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REVIEW PAPER

Nitrogen signalling in *Arabidopsis*: how to obtain insights into a complex signalling network

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Abstract

It is well known that nitrogen (N) and N status can be sensed by plants to regulate their development, physiology, and metabolism. Based on approaches efficiently used for fungi and algae, plant researchers have been trying, but with little success, to elucidate higher plants N signalling for several years. Recently, the use of new strategies such as transcriptomics, comparative reverse genetics, and new forward genetic screens have unravelled some players within the complex plant N signalling network. This review will mainly focus on these recent advances in the molecular knowledge of N sensing in plants such as the dual function of the nitrate transporter CHL1, the roles of the transcription factors LBD37/38/39 and NLP7 or of the CIPK8/23 kinases, as well as the implication of small RNAs, which are at last opening doors for future research in this field.

Key words: *Arabidopsis thaliana*, forward and reverse genetic screens, new technologies, nitrate signalling, transcription factors.

Introduction

Nitrogen (N) is one of the major macronutrients for all living organisms via its incorporation into amino and nucleic acids. Fungi and plants have the capacity to take up inorganic N from their environment, to metabolize it into organic N molecules, and to adjust their development and physiology to both external and internal N concentrations.

Most higher plants use mineral N from the soil, although legumes can fix gaseous N by symbiosis with bacteria. The root system is responsible for this mineral N uptake [mainly nitrate (NO_3^-)] in most cases]. After its transport to the leaves, NO_3^- is reduced to ammonium and incorporated into carbon skeletons originating from photosynthesis in order to produce amino acids. Uptake and transport of NO_3^- are achieved by transmembrane proteins belonging to at least three multigenic families of NO_3^- transporters (NRT1, NRT2, and CLC) (for a review, see Dechorgnat *et al.*, 2011). The reduction of NO_3^- and the synthesis of glutamine are performed by three main enzymes, namely nitrate reductase (NR; two *NIA* genes), nitrite reductase (NIR; *NII* gene), and glutamine synthetase (GS). Both the NO_3^- transport system and the NO_3^- assimilation pathway are under the control of a fine-tuned transcriptional and post-transcriptional feedback by the N status of the plant (for reviews, see Kaiser and Huber, 2001; Meyer and Stitt, 2001; Gojon *et al.*, 2009).

The effect of NO_3^- on plant morphology has been described in barley and tobacco (Drew and Saker, 1975; Scheible *et al.*, 1997). At high concentrations, NO_3^- promotes development of the shoot system whereas NO_3^- depletion enhances the development of the root system. Moreover, the effect of NO_3^- on root morphology has been shown to be independent of its assimilation. Therefore, NO_3^- has been considered not only as a major macronutrient, but as a powerful signalling molecule as well. The NO_3^- signal has both systemic morphological effects, inhibiting the lateral root development when provided at a uniform high concentration, and local morphological effects, enhancing local lateral root elongation when provided in a restricted area of the root (Zhang and Forde, 1999).

Extensive transcriptome studies have characterized the primary NO_3^- signal response (Wang *et al.*, 2000, 2003, 2004;

Scheible *et al.*, 2004; Orsel *et al.*, 2005; Gutiérrez *et al.*, 2007). NO_3^- not only rapidly induces genes responsible for its transport (e.g. *NRT1.1* and *NRT2.1*) and assimilation (e.g. *NIA1*, *NIA2*, and *NII*), but also triggers changes in expression of ~1000 NO_3^--responsive genes in *Arabidopsis*. Processes such as the biosynthesis of amino and nucleic acids, transcription and RNA processing, ribosome and hormone biosynthesis, N assimilation, reductant supply, and trehalose metabolism respond within 20 min to 3 h of NO_3^- induction. More recently, several studies differentiated direct molecular responses to NO_3^- from general responses to N supply, using NR null mutants (Wang *et al.*, 2004) and mutants of the NO_3^- sensor NRT1.1/CHL1 (Muños *et al.*, 2004; Ho *et al.*, 2009; Wang *et al.*, 2009). Taken together, these data suggest that NO_3^- is rapidly and specifically sensed by plant cells and that an NO_3^- signalling pathway adjusts the expression of a large set of genes to adapt cell and organ metabolism and growth to N availability.

Moreover, it is known that the NO_3^--inducible expression of *NIA* and *NII* genes occurs in the presence of a protein synthesis inhibitor, suggesting that the components for NO_3^- signalling and NO_3^--responsive transcription pre-exist in plant cells independently of the presence or the absence of environmental NO_3^- (Gowri *et al.*, 1992).

Since the discovery of NO_3^- as a signalling molecule and with the development of powerful functional genomic tools, researchers have been trying to elucidate the N signalling pathway in plants. The first attempts, based on approaches used successfully in fungi and algae, did not allow identification of N regulators in higher plants. Nevertheless, making use of different strategies has uncovered an increasing number of genes and proteins involved in N signalling.

Forward genetic screens in plants

Regulatory proteins of NO_3^- metabolism have been identified in fungi and in the unicellular alga *Chlamydomonas reinhardtii* using a clever forward genetic screen based on the resistance to chlorate, a toxic analogue of NO_3^-. Fungal mutants able to grow on chlorate appeared to be impaired either in enzymes or coenzymes of the NO_3^- reduction pathway, or in GATA transcription factors (TFs; NIT2 and NIT4 from *Neurospora crassa* and their orthologues AreA and NirA from *Aspergillus nidulans*) directly regulating the transcription of the NR gene (Crawford and Arst, 1993; Marzluf, 1997; Feng and Marzluf, 1998). In a similar way, the NIT2 TF has been isolated from a chlorate resistance screen in *Chlamydomonas* and shown also to regulate transcription of the NR gene (Schnell and Lefebvre, 1993; Camargo *et al.*, 2007). It has been demonstrated that these TFs are responsive to the NO_3^- signal. Indeed, NO_3^- can regulate their activity either by modifying their subcellular localization as for the NirA protein or by enhancing their transcription as for the *NIT2* gene (Berger *et al.*, 2006; Camargo *et al.*, 2007).

In higher plants, several chlorate resistance screens have been performed to identify regulatory genes of NO_3^- metabolism (Braaksma and Feenstra, 1973; Gabard *et al.*, 1987; Wilkinson and Crawford, 1991; Lin and Cheng, 1997). Disappointingly, only mutants affected in NO_3^- transport (*nrt1.1*/*chl1*) and reduction have been isolated in this way. Moreover, proteins homologous to the GATA factors NIT2 and AreA were identified in tobacco, but their involvement in the regulation of NO_3^- assimilation was unclear (Daniel-Vedele and Caboche, 1993).

Forward genetic screens based on reporter gene expression driven by N-regulated promoters have also been performed in plants to search for regulators of the NO_3^- assimilation pathway. Ethyl methane sulphonate (EMS) mutagenesis of plants carrying the tobacco *NII* gene promoter fused to the β-glucuronidase (GUS) coding sequence (Leydecker *et al.*, 2000) was carried out. A class of mutants altered for NO_3^- induction of the reporter gene has been isolated. However, they appeared to be mutated for enzymes involved in NR molybdenum cofactor biosynthesis. Again no new regulatory proteins have been identified in this way and no regulatory moonlighting activity has been shown until now for the enzymes involved in molybdenum cofactor biosynthesis. The tobacco *NII* gene promoter was also fused to the coding region of the luciferase (Luc) reporter gene, which allows a direct visualization and recovery of deregulated mutants (T. Matakiadis *et al.*, unpublished results). Several mutants showing an altered expression of the reporter gene were isolated in this way in mutated *Arabidopsis* seedlings.

More recently Girin *et al.* (2010) followed a similar approach using the *AtNRT2.1* promoter fused to the coding sequence of Luc as the reporter gene. The *AtNRT2.1* promoter is repressed by a feedback mechanism when internal N is high. EMS mutants defective in this repression have been isolated.

Interestingly, a recent new forward genetic screen, based on a synthetic NO_3^--inducible promoter fused to the yellow fluorescent protein (YFP), has allowed the identification of six mutants affected in NO_3^- induction of the YFP marker gene. Identification of two of the mutated genes revealed a nonsense mutation, in either the TF *NLP7* (see below) or the NO_3^- sensor *NRT1.1* genes (Wang *et al.*, 2009). Mutation of the *NRT1.1* locus (*nrg1*) reduced NO_3^- induction of three NO_3^--responsive genes (*NIA1*, *NII*, and *NRT2.1*), and in total NO_3^- regulation of 113 genes was affected in the *nrg1* mutant, including genes involved in NO_3^- assimilation, energy metabolism, and the pentose-phosphate pathway. These results are in agreement with recent data showing that a mutated version of NRT1.1 protein impaired for NO_3^- transport is still able to transduce the NO_3^- signal, confirming the signalling function of NRT1.1 (Ho *et al.*, 2009).

The seemingly disappointing findings from the early years of NO_3^- signalling research, when chlorate resistance screens yielded either *nrt1.1* or NR mutants, might have finally been more successful than previously thought. Indeed they could have been suggesting that NRT1.1/CHL1 was not only a NO_3^- transporter, but, since mutations thereof were clearly repressing NO_3^- assimilation and NR activity, the major regulator of NO_3^- signalling. This evidence was at this time overlooked perhaps because of the conceptual framework imposed by the fungal model of NO_3^- assimilation regulation (Marzluf, 1997).

More proteins involved in N uptake and assimilation might turn out to be dual-activity proteins. Further approaches are needed to obtain evidence for such roles.

Identification of N-responsive *cis*-elements

Better knowledge of the promoter structure responsible for transcriptional regulation by N would allow for prediction of binding sites of TFs and would be the prerequisite for one-hybrid screens in yeast.

Promoters of NO_3^--inducible genes, including *NRT2.1*, *NII*, and NR-encoding genes, have been previously analysed. The promoters of the *Arabidopsis* NR-encoding genes (*NIA1* and *NIA2*) conferred NO_3^--inducible expression to reporter genes (Lin *et al.*, 1994), and further analysis of these promoters via linker scanning mutagenesis led to the definition of a NO_3^--responsive *cis*-element (NRE) (Hwang *et al.*, 1997). Analyses of 5′ deletions of the tobacco *Nii1* promoter fused to the Luc gene demonstrate that NO_3^- regulation is maintained in deletion mutants that retain 0.2 kb of the *Nii1* promoter (Dorbe *et al.*, 1998). Very recently a 43 bp sequence of the *Arabidopsis Nii* promoter has been identified in *Arabidopsis* as a *cis*-element that is both necessary and sufficient for NO_3^--responsive transcription (Konishi and Yanagisawa, 2010). Similarly a 150 bp region within the *Arabidopsis NRT2.1* promoter was found to be sufficient to mediate induction by NO_3^- and repression by N metabolites (Girin *et al.*, 2007). This suggested that this sequence was a closely linked and direct target of regulation by the N status. However, the involvement of this sequence in the activation of transcription by NO_3^- due to binding of specific TFs has not yet been addressed.

NO_3^--inducible genes: plenty of candidates

The initial failure of the chlorate and reporter gene screens fostered the exploration of alternative ways, such as N-inducible genes, to identify N signalling proteins.

Two NO_3^--inducible protein kinases belonging to the family of CBL-interacting protein kinases (CIPK8 and CIPK23) have been identified following transcriptome studies in the *nrt1.1* (*chl1*) mutant. These genes, which are induced by NO_3^- in the wild type, are down-regulated in *nrt1.1* (*chl1*) mutants. Analyses of CIPK8 and CIPK23 knock-out mutants have shown that both participate in the early NO_3^- response phases. However, CIPK8 is a positive regulator for the low-affinity phase of the response while CIPK23 is a negative regulator of the high-affinity phase. At low NO_3^- concentrations CIPK23 phosphorylates CHL1 at Thr101, which turns down the high affinity response. The targets of CIPK8 are as yet unknown (Ho *et al.*, 2009; Hu *et al.*, 2009).

The expression of many TFs is regulated by NO_3^- (Wang *et al.*, 2001, 2003; Scheible *et al.*, 2004). However, to date, only two such NO_3^--regulated TFs have been shown to be involved in NO_3^- signalling.

ANR1, an A*rabidopsis* MADS box TF, was initially isolated in a screen for NO_3^--inducible genes in roots. Down-regulation of the *ANR1* gene affects the plasticity of the root system which is no longer able to respond to local application of NO_3^- (Zhang and Forde, 1998). ANR1 was the first regulatory protein described in the N signalling network. Very recently three NO_3^--inducible TFs from the LBD [lateral organs boundaries (LOB) domain] family were identified among NO_3^--induced TFs as signalling candidates. They have been shown to be negative regulators of N availability signals as well as of anthocyanin biosynthesis (Rubin *et al.*, 2009). Expression of *LDB 37/38/39* is up-regulated by NO_3^- and to a lesser extent by ammonium and glutamine. *lbd37*, *lbd38*, or *lbd39* mutants accumulate anthocyanins when grown in N-sufficient conditions and show constitutive expression of anthocyanin biosynthetic genes. The *LBD* genes also repress many other known N-responsive genes, including key genes required for NO_3^- uptake and assimilation.

To date, the effect of NO_3^- supply to N-starved plants has been studied as early as 20 min after resupply of NO_3^-. Recent studies from our laboratory showed that expression levels of many genes changed as early as 5 min after resupply of 3 mM NO_3^- to 14-day-old seedlings which have been starved for N during 3 d (Fig. 1). A total of 49, 349, and 207 genes which were significantly up-regulated were detected when comparing 0–5, 5–10, and 10–20 min of NO_3^- induction, respectively (unpublished results). These comparisons allow transient expression changes to be easily determined during the 20 min interval. Interestingly, induction only occurred for three genes during the entire 20 min. Many of the genes induced between 0 and 5 min or 5 and 10 min are down-regulated later on and would not be detectable in samples taken at 20 min of induction. Genes which are induced at 20 min (compared with T0) overlap satisfactorily with those described before (Wang *et al.*, 2003). However, several genes whose expression varied only during the very early NO_3^- response have been identified and their role in NO_3^- signalling needs to be further studied. While awaiting further functional studies of NO_3^--controlled putative regulatory genes, other approaches have been successful in identifying molecular players in N signalling (see the following paragraphs).

Phylogenic and comparative approaches: what can we learn from others?

Even though studying N-inducible genes is a powerful tool to identify N signalling proteins, some key players might not be controlled at the transcript level by N. For example, this is the case for the TF NirA from *A. nidulans* which activates NR expression upon NO_3^- induction. *NirA* transcript accumulation is NO_3^- independent, but its translocation to the nucleus is triggered by NO_3^- (Berger *et al.*, 2006). Other approaches have been developed in plants with the aim of obtaining constitutively expressed regulators. One of them is a phylogenic and comparative approach aiming to find plant homologues of known N regulators from different species or from different plant N-regulated processes.

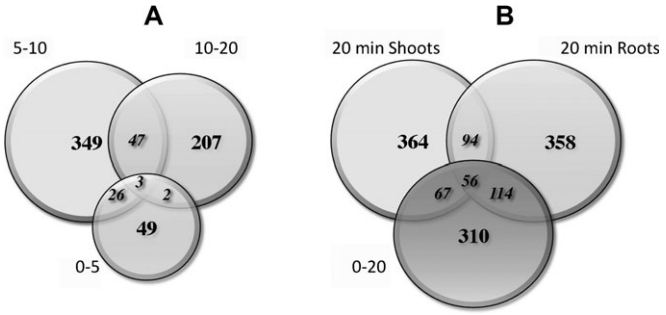

Fig. 1. Transcriptome analysis of short-term nitrate regulation in *Arabidopsis*. *Arabidopsis* seedlings were grown in liquid culture for 10 d in N-replete conditions and then starved of N for 3 d. After 5, 10, or 20 min of nitrate (3 mM) induction, plants were harvested, total RNA extracted, and transcriptome variations were analysed on CATMA arrays. No KCl control was included, given the fact that addition of chloride could also influence gene expression. (A) The total number of statistically significant up-regulated genes is given for each time point comparison. The number of overlapping genes is indicated in each intersection. (B) The total number of statistically significant up-regulated genes after 20 min of nitrate induction (bottom) is compared with the total number of statistically significant up-regulated genes in the experiments performed by Wang *et al.* (2003; top). The same cut-off ratio of 2 was used to select for differentially up-regulated genes. In the latter experiments, differentially expressed genes were detected after 20 min of nitrate induction in either *Arabidopsis* roots or shoots using Affymetrix arrays.

This approach was already undertaken ~20 years ago with the aim of identifying plant homologues of the *Neurospora crassa NIT2* gene, which is the major N regulatory gene in this fungus. The NIT2 protein contains a single zinc finger motif and this DNA-binding domain recognizes the promoter region of *N. crassa* N-related genes as well as fragments derived from the tomato *NIA* gene promoter (Jarai *et al.*, 1992). An NIT2-like protein (named NTL1 for nit-2-like) was isolated from *Nicotiana plumbaginifolia* (Daniel-Vedele and Caboche, 1993). In *Arabidopsis* an NO_3^--inducible member of the GATA TF family has been shown to impact chlorophyll synthesis and glucose sensitivity (Bi *et al.*, 2005), but no clear function for GATA TFs in NO_3^- regulation of gene expression has been described yet.

Only recently a comparative approach has been successfully used to identify the TF NLP7 (Nin-Like Protein 7). NLP7 is one of the nine members of the *Arabidopsis* NIN-like family of RWP-RK TFs that are homologous to both the *Lotus* NIN (Nodule Inception) protein, involved in early steps of the N-regulated symbiosis between rhizobia and legume roots (Schauser *et al.*, 1999), and the NIT2 protein which regulates NR expression in *Chlamydomonas* (Camargo *et al.*, 2007). No molecular function has yet been described for NIN proteins from legumes. In contrast, NIT2 has been shown to bind to the promoter of the *NIA* gene in *Chlamydomonas*. It has been shown that the NLP7 protein is involved in NO_3^- and N starvation responses as a positive regulator of NO_3^--inducible genes and NO_3^--induced stomatal opening. Conversely, NLP7 was found to be a negative regulator of N starvation-inducible genes (Castaings *et al.*, 2009). *nlp7* mutants showed a reduced NO_3^- induction of the NO_3^--regulated genes *NIA1*, *NIA2*, *NRT2.1*, and *NRT2.2*. In addition, these mutants display a constitutive N starvation phenotype, possibly due to an impaired N signalling. The NLP7 protein is located in the nucleus of many tissues involved in N transport (e.g. root hairs, emerging lateral roots, and vascular tissues of stems). In addition, NLP7 is expressed in stomata, which is in agreement with its role in controlling stomatal opening. Recent data from our laboratory showed transactivation of the *NRT2.1* promoter by NLP7 when co-transfecting *Arabidopsis* protoplasts (unpublished data).

Taken together, N regulation in fungi and higher plants seems to involve different regulatory proteins. The unicellular alga *Chlamydomonas* might be a better model when searching for candidate genes. However, plants, in contrast to algae and fungi, are multicellular organisms and regulation circuits might be specific for different cell types. Cell-specific approaches should thus be used to identify new N regulators in higher plants.

The promise of new technologies and systems biology

New technologies such as cellular transcript profiling and next-generation sequencing are opening doors for dissecting and understanding the N signalling network. Indeed, such techniques have led to the identification of microRNAs (miRNAs) involved in the N regulatory pathway, adding another level of complexity to N signalling. Cellular transcript profiling of five *Arabidopsis* root cell types in response to NO_3^- uncovered highly localized regulation which was largely hidden from previous global transcriptomic analyses. Such cell-specific regulation includes miR167 and its target ARF8, an auxin-response factor. This transcriptional circuit controls the plasticity of the pericycle cells that gives rise to the emergence of lateral root primordia in response to N (Gifford *et al.*, 2007). Moreover, 454 sequencing technology identified miR393 and one of its targets, the auxin receptor AFB3, as molecular players mediating NO_3^- regulation of primary and lateral root growth (Vidal *et al.*, 2010). More links between miRNA regulation and responses to NO_3^- are waiting to be deciphered. Pant *et al.* (2009) showed by quantitative PCR that at least two other miRNAs (miR169 and miR398) were regulated by NO_3^- and/or NO_3^- starvation, but no information on cell type-specific expression is available yet. Further analyses of cell type-specific regulatory networks are needed. For example, it turned out that *NLP7* is expressed close to vascular tissue, which raises the question of local and systemic N signalling and the dissection of these signalling pathways when using whole organs.

The use of systems biology has already allowed the identification of two master regulators of the organic N response. Network analysis highlighted a high degree of connectivity for two TFs, CCA1 (circadian clock-associated 1)

and GLK1 (golden-2-like transcription factor), and their biological role was confirmed in overexpressing lines or by promoter binding studies (Gutiérrez *et al.*, 2008). No such detailed analysis has yet been performed for the early events in NO_3^- induction.

However, systems biology has already allowed the connection of the N signalling networks to other nutrient or hormonal pathways (Krouk *et al.*, 2009; Nero *et al.*, 2009). Interference by other nutrients, such as carbon, potassium, and sulphur, with the NO_3^- regulatory pathway has been studied at a global level (Koprivova *et al.*, 2000; Thum *et al.*, 2008; Armengaud *et al.*, 2009), and the newly discovered players such as LBD37/38/39, CIPK23, miR167, and miR393 highlight the existing interactions between several metabolic or signalling pathways. Indeed LBD37/38/39 connect N and secondary metabolism, CIPK23 is also involved in potassium signalling, and the integration of N and hormone signalling can result from miRNA regulation of gene expression. Furthermore, the well-known NRT1.1 NO_3^- transporter was shown recently also to facilitate auxin transport (Krouk *et al.*, 2010), which provides an exciting explanation for the cross-talk between NO_3^- availability in the soil and the control of root growth and development.

In this review the main recent findings on the molecular players in N signalling have been described. Early forward screens based on chlorate resistance, which only identified proteins with enzymatic or transport activities, have to be revisited after the recent discovery that the NO_3^- transporter NRT1.1/CHL1 has a second role in regulation. Global transcriptomic analyses led to the identification of four TFs (ANR1, LBD37/38/39) and two protein kinases (CIPK8 and CIPK23) which are regulated at the transcriptional level by NO_3^- and are involved in N signalling. Despite the high number of NO_3^--regulated genes, this is rather a low yield, but very short-term NO_3^--regulated genes still need to be characterized. Candidate gene approaches based on

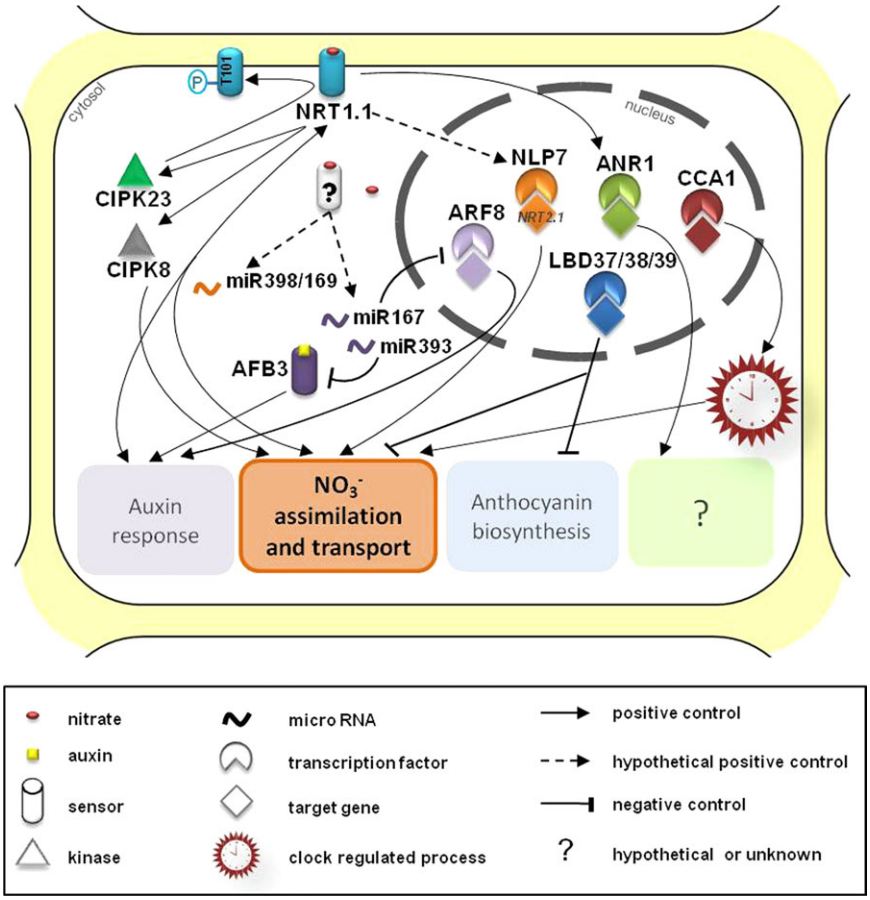

Fig. 2. Molecular players in N signalling and regulation. Schematic representation of players involved in N signalling. For simplicity, all players are presented in the same cell, which might not be the case in a plant. The dual-affinity nitrate transport NRT1.1 has a double role for transport and signalling of nitrate. The CBL-interacting protein kinase CIPK23 regulates the affinity of NRT1.1 for nitrate. CIPK8 is involved in the nitrate-regulated mRNA accumulation of several nitrate-regulated genes. The putative transcription factor (TF) NLP7 is also necessary for full nitrate induction of gene expression. One direct target promoter seems to be the promoter of NRT2.1, a high affinity nitrate transporter. The LOB domain-containing TFs LDB37/38/39 are negative regulators of nitrate-mediated gene expression and they are involved in the regulation of anthocyanin synthesis. ANR1, another TF, regulates lateral root growth in response to nitrate. Regulation by the circadian clock pathway takes place via CCA1, a TF regulated by reduced N compounds such as glutamine. Regulation by microRNAs (miR393 and miR167) connects auxin to nitrate signalling via the auxin-responsive TF ARF8 and the auxin-binding protein ABF3. Further miRNAs are regulated by the N status of the plant (miR169 and miR398) and might play regulatory roles.

homology to fungal N regulators have not been successful. One new TF (NLP7) involved in N signalling has been identified by homology to a *Chlamydomonas* regulatory gene. However, differences in N regulation might be expected between uni- and multicellular organisms, such as higher plants. Cell-specific transcriptome profiling successfully identified new regulatory circuits, and next-generation sequencing revealed the importance of miRNA for N regulation. Furthermore, systems biology connected N signalling to other nutrient or hormonal pathways.

The N signalling network has gained new levels of complexity during very recent years (Fig. 2) and is as yet far from understood. Indeed many regulatory elements are probably still missing in the NO_3^- response pathway, and the connections which exist between this signalling pathway and others such as those responding to nutrients, stresses, or hormones remain to be established. In the future, global approaches will probably help to assemble together the individual N signalling elements discovered using different methods including cell-specific techniques and to build a coherent and comprehensive plant N signalling network.

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