external changes in nitrate concentration and perception may be managed in a tissue-specific manner. Current data suggest lateral root cap, stele and/or pericycle as initial sites of nitrate sensing but other cell-types may be involved in the early stages of plant nitrate responses. Additional data dissecting early transcriptional responses to N with cell-type resolution might be required for a better understanding of the spatial and temporal nature of the plant N response. How are the local events then integrated across the whole organism to articulate coherent responses to N is the next frontier. We still know very little about the systemic N signaling pathways in plants. Understanding N responses at the organism level will be useful to modify plant metabolism, physiology and growth and developmental programs to improve N use efficiency and productivity in crops.

Conflict of interest statement

The authors declare that there are no conflicts of interest related to this publication.

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