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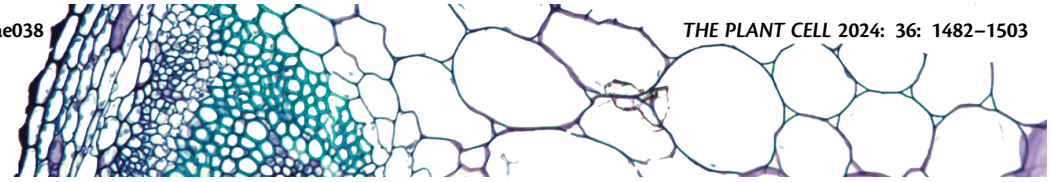

Review

Nitrogen sensing and regulatory networks: it's about time and space

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Abstract

A plant's response to external and internal nitrogen signals/status relies on sensing and signaling mechanisms that operate across spatial and temporal dimensions. From a comprehensive systems biology perspective, this involves integrating nitrogen responses in different cell types and over long distances to ensure organ coordination in real time and yield practical applications. In this prospective review, we focus on novel aspects of nitrogen (N) sensing/signaling uncovered using temporal and spatial systems biology approaches, largely in the model Arabidopsis. The temporal aspects span: transcriptional responses to N-dose mediated by Michaelis-Menten kinetics, the role of the master NLP7 transcription factor as a nitrate sensor, its nitrate-dependent TF nuclear retention, its "hit-and-run" mode of target gene regulation, and temporal transcriptional cascade identified by "network walking." Spatial aspects of N-sensing/signaling have been uncovered in cell type-specific studies in roots and in root-to-shoot communication. We explore new approaches using single-cell sequencing data, trajectory inference, and pseudotime analysis as well as machine learning and artificial intelligence approaches. Finally, unveiling the mechanisms underlying the spatial dynamics of nitrogen sensing/signaling networks across species from model to crop could pave the way for translational studies to improve nitrogen-use efficiency in crops. Such outcomes could potentially reduce the detrimental effects of excessive fertilizer usage on groundwater pollution and greenhouse gas emissions.

Introduction

This special *Plant Cell* issue commemorates 100 years of the American Society of Plant Biologists. Our review focuses on the spatial dynamics of nitrogen sensing and signaling networks: it's about time. Aptly, more than 100 years ago, Michalis-Menten's classic paper published in 1913 sought "to achieve the final aim of kinetic research; namely, to

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obtain knowledge of the nature of a reaction from a study of its progress" (Michaelis and Menten 1913; Michaelis et al. 2011). Inspired by this, our review focuses on systems biology studies conducted in real time and space. Such studies have uncovered the mechanisms by which plants sense and respond to nitrogen (N) signals within minutes to evoke changes in N-signaling networks in specific cell types that influence plant growth and development. Such discoveries of N-sensing/signaling reside in Pasteur's quadrant, a field of inquiry that aims to gain a fundamental understanding of a scientific problem while also providing immediate societal benefits (Stokes 1997)—in this case, improvements in nitrogen use efficiency (NUE).

The advent of synthetic fertilizers has brought significant advantages to agricultural practices by boosting crop yield, but at both economic and environmental costs (Menegat et al. 2022). Approximately half of applied fertilizers are effectively used by plants, while the remaining portion is prone to runoff, resulting in groundwater contamination and eutrophication (Bijay-Singh and Craswell 2021). Moreover, excess fertilizer application can lead to the production of nitrous oxide, a potent greenhouse gas (Mahmud et al. 2020; Menegat et al. 2022). Considering these challenges, a key objective of nitrogen research is to develop plants with enhanced NUE. Achieving this objective would not only reduce the need for excessive fertilizer usage but also support optimal plant growth in nitrogen-limited soils worldwide.

Nitrogen—the rate limiting element for plant growth—is often found in the soil as nitrate (NO_3^-) and/or ammonium (NH_4^+). Organic forms such as amino acids and urea can also play important roles in specific contexts (Yang et al. 2021b). Nitrate—the main form of nitrogen found in aerobic soils—also acts as a N-signal sensed by a nitrate transceptor in roots (Crawford and Forde 2002). As such, nitrate sensing/signaling has been widely studied by using biochemical, molecular genomics, genetics, and systems biology approaches (Krouk et al. 2010; Gaudinier et al. 2018; Wang et al. 2018; Vidal et al. 2020; Lamig et al. 2022). Herein, we explore studies that use systems biology approaches to examine the temporal and spatial mechanisms behind nitrogen sensing and signaling, largely in the model Arabidopsis. We especially highlight progress in this area published after the "Nitrate in 2020" *Plant Cell* Review, which includes an extensive timeline of milestone publications on nitrate signaling up to 2020 (Vidal et al. 2020). In addition to studies that explore the primary N-response (nitrate sensing/signaling), we include temporal studies that examine the plant response to ammonium nitrate, the source of nitrogen in the widely used Murashige and Skoog cell culture medium (Murashige and Skoog 1962; Varala et al. 2018; Brooks et al. 2019; Swift et al. 2020; Alvarez et al. 2021). Plants respond differently to sole sources of nitrate versus ammonium; therefore, we recommend referring to the following excellent reviews for details on specific ammonium responses not covered herein (Hachiya and Sakakibara 2017; Liu and Von Wirén 2017).

For further insights into advances in nitrogen sensing/signaling, we recommend recent reviews that encompass other aspects such as nitrogen transport (Tegeder and Masclaux-Daubresse 2018; Wang et al. 2018), local and systemic nitrogen signaling (Zhang et al. 2020), post-translational modifications and nitrogen signaling components (Muratore et al. 2021; Wang et al. 2021a), nitrogen-dependent developmental responses (Weber and Burow 2018; Fredes et al. 2019), nitrogen regulation of root system architecture (Jia and von Wirén 2020; Hu et al. 2021), nitrogen interactions with other nutrients (Oldroyd and Leyser 2020; Li et al. 2021), nitrogen and hormone interactions (Sakakibara 2021; Xing et al. 2023), and nitrogen responses under abiotic stress (Araus et al. 2020; Plett et al. 2020).

This review briefly touches on nitrogen response networks in crops (Ueda et al. 2020) and translational studies of nitrogen signaling networks—from model to crop (Obertello et al. 2015; Cheng et al. 2021). We also recommend more extensive recent reviews on crops for a comprehensive understanding of nitrogen signaling in agricultural contexts (Jia and von Wirén 2020; Hou et al. 2021; Sandhu et al. 2021; Gao et al. 2022; Hu et al. 2023).

This review focuses on novel insights gained from systems biology approaches to uncover the temporal and spatial mechanisms of nitrogen sensing and signaling. These include the discovery that Michaelis-Menten kinetics mediates N-dose dependent transcriptome responses (Swift et al. 2020). This finding echoes earlier time-based studies that showed that Michaelis-Menten kinetics mediate N-dose dependent nitrogen uptake (Ho et al. 2009; McNickle and Brown 2014) and plant growth responses (Lana et al. 2005). Furthermore, we examine recent studies that have uncovered the time-dependent mechanisms involving the master transcription factor (TF) NLP7, as a nitrate sensor (Liu et al. 2022), the nitrate-dependent nuclear localization of NLP7/6 (Marchive et al. 2013; Guan et al. 2017; Liu et al. 2017; Cheng et al. 2023), the "hit-and-run" model of transient interactions of NLP7-target genes (Alvarez et al. 2020), and the regulation that NLP7 exerts over a temporal cascade of downstream TF2s—uncovered using a method called "network walking" (Alvarez et al. 2020; Brooks et al. 2019, 2020).

We also explore new spatial approaches that can identify how nitrogen sensing, transport, and signaling are governed by cell type specificity in different organs. This includes the cell type-specific signaling responses to nitrate (Chen et al. 2022; Contreras-López et al. 2022), as well as studies that examine nitrate root-to-shoot communication and how plants integrate the shoot and root nitrogen status to systematically regulate nutrient uptake in the roots (Tabata et al. 2014; Ohkubo et al. 2017; Ota et al. 2020; Abualia et al. 2022).

To facilitate and inspire future advances in nitrogen research in space and time, we review advancements in single-cell sequencing technology that can be applied to plant N-sensing/signaling (Rich-Griffin et al. 2020; Cole et al. 2021). For example, the use of single cell (sc) RNA-seq assays

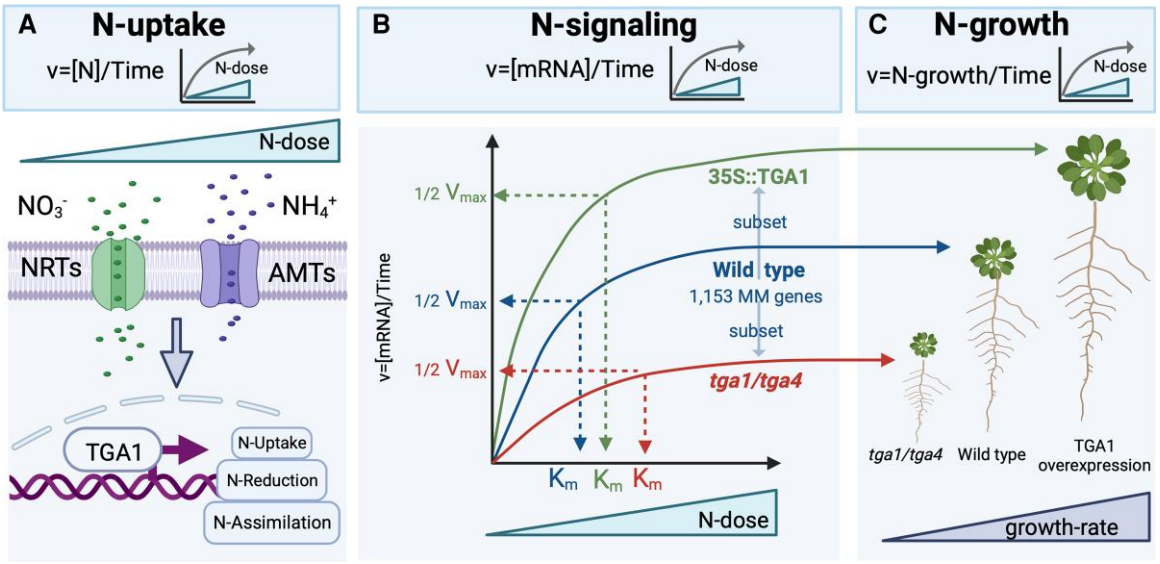

Figure 1. The N-dose-dependent regulation of N-uptake, N-signaling, and N-growth follows Michaelis-Menten (MM) kinetics. **A)** The rate of N-uptake by NRTs and AMTs is regulated by MM kinetics (Ho et al. 2009; McNickle and Brown 2014). **B)** Swift et al. (2020) demonstrated that the transcriptional response to N-dose also follows MM kinetics in Arabidopsis wild-type plants (Swift et al. 2020). Moreover, TGA1 overexpression and *tga1/4* mutant analysis revealed that a portion of this MM-mediated N-dose transcriptional response is mediated by the master transcription factor TGA1, which affects plant growth rate (Swift et al. 2020). **C)** N-dose-regulated growth responses measured by biomass is also regulated by MM kinetics (Lana et al. 2005). Thus, transcriptome kinetics responding to changes in N-dose has the potential to enhance plant growth. Figure adapted from Swift et al. (2020). Figure created with BioRender.com.

could enable (i) tracking of the nitrogen signal from root-to-shoot and (ii) determination of cell fate trajectories using pseudotime analysis (Denyer et al. 2019; Shahan et al. 2022; Nolan et al. 2023). Additionally, we explore how to use computational methods such as machine learning of the gene-to-NUE trait across a model and crop (Cheng et al. 2021) and how artificial intelligence (Gao et al. 2021) may augment experimental nitrogen research endeavors.

Overall, this ASPB Centennial review provides an overview the spatiotemporal dynamics of nitrogen sensing and signaling as an integrated system in plants. These temporal based systems biology approaches can also be applied to study any sensing and signaling network in plant and crop biology.

Nitrogen dose sensing as a function of time

How an organism senses and responds to changes in nitrogen nutrient dose is a basic unanswered question in biology with special relevance to agriculture. Exploiting time to uncover mechanisms underlying N-sensing/signaling in plants derives inspiration from the now classic Michaelis-Menten (MM) paper, which aimed "to obtain knowledge of the nature of a reaction from a study of its progress" (Michaelis and Menten 1913; Michaelis et al. 2011). Importantly, MM kinetics have also previously been shown to mediate nitrogen uptake (Fig. 1A; Ho et al. 2009; McNickle and Brown 2014) and plant growth (Fig. 1C; Lana et al. 2005). Inspired by this, Swift et al (2020) applied the MM kinetic concept to study the molecular basis for N-dose sensing in Arabidopsis, exposing seedlings to a matrix of four increasing N-doses of ammonium nitrate over five time points (Ahmed 2020; Akmakjian and Bailey-Serres 2020; Swift et al. 2020). Modeling of the resulting RNA-seq data revealed that 3,818 genes increased or decreased their expression in proportion to N-dose over time. Moreover, they found that for a subset of these genes, the N-dose-dependent gene responses mirror simple enzyme kinetics described by Michaelis-Menten (MM) in 1913, where changing levels of enzyme abundance will affect the maximum rate of reaction (V_{max}; Michaelis and Menten 1913; Michaelis et al. 2011; Swift et al. 2020). Specifically, N-dose response genes whose expression pattern significantly fit the MM model allowed for estimating the maximum rate of transcript change (V_{max}), as well as the N-dose at which half of V_{max} was achieved (K_m; Fig. 1B; Swift et al. 2020). Indeed, the classic MM kinetic model was able to explain the expression of 30% of N-dose responsive genes in Arabidopsis (1,153 MM modeled N-dose responsive genes; Fig. 1B), whereas the remaining 70% genes could be explained by more complex kinetics and/or other regulatory mechanisms (Swift et al. 2020). This finding suggests that transcription factors (TFs) that regulate MM response genes can be analogized as catalytic enzymes in the MM model since they establish the rates at which transcription takes place in response to N-dose (Swift et al. 2020). To support this, in vivo studies showed that the overexpression of TGA1, an early N-responsive TF, led to an increase in V_{max} of N-dose responsive mRNAs (Fig. 1B), which was translated as an accelerated plant growth in response to N (Fig. 1C; Swift et al.

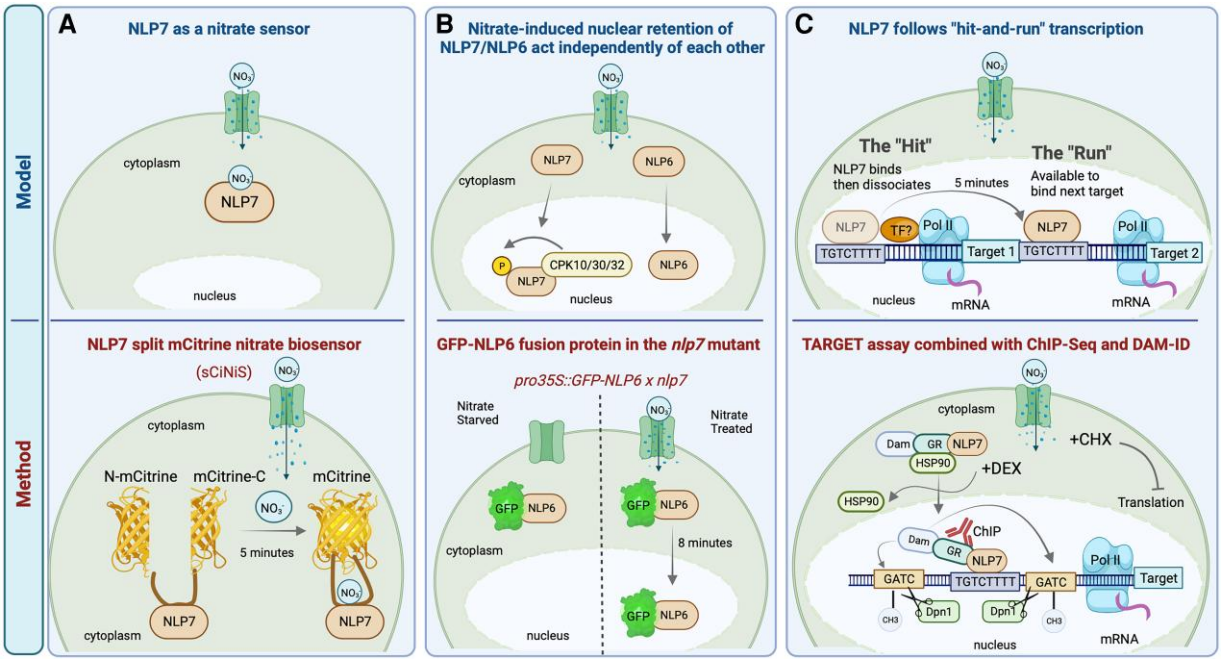

Figure 2. Time- and space-dependent modes of action for NLP7 as a master regulator of nitrate signaling. **A)** NLP7 binds to nitrate and acts as a nitrate sensor as determined using the genetically encoded split mCitrine-NLP7 nitrate biosensor (sCiNiS) assay (Liu et al. 2022). Fluorescent signal was detected 5 min after nitrate treatment in both mesophyll and primary root tip cells (Liu et al. 2022). **B)** Both NLP7 and NLP6 accumulate in the nucleus in response to nitrate as determined with TF-fusion proteins expressed in their respective mutant backgrounds, showing that accumulation of either TF in the nucleus is independent of each other, but dependent on nitrate (Marchive et al. 2013; Guan et al. 2017; Liu et al. 2017; Cheng et al. 2023). **C)** The "hit-and-run" model of transcription posits that a pioneer TF transiently binds to the promoter of a target gene to open the chromatin and allow for other partner TFs to bind the promoter, thereby making NLP7 available to bind the next target gene (Para et al. 2014; Alvarez et al. 2020). The TARGET assay combined with ChIP-seq and DamID was used to identify these highly transient NLP7 target genes (Alvarez et al. 2020). Figure created with BioRender.com.

2020). Uncovering the molecular mechanisms that underlie the transcriptome kinetics responding to changes in N-dose now connects N-uptake (transport) to output (biomass), and thus has the potential to enhance plant growth and improve N-use efficiency in crops (Fig. 1).

Time- and space-dependent modes of action for NLP7 as a master regulator of nitrate signaling

NLP7 is a master regulator of the early nitrate response, acting as a transcriptional regulator of genes involved with nitrate transport, nitrate assimilation, and signal transduction (Marchive et al. 2013; Alvarez et al. 2020). New time-based studies have shown that NLP7 is not only a master transcription factor for mediating nitrate responses, but it can also bind nitrate and act as an intracellular nitrate sensor, as identified using the split mCitrine-NLP7 nitrate biosensor (sCiNiS; Liu et al. 2022; Fig. 2A). The fluorescence signal for NLP7 binding of nitrate was detected after 5 min of nitrate treatment in mesophyll cells of cotyledons and also in primary root tips, showing that NLP7 acts as an intracellular nitrate sensor to initiate nitrate responses (Liu et al. 2022). The nitrate-binding domain on NLP7 is an evolutionarily ancient domain that is conserved among plant NLPs and bacterial nitrate sensors such as NreA (Niemann et al. 2014). Nitrate directly interacts with NLP7 through its amino terminus, inducing its conformational change to activate transcription (Liu et al. 2022; Fig. 2A).

The role of NLP7 as a nitrate sensor is an additional level of NLP7 regulation to the known post-translational modifications that regulate NLP7 in the nucleus in response to nitrate (Fig. 2B; Marchive et al. 2013; Guan et al. 2017; Liu et al. 2017). Once nitrate is transported inside the cell by NRT1.1, a rapid wave of Ca + 2 causes the activation of group III calcium-sensor protein kinases (CPKs) in seconds, which in turn phosphorylates NLP7 to retain it in the nucleus, activating early nitrate-response genes within minutes (Marchive et al. 2013; Liu et al. 2017; Fig. 2B). A recent study by Cheng et al. (2023) found that NLP7 and another NLP family member, NLP6, are both retained in the nucleus in response to nitrate (Cheng et al. 2023; Fig. 2B). Moreover, they showed that nitrate-dependent nuclear accumulation of NLP7 and NLP6 act independently of each other. To do this, they constructed translational fusion proteins for both GFP-NLP6 and GFP-NLP7, expressed in *nlp7* or *nlp6* mutant background, respectively, accumulated in the nucleus in response to nitrate

Table 1 . Systems biology techniques and tools applied to studying TFs and their targets involved in nitrogen sensing and signaling

Technique/tool	Description	References
TARGET (TF → direct target regulation in plant cells)	Transient Assay Reporting Genome-wide Effects of Transcription factors (TARGET) is a plant cell-based assay used to identify direct TF target gene interactions with timed nuclear entry of the TF (Fig. 2).	Bargmann et al. (2013), Brooks et al. (2019), Alvarez et al. (2020); Brooks et al. (2023)
DamID-Seq (TF → Target interaction in vivo)	DNA adenine methyltransferase identification (DamID) uses DNA methylation of promoters to detect highly transient TF-DNA binding interactions.	Steensel and Henikoff (2000), Alvarez et al. (2020)
DAP-seq (TF → target interaction in vitro)	DNA affinity purification sequencing (DAP-seq) is a high-throughput TF-DNA binding assay that uses genomic DNA and TFs expressed in vitro.	O'Malley et al. (2016)
Precision/Recall (AUPR) (Validation of inferred TF → target interactions)	Precision/Recall (PR) analysis with area under precision recall (AUPR) curve uses validated TF-target gene data (TF-binding and/or regulation data) to determine the PR of predicted TF-target genes in gene regulatory networks (GRNs) (Fig. 3A).	Brooks et al. (2019), Shanks et al. (2022), Brooks et al. (2021)
Network Walking (TF_1 → direct TF_{2s} → indirect TF_1 targets)	Network walking is a GRN method that charts a path from the direct target genes of a TF_1 to their indirect target genes via a TF_2 (Fig. 3B).	Brooks et al. (2019, 2021)

and in the absence of either endogenous NLP7 or NLP6 proteins (NLP6 experimental setup is shown as an example; Fig. 2B; Cheng et al. 2023). While previous reports show that NLP7 and NLP6 heterodimerize in the cytosol in response to nitrate (Guan et al. 2017), Cheng et al. (2023) show that the nuclear retention of NLP7 and NLP6 in response to nitrate is independent of each other (Fig. 2B; Guan et al. 2017; Cheng et al. 2023).

Recent studies also implicate NLP7 in initiating a cascade of early N-responsive downstream transcription factors (Alvarez et al. 2020). Specifically, gene expression changes in response to nitrogen occur rapidly (minutes to hours) and are divided into primary and secondary responses (Medici and Krouk 2014; Alvarez et al. 2021). Primary N-response genes are (i) rapidly induced by nitrate (minutes), (ii) do not require de novo protein synthesis, and (iii) are typically involved in nitrate transport, assimilation, and signaling (Medici and Krouk 2014). Secondary N-response genes are induced later (hours) and depend on the transcriptional products of the primary response genes. How the primary and secondary nitrogen response is regulated was recently revealed to involve rapid, transient protein–DNA interactions by TFs that follow the "hit-and-run" model of regulation (Fig. 2C), which includes the TFs bZIP1 (Para et al. 2014; Doidy et al. 2016) and NIN LIKE PROTEIN 7 (NLP7; Alvarez et al. 2020). As a pioneer or triggering TF, NLP7 is at the top of the nitrate signaling hierarchy following the "hit-and-run" model of transcriptional control (Fig. 2C). It was shown that the transient TF2 targets of NLP7 initiate a temporal cascade of genome-wide changes in the nitrate response in planta (Marchive et al. 2013; Alvarez et al. 2021).

The "hit-and-run" model suggests that a TF trigger/pioneer can form a stable transcriptional complex (the "hit"), allowing transcription to continue even after the initiating TF is no longer bound (the "run"; Fig. 2C; Schaffner 1988; Para et al. 2014; Doidy et al. 2016; Alvarez et al. 2020). Genome-wide evidence for the "hit-and-run" model of transcription for transient TF–target gene interactions was validated for two master TFs involved in the nitrate response, first identified with bZIP1 and more recent evidence for NLP7 (Para et al. 2014; Doidy et al. 2016; Alvarez et al. 2020). Time-series ChIP-seq experiments showed that bZIP1 and NLP7 were transiently bound to early nitrate-response genes, and 4-thiol-uracil labeling of nacent mRNA confirmed the active transcription of these hit-and-run targets (Para et al. 2014; Doidy et al. 2016; Alvarez et al. 2020).

Importantly, the plant cell-based TARGET assay (Transient Assay Reporting Genome-wide Effects of Transcription factors) used in these studies can capture early and transient TF-target gene regulation events often undetected *in planta* (Para et al. 2014; Doidy et al. 2016; Brooks et al. 2019; Alvarez et al. 2020). The TARGET TF-assay involves transient expression of a TF fused to a glucocorticoid receptor (GR) in plant cell protoplasts. The TF-GR protein is held in the cytoplasm by HSP90 binding to the GR domain (Bargmann et al. 2013; Fig. 2C, Table 1). The addition of the GR-ligand dexamethasone (DEX) displaces HSP90 binding, allowing nuclear entry of the TF-GR fusion protein. When DEX treatment is performed in the presence of cycloheximide, to inhibit the synthesis of proteins encoded by direct target genes (e.g. TF2), direct targets of a TF can be identified with RNA-seq, compared to empty vector (Fig. 2C, Table 1; Bargmann et al. 2013; Brooks et al. 2019, 2023).

The plant cell-based TARGET TF perturbation system allowed the identification of transient NLP7 targets that were undetected by time-series chromatin immunoprecipitation (ChIP; Alvarez et al. 2020). By coupling the DNA adenine methyltransferase identification (DamID) method (Gutierrez-Triana et al. 2016) to the TARGET TF perturbation system, it was possible to capture NLP7 binding to highly transient targets that were missed by time-course ChIP (Fig. 2C, Table 1; Alvarez et al. 2020). DamID uses a fusion protein of DNA adenine methyltransferase (Dam) to detect TF-DNA binding events by leaving a stable methylation mark at the adenine base in the $GA^{me}TC$ sequences near (within 1 kb) to protein-DNA binding sites as soon as the

TF touches down on the promoter (e.g. even transiently). This adenine methylation at GA^{me}TC allows for the binding and DNA cleavage using the DpnI restriction enzyme. DpnI fragments are mapped to the promoter regions to identify genes "touched" by the TF. Thus, this DNA methylation approach overcomes the limitations of biochemical methods such as ChIP-seq and other antibody-based techniques that are biased for stably bound TF-DNA interactions (Fig. 2C, Table 1; Steensel and Henikoff 2000; Alvarez et al. 2020). Using the TARGET and DamID-Seq methods, the study by Alvarez et al. (2020) confirmed that transient interactions of NLP7 initiate active transcription of its targets, consistent with a "hit-and-run" transcription model (Fig. 2; Alvarez et al. 2020). Overall, the multiple levels of NLP7 regulation highlight the important role of NLP7 in primary nitrate response to ensure a fast and broad adaptation by the plant to fluctuating nitrate levels (Fig. 2).

Temporal nitrogen response networks: generation and validation

In addition to the master TFs discussed above, TGA1, bZIP1, and NLP7, which are critical for signaling N-dose over time, gene network analysis studies and mutant screens have identified 40-plus TFs that are involved in propagating the nitrate signal (for review, see Vidal et al. 2020). Thus, we must understand the temporal regulatory connections between these TFs and the nitrate-responsive genes they control to obtain a complete temporal picture of nitrate signaling events. Combining computational and experimental approaches that consider time in gene expression analysis has proven to be a powerful approach to uncovering the temporal mechanisms of transcriptional responses in plants.

The goal of gene regulatory network (GRN) inference models is to connect a regulator (i.e. TF) to each of the genes it regulates in the genome. As causality moves forward in time, time-series experiments are a valuable resource to infer GRN models that can predict TF-target gene relationships at future untested time points, a main goal of systems biology. To account for the different times of captured gene regulation in time-series data, specialized network inference algorithms have been developed to account for the added factor of time in the data and can be based on correlation: time-lagged, random forest (DynGenie3 and Outpredict), and other regression models (Huynh-Thu and Geurts 2018; Nguyen and Braun 2018; Cirrone et al. 2020).

Over the last 10 years, several studies have exploited time-dependent responses to investigate nitrogen signaling networks in Arabidopsis using fine-scale time series (Krouk et al. 2010; Patterson et al. 2016; Walker et al. 2017; Varala et al. 2018; Brooks et al. 2019; Alvarez et al. 2021). In multiple of these time-based N-response network studies, a state-space model, which is a model that uses first-order differential or difference equations to describe a system, called Dynamic Factor Graph, was applied to fine-scale nitrogen response time series (i.e. many time points close together) datasets to predict regulatory interactions between N-responsive TFs and N-responsive genes in shoots (Varala et al. 2018) or roots (Krouk et al. 2010; Brooks et al. 2019). In general, state-space models are algorithms that model dynamic data (e.g. gene expression) by assuming that data are generated from underlying "hidden states" (Krouk et al. 2010; Brooks et al. 2019). In the case of time series experiments, the data of gene expression consider several time points (e.g. every 5 min, from 5 to 20 min) as consecutive hidden states that form a Markov chain (a mathematical system that statistically modulates random processes). Consequently, each transition in the Markov chain corresponds to a stationary (time) dynamic model. The resulting GRNs revealed the temporal networks operating in each tissue and implicated a hierarchy to the TFs involved (Vidal et al. 2020).

In the first fine-scale time-series study of nitrate signaling, Krouk et al. examined very early (3 to 20 min) gene expression responses to nitrate supply in roots (Krouk et al. 2010), whereas 20 min is the earliest time point that had previously been examined at the genomic level (Wang et al. 2000). The Krouk et al. (2010) study demonstrated that nitrate-triggered gene expression responses occur within as early as 3 min, and that transient changes are missed if plants are only sampled at later time points. In a subsequent study, Varala et al. performed a time-series experiment that included ammonium nitrate treatments across early-to-late time points (starting from 5 min for up to 120 min) and identified 2,737 genes responding to nitrogen as a function of time (NxTime) response genes in shoots (Varala et al. 2018) and 1,458 NxTime response genes in roots (Brooks et al. 2019). Moreover, the concept of "just-in-time" (JIT) analysis developed and deployed in these two studies identified the first time point that a gene was induced >1.4 fold by N-treatment. The JIT analysis bins NxTime genes that are differentially regulated by N for the first time point in the time-series experiment. Analysis of these JIT genes uncovered not only a temporal cascade of enriched cis-elements at each consecutive time point but also GO terms resulting from N-signaling that evolves over time (Varala et al. 2018; Brooks et al. 2019).

A strength of the N-response time-series GRN generated in shoots (Varala et al. 2018) and roots (Brooks et al. 2019) was in assessing the precision and recall accuracy of the TF→target gene GRN predictions using AUPR (area under the precision-recall curve) analysis (Fig. 3A; Table 1). The benefit of AUPR analysis is that it uses validated TF–target interactions to empirically determine precision cutoffs for the TF-target gene predictions in the GRN. By contrast, other methods arbitrarily select the top 1% to 10% of interactions —to prune the GRNs for higher-confidence TF-target edge predictions (Fig. 3A). To conduct the AUPR analysis, the TF–target gene interactions predicted by the GRN for each TF and target gene are ranked based on an edge score computed by each network inference method (Fig. 3A). Next, to determine the accuracy of these predictions, the inferred

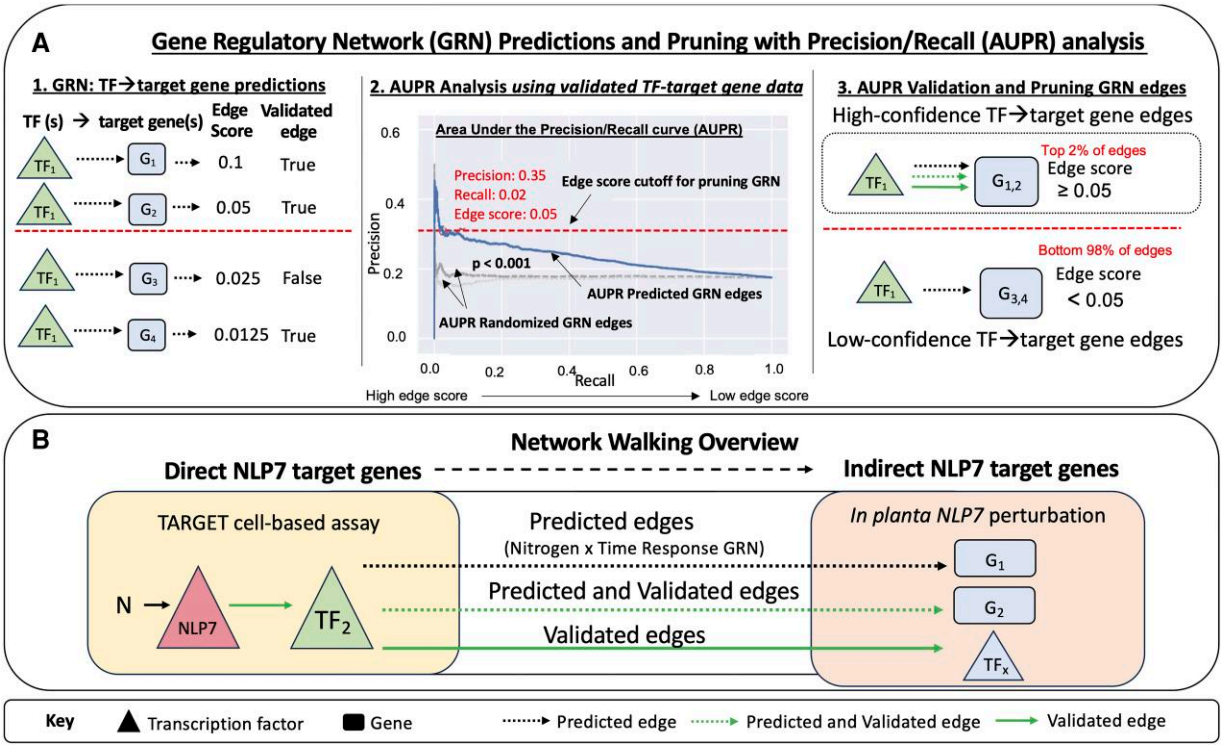

Figure 3. Determining high-confidence GRNs by AUPR and use in network walking. **A)** (A1) The predicted TF–target gene interactions are first ranked according to edge score, and then compared to validated TF–target gene interaction data to calculate precision and recall. (A2) The values are then plotted on the AUPR curve to select a cutoff TF-target edge score. The edges in the predicted network (blue line) were significantly more likely to be true (i.e. validated) edges than when the edge order ranking was randomized (gray lines). The graph is a screenshot from the automated AUPR analysis feature in connectf.org (Brooks et al. 2021). (A3) The edge score cutoff is used to "prune" the network for high-confidence interactions. **B)** Network walking charts a path between direct to indirect target genes of a TF_1 via TF_2s (Brooks et al. 2019, 2021). In this example, the TF NLP7 directly regulates TF_2s as identified with the TARGET cell-based assay (Alvarez et al. 2020). The target genes for each TF_2 can be determined using predicted GRN edges from the NxTime network and/or using validation data from methods such as the TF-TARGET assay and/or TF-target binding by DAP-seq (Table 1). Bottom panel adapted from Brooks et al. (2019).

TF–target gene interactions are compared with validated TF–target gene interactions, for a subset of TFs in the network, as determined by methods such as DAP-seq for TF-target binding interactions in vitro (O'Malley et al. 2016) or direct TF-target gene regulation based on the TARGET TF assay in protoplasts (Table 1, Fig. 3A; Varala et al. 2018; Brooks et al. 2019). This analysis then determines which predicted TF-target edges are supported by experimentally validated data. The validation data are then used to calculate the precision and recall for predicted TF–target gene interactions in the GRN (Schrynemackers et al. 2013). These values are used to produce the AUPR curve, which is then used to select a cutoff edge score for the GRN predictions (Fig. 3A). The selected cutoff edge score from the AUPR curve is used as a threshold to "prune" for high-confidence edge predictions in the GRN. Using the above outlined time-series N response GRNs as examples for AUPR analysis, ConnecTF (connectf.org) is a web-based platform that offers automated AUPR functions where researchers can upload their own networks, select a precision cutoff, and download the high-confidence TF-target gene predictions (Table 1, Fig. 3A; Brooks et al. 2021).

The use of validated TF-target data to prune GRNs for high-confidence TF-target gene predictions is important for identifying key regulatory control points in the absence of comprehensive validated TF-target data. Although there is now experimentally validated TF-target gene binding and regulation data for over 500 Arabidopsis TFs, primarily from DAP-seq (O'Malley et al. 2016), this is still only approximately a quarter of all predicted TFs in Arabidopsis (see data housed in ConnecTF, Brooks et al 2021). This means that for any given signaling pathway studied, it is likely that most of the TFs involved do not have validated target genes. For example, the N-response time-course experiments described above revealed that 326 TFs respond to N-treatment in roots and/or shoots (Varala et al. 2018), but only 95 of those TFs have experimentally validated target genes. Furthermore, for the TFs that lack experimental validation data, it is unclear if the TFs are activators or repressors of their target genes in the network. To address this question, the authors of Hummel et al. (2023) used synthetic biology approaches coupled with this systems biology analysis to determine which TFs in the Varala et al. NxTime GRN are activators or repressors of N-responses by using the reporter genes,

nitrate reductase 1 (NR1) and nitrite reductase 1 (NIR1; Varala et al. 2018; Hummel et al. 2023).

A further complication to interpreting TF signaling pathways is that *in planta* gene expression responses in TF mutants and constitutive TF overexpressors reflect both direct and indirect effects of the TF being perturbed. To determine how the N-responsive TFs work to propagate the N signal in a temporal network, Brooks et al. developed a "network walking" approach to chart a temporal network path for a TF of interest (Fig. 3B, Table 1; Brooks et al. 2019, 2021). Network walking connects direct target genes of a focus TF of interest (i.e. genes identified in plant cells with the TARGET TF assay) with their indirect target genes (i.e. genes identified only *in planta*) via their directly regulated TF_2s (Fig. 3B). In the network walking approach, the TF_2s directly regulated by the focus TF being perturbed are then used to explain the response of the indirect target genes *in planta*. Because many of these TF_2s lack experimental data, the high-confidence TF-target gene predictions from the time-series inferred network are crucial to identify the most important TF_2s that mediate the N-response signaling pathway and guide further studies (Fig. 3B; Brooks et al. 2019, 2021). For example, the network walking approach was used to chart a path between direct and indirect target genes for the N-response TFs, TGA1 (Brooks et al. 2019), CRF4 (Brooks et al. 2019), and NLP7 (Alvarez et al. 2020; Brooks et al. 2021).

Learning nitrogen-dependent gene regulatory networks at a temporal level has helped to unravel how shoots integrate multiple root-derived signals. The fine-scale (i.e. many timepoints close together) time-series N-response data from Varala et al. have been particularly useful for shoot and root network comparisons as gene expression was measured from both organs for the same sets of Arabidopsis plants (Varala et al. 2018). This study found a significant overlap between shoot and root N-responsive genes and TFs, and yet a large set of genes were also specific to each organ (Varala et al. 2018). The timing of expression between the overlapping shoot and root genes often differed, suggesting that the N-responsive signaling networks had some degree of organ specificity. Additionally, a subset of N-responsive TFs displayed organ specificity in their N-responsive target genes using the TARGET assay (Brooks et al. 2019). For example, CRF4 regulated early N-responses specifically in the shoot, whereas LBD37 regulated N-responses specifically in the root (Brooks et al. 2019). Furthermore, these fine-scale time-series N-response data (Varala et al. 2018) were used to identify the causal relationship of N-responsive genes between organs using Granger-causal analysis (Heerah et al. 2021). Using this analysis, Heerah et al. predicted 1,007 root- and shoot-expressed genes that influenced gene expression in the other organ (Heerah et al. 2021). Interestingly, the list of predicted genes included a significant number (384 genes) of causal genes that are known or predicted mobile transcripts (Heerah et al. 2021). These GRN findings show a coordination between root and shoot N-responses that can be used to determine how these responses coordinate physiological outcomes.

The dynamics of nitrogen responses in specific cell types

The plant's ability to sense and respond to the fluctuating N status of the soil is governed by cell type-specific responses (Jia and von Wirén 2020; Liu et al. 2020; Hu et al. 2021). Three studies have examined cell type-specific nitrogen responses in roots by treating GFP-marked cell lines with nitrogen followed by FACS and transcriptomic analysis (Gifford et al. 2008; Walker et al. 2017; Contreras-López et al. 2022). Consistently, these studies found that nitrate responses in the root are largely cell type-specific and highlight the need for routine cell type studies, as whole root studies will miss a significant portion of the plant response to an environmental stimulus such as nitrate.

The most recent study to examine cell type-specific nitrate responses identified 5,231 differentially expressed genes and a rapid transcriptome reprogramming, with 1,572 genes responding early 12 min after nitrate treatment (Contreras-López et al. 2022). Moreover, 42.5% of regulated genes were localized in the endodermis cell type, suggesting that the endodermis might have a role as a regulatory hub for nitrate signaling since it is embedded with the Casparian strip, being a nutritional checkpoint for the vascular system (Palmgren 2018; Contreras-López et al. 2022). Analysis of gene ontology (GO) terms found that nitrate responses initiate in the epidermis and cortex as outermost cell types, followed by innermost cell types in later time points (Fig. 4A), which is in line with nitrate uptake and transport (O'Brien et al. 2016; Contreras-López et al. 2022). The first biological processes to be enriched include "response to carbohydrate stimulus," "glycolysis, "response to reactive oxygen species," "response to lipid," "response to abscisic acid," and "response to nitrate," which are initiated from the epidermis and then move toward inner cells (Fig. 4A). For instance, the nitrate transceptor *NRT1.1/NPF6.3* and *NRT2.1* are rapidly induced by nitrate in the epidermis and then within all cell types at later time points (Fig. 4B; Contreras-López et al. 2022). This is in line with previous studies that identified the cell type specificity of nitrate transporters in the epidermis (Tegeder and Masclaux-Daubresse 2018; Wang et al. 2018; Lhamo and Luan 2021; Muratore et al. 2021). At later times, expression of genes involved in "nitrate assimilation" are enriched from inner cell types toward the epidermis (Fig. 4B), while expression of genes involved in "root system development" are localized in epidermis and endodermis, consistent with their role in lateral root growth and root hair development, showing a localized and transient response to nitrate (Ramakrishna et al. 2019; Liu et al. 2020; Fig. 4B). Furthermore, by integrating the spatiotemporal transcriptomic data with TF-target gene interactions, Contreras-

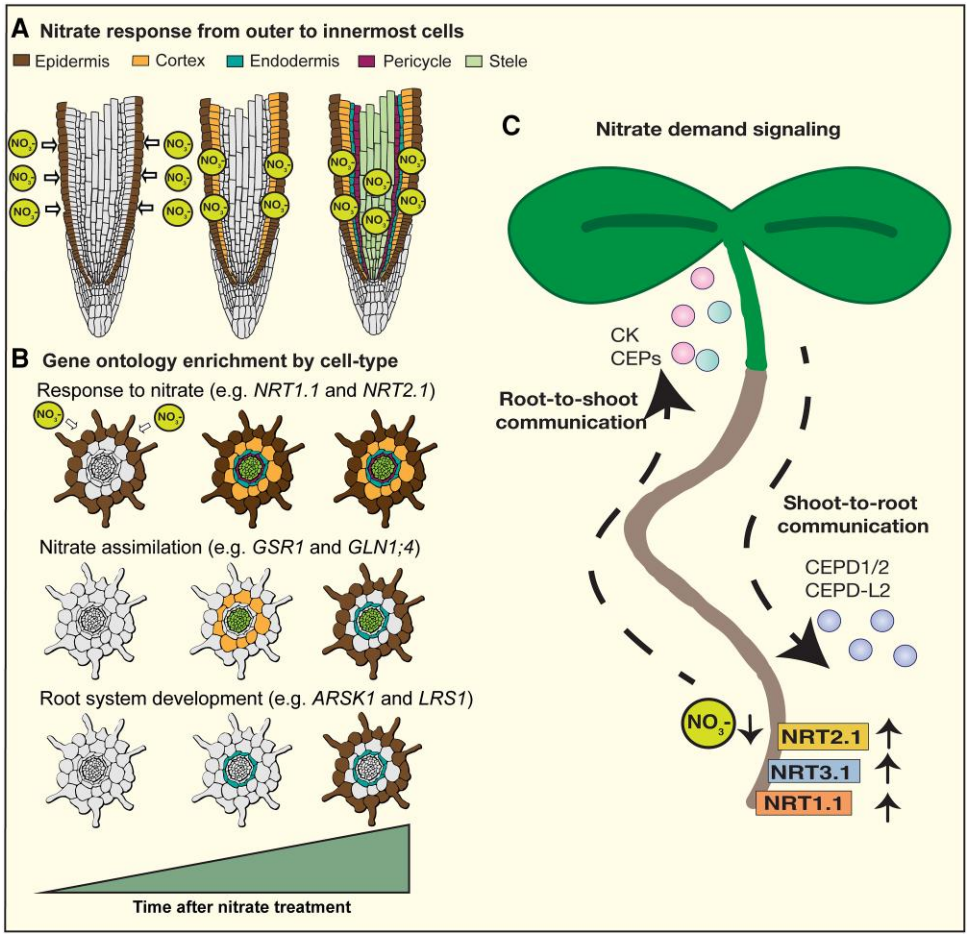

Figure 4. Spatiotemporal responses after nitrate treatments in Arabidopsis root cells are highly dynamic and localized. **A)** During nitrate treatments, the first cell type to respond is epidermis, followed by cortex. Consistent with their outermost location and first layers of nitrate acquisition. At later times of treatment, nitrate responses are present in all major root cell types (Contreras-López et al. 2022). **B)** Transverse view of root cells shows gene ontology (GO) enrichment after nitrate treatments. The first enriched GO term is "response to nitrate," moving from epidermis toward innermost cell types. At later times, "nitrate assimilation" and "root system development" go from inner to outermost cell types. Transcriptomic analysis and GO terms were obtained from sorted root cells by Contreras-López et al. (2022). **C)** Nitrate-demand signaling model. When roots are grown on limited nitrate levels, C-terminally encoded peptides (CEPs) and tZ-type cytokinins (CK) are translocated to the shoot, increasing the expression levels of *CEPD1/2* and *CEPD-L2*. In turn, shoot-derived CEPD1/2 and CEPD-L2 descend back to the root and increase the expression of nitrate transporters *NRT3.1* and *NRT1.1/NPF6.3* and *NRT2.1* to compensate for the lack of nitrate in the soil. This highly coordinated system results in plant growth adaptation according to the changing nutrient levels (Tabata et al. 2014; Ohkubo et al. 2017; Ota et al. 2020).

López et al. found that 62% of TF–target interactions were predicted to occur in the endodermis, being an important cell type for transcriptional regulation. The transcription factors ABF2 and ABF3, previously investigated for their role in ABA-mediated signal transduction, were revealed to be master regulators of nitrogen responses in the endodermis, displaying lateral root growth inhibition in *abf2*, *abf3*, and *abf2/3* plants in response to nitrate (Contreras-López et al. 2022). This phenotype is, in part, due to an altered development of lateral root primordium. Overall, these results highlight the importance of spatiotemporal analysis to uncover how the nitrate signal is dynamically propagated in the root and reveal new molecular mechanisms controlling nitrogen responses in specific cell types, which otherwise would be missed.

Complementary with the identification of cell-type transcriptional responses to nitrate over time, the authors of Chen et al. developed a nitrate biosensor to visualize the spatial and temporal distribution of nitrate in the Arabidopsis root (Chen et al. 2022). To accomplish this, Förster resonance energy transfer (FRET) sensors were developed as a (i) fusion fluorescent protein possessing a sensor domain (FRET acceptor protein) and a (ii) fused FRET donor fluorescent protein. Once the donor protein is excited, energy is transferred to the FRET acceptor protein. When the sensor domain from the acceptor protein interacts with its target molecule, a conformational change occurs. This conformational change, in return, alters the efficiency of energy transferred from the FRET fusion donor protein to the FRET fusion acceptor protein. Hence, by measuring the ratio change, as the

change between the fluorescence intensity of the donor and the acceptor protein, it is possible to report the concentration of the target (Chen et al. 2022). The FRET sensor developed by Chen et al. used the bacterial protein NasR (NitraMeter3.0), which is a soluble receptor protein containing a nitrate and nitrite sensing domain as a FRET acceptor protein fused to a modified Aphrodite (edAFP) protein. On the other hand, a modified cyan fluorescent protein was used as a FRET donor protein (edeCFP; Chen et al. 2022). When Arabidopsis plants expressing the nitrate biosensor were exposed to exogenous nitrate treatments for 5 min, the fluorescence emission ratios increased in the epidermis, cortex, pericycle, and stele cells, with the highest emissions ratio increase in the cortex cells, suggesting a higher nitrate uptake or transport function in this cell type. These results are in line with the reports of nitrate import and signaling in multiple root cell types (Gifford et al. 2008; Walker et al. 2017; Contreras-López et al. 2022). Additionally, the mutant for the nitrate transceptor, *nrt.1.1*/*nfp6.3*, displayed lower emission ratios in all root zones, supporting its role as a major nitrate transporter (Chen et al. 2022). The emissions ratio of the endodermal cell layer remained high when roots were grown under low nitrate conditions and increased slowly compared to other cell types, which coincides with the previous result of the endodermis as a nitrate regulatory hub for plants to respond and adapt to their environment (Chen et al. 2022; Contreras-López et al. 2022).

In addition to cell type-specific nitrate responses in the root regulating plant growth and development, there are also nitrate responses localized in the shoot. For example, NRT1.1/NPF6.3 and NLP7 drive stomatal opening by controlling the entry of nitrate into guard cells, resulting in nitrate-induced depolarization and increased nitrate levels during stomatal opening (Guo et al. 2003; Castaings et al. 2009). Indeed, *nrt1.1*/*npf6.3* and *nlp7* plants are impaired in nitrate content, reducing stomatal opening and water loss, resulting in improved drought tolerance (Guo et al. 2003; Castaings et al. 2009; Araus et al. 2020). However, the role of nitrate signaling mediated by NRT1.1/NPF6.3 and NLP7 in the control of stomatal opening remains to be elucidated.

These studies have shown us how cell type-specific nitrate responses can modulate root and shoot growth, raising the need to implement single-cell level approaches to understand organ-level plasticity. As an in silico approach at single-cell resolution, Lhamo and Luan (2021) profiled putative nitrate transporters in root cell types to understand nitrate uptake and translocation from the soil (Denyer et al. 2019; Ryu et al. 2019; Lhamo and Luan 2021). The dual-affinity transporter *NRT1.1*/*NPF6.3* and high-affinity transporters *NRT2.1*, *NRT2.2*, *NRT2.4*, and *NRT2.5* were highly expressed in epidermis and root cap cells, concomitant with the role of sensing NO_3^- changes in soil (Ho et al. 2009) and uptake function, respectively (Lhamo and Luan 2021). *NPF1.1* and *NPF1.2* were expressed in procambium cells, indicating that they could be participating in loading NO_3^- to phloem and xylem cells in developing roots (Jouannet et al. 2015; Lhamo and Luan 2021). These results indicate that in the future we will be able to generate maps of local and systemic nitrate sensing/signaling from root to shoot and vice versa by using single-cell approaches.

Nitrogen responses across organs: root-to-shoot communication

Because nitrogen availability in the soil changes constantly, plants have developed communication systems that regulate nitrate uptake from the root according to the nutritional state of the soil and the plant. In response to changes in nitrate availability, the nitrate-response targets the fast reallocation of resources to rebalance biomass between below- and above-ground organs, as well as the regulation of physiological activities such as root nitrate transport. For example, heterogeneous nitrate supply leads to greater development, growth, and nitrate transport stimulation in the roots that are locally exposed to nitrate (Ruffel et al. 2011). Such integrated/adapted responses result from a combination of (i) continuous and long-distance exchange of signals through the vascular system and (ii) organ-specific GRNs. Currently, the challenge is to understand how these multiple signals interact and converge toward regulating central physiological and developmental processes in respective organs.

Nitrate-related long-distance signals are just starting to be understood and as of now belong to the following classes of molecules: hormones (e.g. root-to-shoot trans-zeatin cytokinin signal; Poitout et al. 2018), small peptides (e.g. root-to-shoot C-terminally encoded peptides; CEP; Tabata et al. 2014), and microRNAs (so far only functionally characterized in legumes to regulate the nodulation; Gautrat et al. 2021). The coordination of root/shoot communication and growth responses may rely on other types of long-distance signals that remain to be characterized, such as ions or metabolites. However, connections between the known systemic signals and the discovered nitrate-related local and systemic signaling pathways are starting to be proposed, thereby improving our mechanistic understanding of the nitrate signaling network. In addition to the nitrogen-regulated systemic response of root activity, it has been known for a long time that root nitrate supply is a main input for shoot growth. Interestingly, recent findings indicate that once again, multiple signals likely co-exist to properly coordinate the activity of this aerial organ. Several studies have reported that nitrate in the root induces the synthesis of cytokinins (CK) like tZ-type, which are then translocated to the shoot (Osugi et al. 2017; Poitout et al. 2018). Interestingly, this "CK neo-synthesis" in the root has recently been shown to be under the control of the master nitrate signaling regulator NLP7, contributing with CK translocation to the shoot and upregulation of cytokinin response factors (CRF; Abualia et al. 2022). In this manner, CRFs directly induce the expression of auxin transporters (*PINs*), resulting in auxin transport and shoot growth (Abualia et al. 2022).

A very sophisticated example of interaction between systemic signals and nitrate signaling is the case of CEP peptides. CEPs act as root-derived peptides that ascend the nitrate starving signal to the shoot, where the production of a nitrate descending signal induces the expression of root nitrate transporters *NRT2.1*, *NRT3.1* and the transceptor *NRT1.1/NPF6.3* to compensate for the lack of nitrate in the soil (Fig. 4C; Tabata et al. 2014). Years later, Ohkubo et al. identified the nitrate descending signal as a polypeptide named CEP Downstream 1 (CEPD1) and CEPD2 (Fig. 4C; Ohkubo et al. 2017). In 2020, it was also established that CEPD-like 2 (CEPD-L2), together with CEPD1 and CEPD2, contributes to the nitrate demand systemic signaling (Ota et al. 2020). Interaction between tZ-type CK and these peptides also occurs as a response to nitrate starvation. Indeed, the presence of root-synthesized tZ-type CK is necessary in shoots to induce maximal expression levels of shoot-to-root CEPD1/2 and CEPD-L2 peptides, which are mainly induced by the nitrate starvation signal (Fig. 4C; Ota et al. 2020). Moreover, CEPD-L2 positively regulates the expression of high-affinity nitrate transporters and NRT1.5, which loads nitrate into the xylem layer, demonstrating that these peptides have an important role in nitrate uptake and translocation to the shoot under starving conditions (Ota et al. 2020). Altogether, these new findings illustrate that shoots can also perceive and integrate nitrate-related signals, first by receiving a nitrate status signal (e.g. starvation) from the root and responding by sending another signal back to the root (e.g. peptides) to optimize their activity. For future studies, moving on from organ-specific nitrate-responses to cell-specific nitrate-response networks using single-cell data will aid in our understanding of root-shoot-root communication in response to N.

New aspects of N-response in time and space: single-cell analysis

Single-cell RNA-sequencing (scRNA-seq) has emerged as an important tool to better understand dynamic cellular processes such as spatiotemporal gene expression and developmental trajectories from heterogeneous cell populations in a single snapshot (Fig. 5). As outlined above, previous studies that have examined the response of nitrate in specific cell types over time have relied on the use of GFP-marked cell lines followed by bulk RNA-seq (Walker et al. 2017; Contreras-López et al. 2022). The use of single-cell sequencing over GFP-marked lines offers the following advantages: (i) to profile nitrate responses in all the cell types composing an organ, (ii) to track nitrate responses in cell types according to their developmental time in a single snapshot, (iii) to examine nitrate responses in all cell types over time in a single experiment without the need for individual experiments for each GFP-marked cell line, and (iv) to examine the effect of nitrate specific cell types of mutants without the need to develop multiple mutant lines crossed with specific GFP-marked cell lines. Despite these promising advantages, single-cell (sc) RNA-sequencing data are still highly sparse due to cell dropouts. To capture the dynamic cell-specific response to nitrate that regulates multiple plant developmental processes, future studies should examine the cell type-specific responses over multiple time points using single-cell sequencing.

The root has been widely used as a model for scRNA-seq due to its wide characterization, availability of reporter lines and cell type marker genes (Denyer et al. 2019; Jean-Baptiste et al. 2019; Ryu et al. 2019; Zhang et al. 2019; Shahan et al. 2022). Indeed, the root is distributed in different developmental zones, including the less differentiated meristem zone, elongation zone, and most differentiated maturation zone (Fig. 5A). Therefore, we can analyze a gradient of cell differentiation from the root in a single experiment (Fig. 5B). Single-cell transcriptomes offer the unique opportunity to generate computational "developmental trajectories" (Fig. 5C), in which we can order cell type progression from the beginning of cell fate until the final development of mature cell types. Once the developmental trajectory of a specific cell type is established, gene expression throughout development as "pseudotime" can be graphed (Fig. 5D). For example, Denyer et al. observed that during trichoblast development, genes expressed at the beginning of cell fate (e.g. meristematic cells) were enriched for biological processes such as DNA replication, cell proliferation, and ribosomal functions, whereas more differentiated trichoblast cells were enriched in expression of genes controlling unidimensional growth, root hair elongation, and maturation (Fig. 5C; Denyer et al. 2019). Furthermore, pseudotime trajectories coupled with GRNs also contributed to identifying the developmental time-regulated TFs that modulate the expression of target genes in a spatiotemporal manner (Denyer et al. 2019). Using the same trichoblast developmental trajectory, we find that NRT1.1/NPF6.3 expression exhibits a gradual increase as cells become differentiated into mature trichoblasts, which supports the function of nitrate signaling and uptake in this cell type (Fig. 5, C and D). These current single-cell transcriptomic profiles represent plants grown in standard MS media; therefore, expression profiles under changing nitrate conditions need to be examined in future studies.

Multiple studies in Arabidopsis have created root and/or shoot cell atlases from scRNA-seq data with web-based platforms to examine the cell-specific expression profile for genes of interest (Table 2). To develop a comprehensive Arabidopsis root single-cell expression atlas, Shahan et al. analyzed single-cell data for 110,427 Arabidopsis root cells, including root data from previously published single-cell studies, to determine cell-specific gene expression over developmental time in each cell type (Table 2; Denyer et al. 2019; Ryu et al. 2019; Shahan et al. 2022). In addition to single-cell studies, single-nuclei (sn) sequencing has also been used as an alternative method to avoid the limitations of developing protoplasts. To provide a holistic view of the Arabidopsis

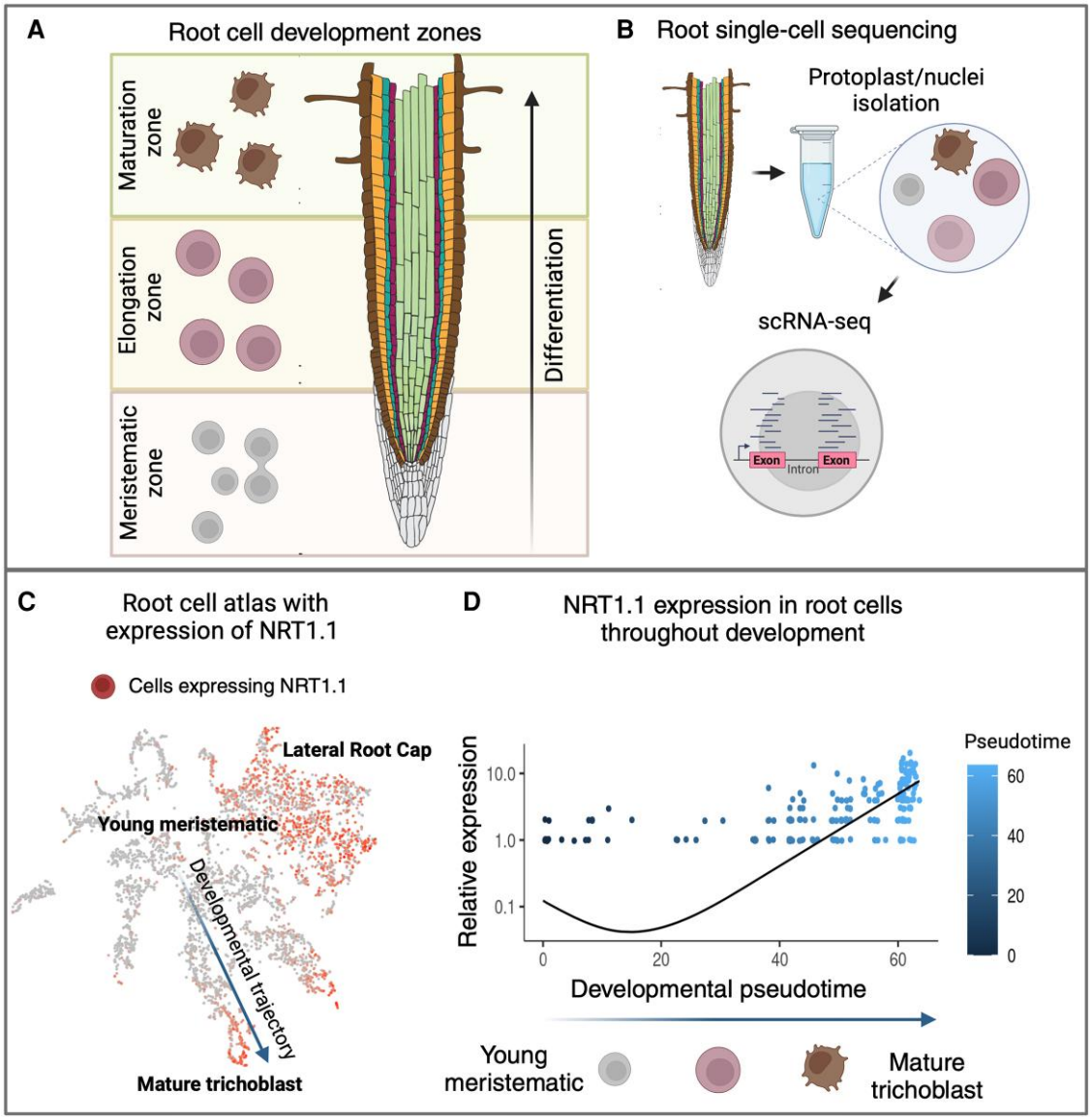

Figure 5. Investigating spatiotemporal gene expression using single-cell RNA sequencing in Arabidopsis thaliana. **A)** Longitudinal view of the root shows different developmental zones from young (meristematic) to mature (maturation zone), which is used as a model for single-cell analysis to construct developmental trajectories in a single experiment (Denyer et al. 2019; Rich-Griffin et al. 2020). **B)** Thousands of protoplasts or nuclei at different developmental stages are used for single-cell library construction (Swift et al. 2022). **C)** Computational analysis of scRNA-data allows the construction of "developmental trajectories" of root cells expressing a gene of interest (red dots), NRT1.1/NPF6.3 using the Plant scRNA-seq Browser with representative screenshots from this tool (Denyer et al. 2019; Ma et al. 2020) (Table 2). **D)** "Pseudotime" expression of *NRT1.1/NPF6.3* from young meristematic cells to mature cells show that NRT1.1 expression is highly expressed in differentiated trichoblast (Denyer et al. 2019; Ma et al. 2020).

transcriptional response over plant development, Lee et al. analyzed single-nuclei data for 801,276 nuclei that represented seed-to-seed development across all major organs during the Arabidopsis life cycle (Lee et al. 2023; Table 2). Furthermore, other researchers have developed web-based interfaces to explore gene expression in smaller-scale single-cell experiments, which include analysis of hormone-treated tissues and/or developmental trajectory analysis (Table 2; Ma et al. 2020; Denyer et al. 2019; Ryu et al. 2019; Wendrich et al. 2020b; Kim et al. 2021; Graeff et al. 2021).

The accessibility to single-cell datasets such as these provides useful tools to form hypotheses on nitrate signaling and explore the spatiotemporal profiles of nitrate-responsive genes in roots/shoots under non-stress cell conditions (Fig. 5). For instance, to understand nitrate uptake and translocation from the soil at single-cell resolution, Lhamo and Luan profiled putative nitrate transporters in root cell types using published single-cell transcriptomic data from Arabidopsis (Denyer et al. 2019; Ryu et al. 2019; Lhamo and Luan 2021). Additionally, we can use the tools outlined in

Table 2 . Web browsers available to visualize cell type-specific gene expression from single cell/nuclei datasets in Arabidopsis

Name	Description	URL	References
Arabi Atlas data	Single-cell gene expression in specific root cell types over developmental time for each cell type	https://phytozome-next.jgi.doe.gov/tools/scrna/	Denyer et al. (2019), Ryu et al. (2019), Goodstein et al. (2012) Shahan et al. (2022)
Arabidopsis Developmental Atlas Viewer	Single nuclei transcriptome data for cell type-specific expression of genes throughout plant development stages	http://arabidopsisdevatlas.salk.edu/	Lee et al. (2023)
Plant sc Atlas	Resource and visualization tools for multiple single cell datasets in roots and shoots	https://bioit3.irc.ugent.be/plant-sc-atlas/	VIB Ghent University, Wendrich et al. (2020b), Graeff et al. (2021), Yang et al. (2021a), Nguyen et al. (2023)
The Plant scRNA-seq Browser	Single cell gene expression in roots and shoot cell types including trichoblast, atrichoblast, and cortex pseudotime	https://www.zmbp-resources.uni-tuebingen.de/timmermans/plant-single-cell-browser/	Ma et al. (2020), Denyer et al. (2019), Kim et al. (2021)

Table 2 to examine the expression profiles of genes of interest for nitrate response, such as the transceptor *NRT1.1/NPF6.3*, in roots (Fig. 5, C and D). We find that *NRT1.1/NPF6.3* is expressed in trichoblast, atrichoblast, and lateral root cap, which is also supported with previous studies (Guo et al. 2003; Yang et al. 2008; Denyer et al. 2019). This is also in line with the function of NRT1.1/NPF6.3 in nitrate uptake and signaling; the location of NRT1.1/NPF6.3 expression in the outer cell layers allows for easy access to nitrate in the soil (Contreras-López et al. 2022).

Building on the "pseudotime," cell type-specific gene expression can be monitored in "real time" by adding time-series response data taken throughout development or in response to environmental stimuli (Swift et al. 2022). For example, Nolan et al. generated brassinosteroid (BR) treated time-series expression data of 210,856 single-cell transcriptomes and used GRNs analysis to identify new TFs that activate cell wall-regulated genes in cortex cells to promote elongation (Nolan et al. 2023). Using this approach, it is possible to determine the dynamic nature of how nitrate regulates developmental processes such as lateral root and root hair development in time and space. We propose future studies that incorporate both single-cell profiling and time-series nitrate response assays. Using time-series nitrogen response single-cell data can create cell type-specific developmental trajectories and GRNs to identify the TFs that regulate cell development during early and late nitrate responses, together with their predicted target genes—thus, enhancing our understanding of how the plant assimilates and responds to nitrate supply to engineer plants with enhanced NUE at the single cell type level.

Following pseudotime and GRN analysis, scRNA-seq of mutant plants will provide new layers of information on nitrate transport and signaling. To date, there are a few studies including single-cell sequencing of mutants to follow changes in developmental trajectories from Arabidopsis roots (Shahan et al. 2022; Nolan et al. 2023). For instance, *SHR* and *SCR* are important TFs for cell identity and differentiation, whereas *BRI1* is an important receptor for BR signaling (Shahan et al. 2022; Nolan et al. 2023). The analysis of *shr* and *scr* single-cell transcriptomes revealed that there is a putative loss of pericycle identity in *shr* mutant and a putative transdifferentiation from cortex to endodermis cells in the scr mutant (Nolan et al. 2023), while Nolan et al. (2023) generated cell-specific *bri1* CRISPR mutants by guiding Cas9 expression into cortex or epidermis cells using as background a *bri1* plant complemented with pBRI1:BRI1:mCitrine. The cortex mutant lines displayed shorter cortex cells in the mature zone but not in the meristem zone, whereas the epidermis mutant lines presented shorter cortex cells in both zones, suggesting that BR signaling is necessary in both the epidermis and cortex to promote cell expansion by modulating cell-wall genes in the elongation zone (Nolan et al. 2023). These results demonstrate that scRNA-seq can address cell identity and signaling pathways in the context of space and time, being also an interesting approach for future N-response studies.

Together with scRNA-seq, single-cell sequencing of accessible chromatin sites will contribute to more resolutive GRNs and will shed light on TFs involved in spatial and temporal regulation during nitrogen responses. Cell type-specific regulation of gene expression is in part modulated by a dynamic chromatin state that responds to development and environment. Changes in the chromatin landscape are reversible and affect the binding of regulatory proteins, such as TFs, controlling gene expression. With single-cell assay for transposase-accessible chromatin (scATAC-seq) as a strategy to uncover putative TF-binding sites during nitrate responses, we can obtain highly resolutive GRNs considering cellular heterogeneity, resulting in cell type-specific accessibility variance and TF-target regulation (Buenrostro et al. 2015). Moreover, together with scRNA-seq approaches, it is possible to correlate chromatin regulation over gene expression across cell types as determined recently by the human ENCODE project and Arabidopsis roots (Buenrostro et al. 2015; Gulko and Siepel 2019; Dorrity et al. 2021; Farmer et al. 2021). Single-cell ATAC-seq in roots has also revealed more accessible and dynamic sites than bulk ATAC-seq, suggesting that a more resolutive network needs to be accompanied by single-cell expression of TFs (Dorrity et al. 2021). Indeed, by integrating snATAC-seq and snRNA-seq from Arabidopsis roots, Farmer et al. led to the identification of

11,858 genes overlapping with chromatin-accessible sites. A high correlation ($P < 10E{-}05$) was obtained when comparing sn/scRNA-seq and snATAC-seq of root marker genes (Farmer et al. 2021). Overall, in the future, both open chromatin sites and gene expression profiles could be used as biological markers for cell type identity and differentiation level under different nitrogen conditions or time series.

Model-to-crop: nitrogen sensing and signaling and its impact on agricultural outcomes

How can we use results from model species such as Arabidopsis to have an impact on NUE in crops? In reference to this question, a follow-up document to the United States White House's Executive Order 14081, a set of goals for "Harnessing Research and Development to Further Societal Goals" was established and includes multiple goals to improve NUE in agricultural practices (The White House Office of Science and Technology Policy 2023). Specifically, the goals in the White House report are highly relevant to current and future NUE studies, which include reducing nitrogen emissions in agriculture by engineering plants with increased nitrogen use efficiency, improving fertilizer practices, and manipulating plant microbiomes to produce plants capable of growing in nutrient-poor land. To accomplish these goals, we review current research that focuses on (i) how we can apply our knowledge of nitrogen in the model system Arabidopsis to crop species and (ii) understanding how microbial communities affect nitrogen uptake and availability in agriculture.

Advances in our base knowledge of nitrogen uptake, transport, and signaling have greatly benefited from studies in the model Arabidopsis; however, we must develop ways to apply this knowledge base to crops. With the substantial progress in omics technology and the availability of bulk- and single-cell RNA-seq datasets accumulated in the past two decades, cross-species comparisons of genetic information have been gaining momentum (Table 3; Katari et al. 2010; Alvarez et al. 2021; Chen et al. 2021; Fu et al. 2022; Xu et al. 2022). The interspecific comparisons, which can be made at the level of genomic sequences, gene expression, co-expression networks, expression atlas, expression quantitative trait loci (eQTL), and gene regulatory networks, open the possibility of knowledge transferring from model organisms to economically important species. For example, in Obertello et al., a cross-species N-regulatory network between rice and Arabidopsis was constructed by (i) identifying the N-responsive differentially expressed genes in one species as the starting point; (ii) constructing the edges using metabolic interactions, protein-protein interactions, and correlated expression; and (iii) only retaining the nodes whose orthologs were also N-responsive in another species (Obertello et al. 2015). The identified rice candidate TFs targeting the conserved N-regulatory network, including *HYPERSENSITIVITY TO LOW PHOSPHATE-ELICITED PRIMARY ROOT SHORTENING 1*/NITRATE-INDUCIBLE GARP-TYPE TRANSCRIPTIONAL REPRESSOR (HRS1/NIGT1; *HRS1 HOMOLOGs* (HHO), OsHHO3 and OsHHO4) and TGA (OsbZIP11) transcription factors were later functionally validated as key regulators of N-deficiency responses *in planta* (Ueda et al. 2020).

In more recent rice studies, nitrogen-dose sensing in the field was examined as the interaction between nitrogen (N) and water (W; Swift et al. 2019). It was discovered that nitrogen dose is sensed as either moles (N-moles), molarity (N/W), or the synergistic interaction with nitrogen and water (N × W; Swift et al. 2019). Notably, it is the interaction between N and W (N/W or N × W) that positively correlates with phenotypic outcomes such as grain yield and water-use efficiency in the field (Swift et al. 2019). These conclusions were determined using linear models that analyzed RNA-seq and phenotypic data from rice exposed to a factorial matrix of N-by-W conditions of different rice varieties in both laboratory and field conditions (Swift et al. 2019). Using this N-by-W expression and phenotype dataset, Shanks et al. 2022 identified the TFs, OsbZIP23, and Oshox22 as regulators of NUE grain yield (NUEg) by developing gene regulatory networks that linked TFs to target genes to field NUEg phenotypes (Swift et al. 2019; Shanks et al. 2022).

Another widely used approach to uncover gene-to-trait relations from the network perspective is by associating co-expression network modules with either functional information or phenotypes. Cross-species weighted gene co-expression network analysis (WGCNA; Langfelder and Horvath 2008) has identified an N-regulatory network conserved between maize and sorghum, despite the difference in genome size and phylogenetic distance between these two species (Du et al. 2020). Notably, *ZmHPP*, a top-ranked TF in regulating the conserved N-regulatory modules, has an Arabidopsis homolog ATNITR2;2 (AT3G47980) that is also a hub gene (nodes with the most edges) in another co-expression network module for nitrogen signaling (Canales et al. 2014). Overall, these results consistently support the importance of conserved N-regulatory networks in regulating the phenotypic response.

Although tools exist to aid in orthology analysis (Table 3; Huerta-Cepas et al. 2019), the cross-species analyses are limited by the ortholog conversion methods, most of which assume the sequence-based orthologs to have similar functions, the so-called ortholog conjecture, which is arguably applicable to all species as orthology inference tends to be more complicated than a straightforward one-to-one relationship due to genome duplication (Gabaldón and Koonin 2013). This phenomenon is especially prominent in plant species that follow a pattern of genome evolution involving polyploidization followed by the loss or partial retention of duplicated genes (Wendel et al. 2016). As a result, plant genomes have highly complex gene families, making the identification of a single ortholog conceptually impossible. Considering that gene expression can serve as a proxy

Table 3 . Web-based platforms for analysis of genomic data across plant species

Name	Description	URL	References
VirtualPlant	Resource that integrates plant genomic data with visualization and analysis tools	http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/	Katari et al. (2010)
EggNOG	Database for orthology relationships, gene evolutionary relationships and functional annotations for multiple species	http://eggnog5.embl.de/#/app/home	Huerta-Cepas et al. (2019)
ConnecTF	Platform that integrates genome-wide studies to develop and validate networks with AUPR analysis	https://connectf.org	Brooks et al. (2021)
ChIP-Hub	Application to explore the plant regulome and epigenome	https://biobigdata.nju.edu.cn/ChIPHub/	Fu et al. (2022)
Plant Single Cell Hub	Single-cell data repository for plant species	http://jinlab.hzau.edu.cn/PsctH/	Xu et al. (2022)
PlantscRNAdb	Database and browser single-cell expression from multiple species including marker gene selection and analysis for specific cell types	http://ibi.zju.edu.cn/plantscrnadb/index.php	Chen et al. (2021)

for gene function, a novel type of approach has been proposed to classify genes based on not only sequence similarity (genes in the same orthogroup) but also expression patterns (Das et al. 2016). Kasianov et al. constructed a pipeline that trained an XGBoost-based machine learning classifier using developmental transcriptome profiles and sequence-based ortholog information and was able to provide functional correspondence between genes from phylogenetically distinct species (Arabidopsis-maize and Arabidopsis-buckwheat; Kasianov et al. 2023). This method has the potential to enable the selection of functionally similar orthologs, which is approximated by the expression patterns, even for species with distinct morphologies. Furthermore, conservative gene function is not always found between species, which is why mutant analysis and validation must be performed.

In Cheng et al. (2021), the authors exploited the evolutionary conservation of the N-responses across species to identify genes of importance to NUE in a model (Arabidopsis) and crop (maize; Cheng et al. 2021). Specifically, this study integrated the sequence-based and expression-based similarity in orthologs with machine learning modeling to infer gene-to-NUE phenotype relations in Arabidopsis and maize. To do this, the N-response differentially expressed genes (N-DEGs) conserved in Arabidopsis and maize, as determined by sequenced-based orthology, were used as gene features (predictors) in the machine learning, gradient boosting-based method XGBoost (Chen and Guestrin 2016). The output of XGBoost gives each gene feature an importance score for how that gene contributes to a trait (i.e. NUE; Cheng et al. 2021). The top-ranked TFs in the XGBoost models that were important in predicting gene-to-NUE relations were functionally validated using the T-DNA loss-of-function mutants in Arabidopsis. Remarkably, a model-to-crop validation was performed using maize *nfya3* mutant whose Arabidopsis ortholog NF-YA6 (AT3G14020) is the top TF in Arabidopsis XGBoost model and displayed enhanced grain NUE compared to wild-type counterparts in the field experiment (Cheng et al. 2021). In addition, the gene regulatory networks were constructed using a random forest-based algorithm GENIE3 to identify the TFs regulating the conserved N-DEGs predictive of NUE. Functional validation using Arabidopsis mutants defective in the TF hubs (nodes with the most edges) displayed higher NUE (Cheng et al. 2021). These results demonstrated the utility of cross-species transcriptome analyses in optimizing machine learning models and constructing GRNs.

Given the importance of developing high-confidence cross-species networks, the ConnecTF platform (connectf.org) was developed for researchers to perform interactive and automated precision/recall analysis (AUPR) on their uploaded networks, as well as to build and visualize networks and compare validated datasets for one or more TFs in Arabidopsis, rice, and maize (Table 3) (Brooks et al. 2021). To facilitate these analyses, the open-source ConnecTF web platform includes the validated TF-target gene data generated using the plant cell-based TARGET system, along with published *in planta* TF perturbation data, ChIP-seq, and DAP-seq data. Examples of how ConnecTF can be used to develop high-confidence networks using AUPR analysis have been published for Arabidopsis (Brooks et al. 2021) and rice (Shanks et al. 2022).

In addition to cross-species networks and machine learning, single-cell RNA-seq data can be used to uncover conserved regulatory programs and key regulators in specific cell types between species (Table 3; Chen et al. 2021; Xu et al. 2022). Cell type-specific N-responses in crops have been examined on plants grown under low N stress conditions using single-cell RNA-sequencing (scRNA-seq). Like Arabidopsis, studies in crops such as maize and rice also show cell type-specific responses to N in the root using single-cell sequencing technology (Wang et al. 2021b; Li et al. 2022). For example, in maize studies, the nitrate assimilation genes such as ZmGS2 (glutamine synthetase 2) and ZmNAR2.1 (high-affinity nitrate transporter) were induced specifically in the epidermis (Li et al. 2022). One limitation of single-cell studies in crop species compared to Arabidopsis is the lack of well-defined cell-specific marker genes derived from cell type-specific studies and transcriptomes. To address this issue, databases that include single-cell studies in multiple plant species offer tools to identify marker genes for specific cell types in multiple crop species (Table 3; Chen et al. 2021; Xu et al. 2022). Additionally, the new PHYTOMap (plant hybridization-based targeted observation of gene expression map) technique was developed as a

fluorescence in situ hybridization method for whole-mount tissue that can be applied to single-cell data for spatial analysis of gene expression and to define marker genes for specific cell types (Nobori et al. 2023). This technique developed in Arabidopsis has the potential to be applied to crops to assist in cluster gene annotation from single-cell studies and marker gene validation without the need to develop time-consuming transgenic reporter lines (Nobori et al. 2023). Furthermore, this method is developing rapidly and can be applied to identify the spatial cell type-specific nitrogen response. How plant host–microbe interactions are affected by nitrogen levels in the soil is another area of nitrogen research that can benefit from single-cell technology as the colonization of microbes is a cell type-specific response (Cole et al. 2021). For example, multiple studies have examined how nodulation in legumes is initiated in the cortex cells (Walker et al. 2017; Mahmud et al. 2020). During nodulation, legumes will recruit microbes that can perform biological nitrogen fixation (BNF), in which microbes use the enzyme nitrogenase to catalyze the conversion of abundant N_2 gas in the atmosphere into ammonia that can be assimilated by the host plant via glutamine synthase to form glutamine (Gautrat et al. 2021). To reduce the reliance on excess synthetic fertilizers, future research is examining how to harness nitrogen fixation by microbes and apply this process to benefit non-legume crops such as maize, rice, and wheat (Mahmud et al. 2020; Wen et al. 2021). One way this can be accomplished is by engineering non-legumes to form nodules with N-fixing bacteria (Mahmud et al. 2020; Wen et al. 2021). Alternatively, crops can be engineered to secrete specific root exudates, which is a mixture of sugars, amino acids, fatty acids, and vitamins, to either recruit beneficial microbes that help improve nitrogen acquisition or to inhibit the colonization of microbes that might compete with nutrient acquisition (Hartman and Tringe 2019). In addition to engineering the crop itself, there is also an effort to use specific microbial inoculants as biofertilizers (Klimasmith and Kent 2022). This can be done either by adding microbes in the soil that will form endosymbiotic relationships with the plant to increase nitrogen fixation or by adding free-living microbes that offer greater nitrogen availability to the plant in the soil (Cole et al. 2021; Klimasmith and Kent 2022).

Nitrogen research in the age of artificial intelligence (AI)

Since Alan Turing first proposed the concept of machines capable of self-learning and self-instruction in 1950, the field of artificial intelligence (AI) has experienced explosive growth. AI, in general, refers to the ability of machines to simulate the intelligence observed in complex organisms.

Increasingly, AI is intersecting with the field of biology. Machine learning and in particular AI, mainly in the form of Deep Neural Net (DNN; Lecun et al. 2015), are transforming the way science is performed. Although traditional model-driven methods still play a valuable role in analyzing biological data, they often lack the capacity to effectively harness vast amounts of available data, including big data, to extract information, forecast data behavior, and comprehend complex data relationships. Particularly over the last decade, we have seen a dramatic increase in the number of large, highly complex datasets being generated from biological experiments, quantifying molecular variables such as gene, protein, and metabolite abundance, microbiome composition, and population-wide genetic variation, to name just a few. Community efforts across research disciplines are regularly generating petabytes of data. The nitrogen signaling field has been very active to use and develop new algorithms to decipher the obvious complexity of the nitrogen signaling pathway even before the rise of the DNN. The nitrogen community has been particularly keen on using several mathematical models to decipher and predict the actual interactions between transcription factors and their target genes or to model solute transport and developmental processes. These approaches ranged from linear models (Gutiérrez et al. 2007; Krouk et al. 2009; Ristova et al. 2016), state-space modeling (Krouk et al. 2010; Brooks et al. 2019; Alvarez et al. 2020), to "ordinary differential equations" (ODEs) embedded in organ and tissue models (Boer et al. 2020; Ötvös et al. 2021). DNNs are very good at image classification and segmentation as they were originally developed for computer vision (Lecun et al. 2015). This is why an important trend is now rising as it relates to the measure of N content on plants from image analysis. Most of the time these approaches use multispectral images to classify the N content of different crops, including maize (Nguyen et al. 2023; Wijewardane et al. 2023), cotton (Xiao et al. 2022), or sorghum (Wijewardane et al. 2023).

Impressive advancements in AI in biology have been made, for example, in precise identification of the three-dimensional structure of biological molecules, such as AlphaFold, a critical task with significant implications for biological research (Jumper et al. 2021; Lin et al. 2023) and now widely used in biology. But the potential for AI to replicate the capacities of living systems, particularly human intelligence, represents a significant achievement and a turning point in how science is performed. AI is now capable of object recognition and decision-making, using cognitive and perceptual abilities akin to those observed in biological systems. A relatively more recent branch of neural networks called "natural language processing" (NLP), originally developed to understand human languages (including translating one language into another), has been applied to biological questions and to published articles or written information and sequence-related data, including DNA or protein sequences. In the context of NLP, self-supervised large language models, such as "generative pre-trained transformer 3" (GPT-3; Brown et al. 2020) or "pathways language model" (PaLM; Chowdhery et al. 2022), have demonstrated impressive abilities to extract meaningful pieces of world knowledge from being exposed to an extremely large quantity of

text (billions of words). A relevant challenge is related to how to access the knowledge encoded by the internal representation of a large AI model. Surprisingly, recent research (Gao et al. 2021) has found that it is possible to steer these large models to output relevant knowledge from a novel target task using just a prompt. Specifically, by using a prompt that provides the model with a human language description or several examples of what one wants them to do, the model can output meaningful knowledge related to a target task. This learning strategy, referred to as contextual prompting, offers a new degree of control to selectively access the knowledge encoded in the internal representations of a large language model. Nevertheless, it remains to be assessed how useful and in which way tools such as NLP models or ChatGPT, trained specifically with scientific literature, will be for scientific research.

AI-based algorithms and programs continue to emerge with diverse applications from basic research to precision farming. Precision farming has the potential to revolutionize various agricultural practices, ranging from soil management and water analysis to accurate modeling of fertilizer requirements, as well as the optimization of pesticides, insecticides, herbicides, yield projections, and overall crop management. These advancements in AI intervention can play a pivotal role in meeting the increasing demands for food from a growing global population. Early prediction and identification of agricultural problems, as well as optimization of production practices, are key areas that can greatly benefit from AI applications. Such approaches not only have the potential to save significant costs but also mitigate environmental impacts, leading to more sustainable agricultural practices.

Conclusions

In this review, we examine how studies of nitrogen sensing and signaling over time and space have begun to uncover the underlying dynamic regulatory networks that mediate changes in plant metabolism and development. We also explore how emerging experimental and computational techniques can be applied to advance nitrogen research. This includes leveraging new experimental approaches such as single-cell sequencing to unravel the relay of nitrogen signaling in specific cell types over time and its effect on crucial processes, such as cell differentiation, to modulate organ development. On the other hand, computational methods such as machine learning and artificial intelligence will augment experimental nitrogen research endeavors to uncover the mechanisms by which plants sense and respond to nitrogen sources in their environment. We also highlight how model-to-crop translational studies can be used for practical gain in enhancing NUE in agriculture.

Author contributions

CS, KR, RG, and GC outlined the review topics, current research areas, and prepared figures and tables. CS, KR, MB, CYC, JMA, SR, GK, RG, and GC contributed to writing review topic sections, editing the paper, and approving the submitted version.

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