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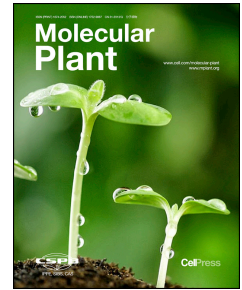
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**Nitrate transport, sensing and responses in plants.**

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## Abstract

Nitrogen (N) is an essential macronutrient that impacts plant growth and development. N is an important component of chlorophyll, amino acids, nucleic acids and secondary metabolites. Nitrate is one of the most abundant N sources in the soil. Because nitrate and other N nutrients are often limiting, plants have developed sophisticated mechanism to ensure adequate supply of the nutrient in a variable environment. Nitrate is absorbed in the root and mobilized to other organs by nitrate transporters. Nitrate sensing activates signaling pathways that impinge upon molecular, metabolic, physiological and developmental responses locally and at the whole plant level. With the advent of genomics technologies and genetic tools, important advances in our understanding of nitrate and other N nutrient responses have been achieved in the past decade. Furthermore, techniques that take advantage of natural polymorphisms present in divergent individuals from a single species have been essential to uncover new components. However, there are still gaps in our understanding of how nitrate signaling impacts biological processes in plants. Moreover, we still lack an integrated view of how all the regulatory factors identified interact or cross-talk to orchestrate the myriad N responses plants typically exhibit. In this review, we provide an updated view of mechanisms by which nitrate is sensed and transported throughout the plant. We discuss signaling components and how nitrate sensing crosstalks with hormonal pathways for developmental responses, locally and globally in the plant. Understanding how nitrate impacts on plant metabolism, physiology and growth and development in plants is key to improve crops for sustainable agriculture.

## INTRODUCTION

Nitrogen (N) is an essential macronutrient and its availability in the soil has a critical role in plant growth and development, and crop yield (Hirel et al., 2007; Krapp et al., 2014; Ruffel et al., 2014; Vidal et al., 2014b; Wang et al., 2012b). Nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) are preferred N forms used by plants, but are in short supply in most ecosystems as well as in agricultural lands. Even though N is not the only essential nutrient, it must come from outside the plant-soil system since unlike other elements it cannot be released from rocks into the soil solution. In agricultural systems, high-yield crop production removes N from the soil and relies heavily on application of large quantities of nitrogenous fertilizers for sustained productivity over time. Unfortunately, a large fraction of the N deposited in fields is not directly absorbed by plants and is lost by leaching (Hirel et al., 2011). Despite significant efforts by the scientific community, N use efficiency for crops has not substantially improved over the last fifty years (Cassman et al., 2002). Beyond the economic costs caused by these large quantities of fertilizers, the high levels of N used in agriculture causes an array of environmental problems (Galloway et al., 2008; Hirel et al., 2007). For example, increased N fertilization can cause eutrophication of terrestrial and aquatic systems, together with global acidification and stratospheric ozone loss (Gruber and Galloway, 2008). As a result of these detrimental environmental consequences, and because of its significant cost for agriculture, understanding how plants sense, uptake, use and respond to N nutrient/metabolites is critical. Unraveling the molecular mechanisms implicated in these processes is the first step to develop biotechnological strategies to improve N use efficiency for sustainable agriculture.

$\text{NO}_3^-$  is one of the most abundant sources of N in natural as well as agricultural systems (von Wiren et al., 2000).  $\text{NO}_3^-$  uptake, transport, and responses have been a major focus of research. In addition to its role as a nutrient,  $\text{NO}_3^-$  can act as a signaling molecule that modulates gene expression and a wide range of processes including plant growth, root system architecture (Alvarez et al., 2012; Krouk et al., 2010a; Vidal and Gutierrez, 2008), leaf development (Rahayu et al., 2005), seed dormancy (Alboresi et al., 2005), and flowering time (Marin et al., 2011).

In this review, we will discuss important milestones in physiological, metabolic and signaling aspects of  $\text{NO}_3^-$  in plants, with a focus on *Arabidopsis* research. We will provide an updated view of  $\text{NO}_3^-$  responses and its local and systemic regulation to integrate metabolic and developmental changes. In addition, we will highlight the most recently characterized molecular components involved in N sensing, signaling, and downstream physiological processes. Finally, we will focus on the importance of genome-wide associated studies in the identification of novel components of  $\text{NO}_3^-$  signaling pathway. There are many excellent recent reviews that the reader may refer to for details on specific topics of interest (Alvarez et al., 2012; Castaings et al., 2011; Giehl and Wirén, 2014; Gojon et al., 2011; Gutierrez, 2012; Krapp, 2015; Krouk et al., 2011; Li et al., 2014; Wang et al., 2012b).

## **NITRATE TRANSPORT**

### **Physiological characterization of $\text{NO}_3^-$ transport systems**

Long before molecular identification of the transporters themselves, the conceptual framework of  $\text{NO}_3^-$  transport in plants arose from physiological studies conducted during the 70's and 80's, and predominantly focused on  $\text{NO}_3^-$  uptake by the young cereal seedling as a model (Hanson, 1978; Morgan et al., 1973). The use of tracers demonstrated that the root uptake of  $\text{NO}_3^-$  is actually the balance between two concomitant opposite fluxes, influx and efflux, likely mediated by different carrier proteins (Morgan et al., 1973). The physiological significance of root  $\text{NO}_3^-$  efflux remains unclear, but it was hypothesized that efflux transporters play a key role in other processes than root uptake, such as secretion into the xylem (Hanson, 1978). Kinetics studies of  $\text{NO}_3^-$  influx as a function of external concentration further revealed that it has a bi-phasic pattern, with a saturable component in the low concentration range (e.g. <0.2-0.5 mM), and a linear one at higher concentrations (Siddiqi et al., 1990). This general observation (in many species and under different environmental conditions) suggested the existence of two separate classes of influx transporters, corresponding to high-affinity transport systems (HATS) and low-affinity transport systems (LATS), respectively (Crawford and Glass, 1998). Furthermore, the regulatory pattern of root  $\text{NO}_3^-$  uptake unraveled additional levels of complexity in the composition of root transport systems. First, the accelerated rate of root  $\text{NO}_3^-$  uptake following first supply of  $\text{NO}_3^-$  was shown

to require *de novo* protein synthesis, indicating the likely induction of novel transporters by  $\text{NO}_3^-$  itself (Hole et al., 1990; Jackson et al., 1973). As this was more obvious for the high-affinity transport systems, this yielded to the proposal that the HATS actually comprise both inducible and constitutive systems (iHATS and cHATS, respectively, (Crawford and Glass, 1998). Second, root  $\text{NO}_3^-$  influx is strongly up-regulated by N-limitation or N-starvation, and on the contrary, down-regulated by high N provision (Lee, 1993). This was interpreted as evidence for a feedback regulation of root  $\text{NO}_3^-$  transporters by the plant's N status (Imsande and Touraine, 1994). Third, root  $\text{NO}_3^-$  uptake is dependent upon photosynthesis, and displays marked diurnal rhythms attributed to a positive regulation by shoot-to-root transport of sugars (Delhon et al., 1995). Because the previously described controls differentially affect influx and efflux, HATS and LATS, a major hypothesis emerging from these physiological studies stated that root  $\text{NO}_3^-$  transport systems are constituted by several (if not many) different carrier proteins, with specific functional properties (directionality,  $V_{\text{max}}$ ,  $K_m$ ) and specific regulatory patterns (induction by  $\text{NO}_3^-$ , feedback repression by N status or stimulation by photosynthesis).

### **Molecular identification and functional characterization of $\text{NO}_3^-$ transporters**

The molecular identification and functional characterization of the genes encoding  $\text{NO}_3^-$  transporters in plants started in the mid-90's, and is still an active research topic, given the multiplicity of candidates. Unlike most other nutrient transporters (for more details, see (Dreyer et al., 1999),  $\text{NO}_3^-$  carriers were not isolated by functional complementation of yeast mutants because *Saccharomyces cerevisiae* is unable to metabolize this N source. Therefore, alternative strategies needed to be employed. So far,  $\text{NO}_3^-$  transporters/channels belonging to four different families (NPF, NRT2, CLC and SLAC/SLAH, see (Krapp et al., 2014) for review) have been found. Only the NPF and NRT2 families will be considered in this review, with a specific focus on *Arabidopsis*.

The first cloned plant  $\text{NO}_3^-$  transporter gene in *Arabidopsis* was *CHL1* (also named *NRT1.1* and now *NPF6.3*). It was isolated from a chlorate resistance screen with T-DNA insertion mutants (Tsay et al., 1993). *NPF6.3* (*CHL1/NRT1.1*) encodes a 590 aa protein with a predicted topology of 12 membrane-spanning domains, and was shown to

be a member of the *NPF* (*NRT1/PTR Family*, (Leran et al., 2014)) gene family (formerly *NRT1/PTR* family), comprising 53 genes in *Arabidopsis*. Phylogenetic studies have revealed that in higher plants, *NPF* families gather a large number of genes (from around 50 to up to 139) and could be divided in 8 to 10 subfamilies (Leran et al., 2014; von Wittgenstein et al., 2014). This is considerably higher than what has been found in most other organisms (e.g. bacteria, yeast, algae, and animals), where only a few *NPF* genes are present. A main feature of the plant *NPF* transporters is that, unlike their non-plant counterparts that mostly transport peptides, there is no substrate selectivity conserved within each family, and even within each subfamily. Up to now, plant *NPF* families (and in particular the *Arabidopsis* one) have been shown to incorporate transporters not only for  $\text{NO}_3^-$ , but also for peptides (Komarova et al., 2008), amino acids (Zhou et al., 1998), nitrite (Sugiura et al., 2007), glucosinolates (Nour-Eldin et al., 2012), auxin (Krouk et al., 2010b), abscisic acid and gibberelins (Chiba et al., 2015; Kanno et al., 2012; Tal et al., 2016). Clearly, *NPF* plant families are characterized by extensive processes of gene amplification and neofunctionalization, with  $\text{NO}_3^-$  transport most probably arising from an ancestral peptide or amino acids transport function (von Wittgenstein et al., 2014). The  $\text{NO}_3^-$  transport gain of function apparently occurred independently in different *NPF* subfamilies, leading to the fact that *NPFs* transporting  $\text{NO}_3^-$  are often phylogenetically more closely related to functionally distinct *NPFs* than to other *NPF*  $\text{NO}_3^-$  transporters. The recent crystallization of the *Arabidopsis* *NPF6.3* (*CHL1/NRT1.1*) indicated that the His356 residue plays a key role in the  $\text{NO}_3^-$  substrate specificity of this protein (Parker and Newstead, 2014), (Sun et al., 2014). Despite this, the protein motifs explaining the variety of substrates that can be transported by *NPFs* remain largely unknown (Leran et al., 2014).

Initially characterized as a LATS involved in root  $\text{NO}_3^-$  uptake (Tsay et al., 1993), *NPF6.3* (*CHL1/NRT1.1*) was later shown to be a dual-affinity transporter, displaying either high- or low-affinity for  $\text{NO}_3^-$  depending on phosphorylation of the T101 residue (Liu and Tsay, 2003). With this exception, other *NPF*  $\text{NO}_3^-$  transporters in *Arabidopsis* such as *NPF4.6* (*NRT1.2/AIT1*), *NPF2.7* (*NAXT1*), *NPF7.3* (*NRT1.5*), *NPF7.2* (*NRT1.8*), *NPF2.9* (*NRT1.9*), *NPF2.3*, *NPF1.1* (*NRT1.12*), *NPF1.2* (*NRT1.11*), *NPF2.13* (*NRT1.7*), *NPF6.2* (*NRT1.4*), *NPF2.12* (*NRT1.6*) and *NPF5.5* are strict LATS. Besides



NPF6.3 (CHL1/NRT1.1), only NPF4.6 (NRT1.2/AIT1) and NPF2.7 (NAXT1) were shown to be involved in root  $\text{NO}_3^-$  uptake (Fig. 1). NPF4.6 (NRT1.2/AIT1) acts as a constitutive LATS for  $\text{NO}_3^-$  influx (Huang et al., 1999), whereas NPF2.7 (NAXT1) mediates  $\text{NO}_3^-$  efflux to the external medium (Segonzac et al., 2007). All other AtNPFs characterized to date are associated with transport steps internal to the plant (Fig. 1). NPF7.3 (NRT1.5) is a bi-directional transporter (influx/efflux) playing a role in  $\text{NO}_3^-$  secretion into the xylem, and thus in root-to-shoot translocation of  $\text{NO}_3^-$  (Lin et al., 2008). NPF7.2 (NRT1.8) and NPF2.9 (NRT1.9) also contribute to control long-distance  $\text{NO}_3^-$  transport to the shoot by mediating the opposite flux and retrieving  $\text{NO}_3^-$  from the xylem sap into the root stele (Li et al., 2010; Wang and Tsay, 2011). NPF2.3, a  $\text{NO}_3^-$  efflux transporter which is expressed in the root pericycle cells, is involved in  $\text{NO}_3^-$  translocation from roots to shoots in response to salt stress (Taochy et al., 2015). NPF1.1 (NRT1.12), NPF1.2 (NRT1.11), NPF2.13 (NRT1.7) and NPF6.2 (NRT1.4) function in shoots (Chiu et al., 2004; Fan et al., 2009; Hsu and Tsay, 2013) and govern  $\text{NO}_3^-$  accumulation in leaf and petiole, in particular by mediating xylem-to-phloem transfer of  $\text{NO}_3^-$  and its remobilization from source to sink leaves (Fig. 1). Both NPF2.12 (NRT1.6) and NPF5.5 contribute to  $\text{NO}_3^-$  transport to and in the seeds. NPF2.12 (NRT1.6) ensures  $\text{NO}_3^-$  supply to the developing seeds (Almagro et al., 2008) and NPF5.5 plays a role in N accumulation in the embryo (Leran et al., 2015b). Finally, several other NPF proteins have been shown to be able to transport  $\text{NO}_3^-$  (NPF1.2, NPF3.1, NPF5.13, NPF5.14 and NPF6.4, reviewed in (Leran et al., 2014), but their role in the  $\text{NO}_3^-$  utilization by the plant has not been elucidated yet.

The *NRT2* gene family of  $\text{NO}_3^-$  transporters was first identified in *Aspergillus nidulans* (Unkles et al., 1991), and later in plants (Filleur and Daniel-Vedele, 1999). As compared to *NPF* families, *NTR2* families analyzed in various species contain a much lower number of genes (from one to 8) (von Wittgenstein et al., 2014), and display a much stronger substrate specificity, as most gene products are only  $\text{NO}_3^-$  transporters. The general structure of *NRT2* proteins (11 to 12 membrane-spanning domains) resembles that of *NPF* proteins, but there is no sequence homology between the two families (von Wittgenstein et al., 2014). A main characteristic of *NRT2* proteins is that they are generally unable to transport  $\text{NO}_3^-$  on their own, but need to interact with the

partner protein AtNAR2.1, which belong to the NAR2 (NRT3) family, to be active (Kotur et al., 2012). It has been proposed that the functional unit is composed of a NRT2 dimer and a AtNAR2 dimer, forming a heterotetrameric protein complex (Kotur and Glass, 2015).

In *Arabidopsis*, 7 NRT2 proteins are present and have all been characterized as influx HATS specific for  $\text{NO}_3^-$ , for most of them in interaction with NAR2.1 (NRT3.1), with the possible exception of NRT2.7 (Chopin et al., 2007). NRT2.1, NRT2.2, NRT2.4 and NRT2.5 play a role in root  $\text{NO}_3^-$  influx, but with markedly different importance and under different conditions. NRT2.1 appears to be by far the main component of the HATS for root uptake under most conditions (with the exception of severe N starvation), as its knock-out mutation results in the loss of up to 75% of the root  $\text{NO}_3^-$  influx (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007). NRT2.2 most often plays a minor role (Li et al., 2007). NRT2.4 displays a particularly high-affinity for  $\text{NO}_3^-$ , and is thus believed to make a significant contribution to  $\text{NO}_3^-$  acquisition at very low external  $\text{NO}_3^-$  concentration (Kiba et al., 2012). Interestingly, NRT2.4 and NRT2.5 are also expressed in shoots, where they contribute to phloem loading of  $\text{NO}_3^-$  (Kiba et al., 2012; Lezhneva et al., 2014). Unlike all other NRT2 proteins which are plasma membrane transporters, NRT2.7 is localized in the tonoplast, and is particularly important for  $\text{NO}_3^-$  accumulation in seeds (Chopin et al., 2007). Although NRT2.3 and NRT2.6 are able to mediate  $\text{NO}_3^-$  transport in *Xenopus* oocytes (Kotur et al., 2012), their function *in planta* remains elusive.

This overview of NPF and NRT2 protein families indicates that at least 20  $\text{NO}_3^-$  transporters are active in *Arabidopsis*, most of them displaying quite specific functions *in planta* (as evidenced by the knock-out mutants phenotypes that suggest a low level of functional redundancy). It is striking to see how the current overall picture for the molecular mechanisms of  $\text{NO}_3^-$  transport in plants matches the predictions made by physiologists more than 30 years ago. Indeed, with only very few exceptions, there are separate transporters for  $\text{NO}_3^-$  influx or efflux and the influx transporters are either HATS or LATS. This tight correspondence between the physiological predictions and the molecular reality is further strengthened by taking regulatory aspects into consideration. In brief, many of the NPF and NRT2  $\text{NO}_3^-$  transporter genes listed above are strongly

regulated at the transcript level by at least one of the main regulatory mechanisms identified by the physiologists, *i.e.*, induction by  $\text{NO}_3^-$ , feedback repression by N status or stimulation by photosynthesis (see Nacry et al., 2013 for review). This has allowed the precise designation of transporters belonging to the transport systems proposed in the 70's and 80's. For instance, the iHATS for  $\text{NO}_3^-$  uptake in *Arabidopsis* is now known to mostly correspond to NRT2.1 and NRT2.2 (Cerezo et al., 2001), whereas the cHATS predominantly relies on NRT2.5 (Kotur and Glass, 2015).

### Unexpected functions of $\text{NO}_3^-$ transporters

Although molecular studies have provided a general validation of the physiological conceptual framework of  $\text{NO}_3^-$  transport in plants detailed above, they also unraveled totally unexpected facets of  $\text{NO}_3^-$  transporter's function. These relate in particular to the role of NPF and NRT2 proteins in  $\text{NO}_3^-$  sensing and signaling, and to the physiological significance of phloem transport of  $\text{NO}_3^-$  (Fig. 1).

The hypothesis that  $\text{NO}_3^-$  transporters also act as  $\text{NO}_3^-$  sensors or transducers has been put forward for both NPF6.3 (CHL1/NRT1.1) and NRT2.1 (Ho et al., 2009; Little et al., 2005; Munos et al., 2004). It is known for years that  $\text{NO}_3^-$  is both a nutrient and a signal molecule triggering a wide range of physiological and developmental responses of the plant. However, the molecular identity of the plant  $\text{NO}_3^-$  sensors remained obscure. The past decade has provided mounting evidence that many of the responses of *Arabidopsis* to  $\text{NO}_3^-$  require a functional NPF6.3 (CHL1/NRT1.1) protein, that is now proposed to be a 'transceptor' (a protein with dual transport/sensing function), according to the concept well documented in yeast for nutrient sensing (Gojon et al., 2011). NPF6.3 (CHL1/NRT1.1) was shown to control not only the short-term induction of gene expression by  $\text{NO}_3^-$  (Ho et al., 2009), but also the long-term feedback repression of gene expression by high  $\text{NO}_3^-$  supply (Bougyon et al., 2015; Munos et al., 2004) and the local stimulation of lateral root development by  $\text{NO}_3^-$  (Krouk et al., 2010b; Remans et al., 2006a). These versatile roles seem to rely on the fact that NPF6.3 (CHL1/NRT1.1) is able to activate several independent mechanisms of  $\text{NO}_3^-$  sensing/signaling, that can be uncoupled by specific point mutations in the protein (Bougyon et al., 2015). Indeed, the mechanisms evoked for the regulatory functions of NPF6.3 (CHL1/NRT1.1) differ

according to the responses to  $\text{NO}_3^-$ . The  $\text{NO}_3^-$  induction of gene expression involves NPF6.3 (CHL1/NRT1.1)-dependent inositol 1,4,5-triphosphate and cytoplasmic  $\text{Ca}^{2+}$  signaling (Riveras et al., 2015), whereas the stimulation of lateral root growth is due to ability of NPF6.3 (CHL1/NRT1.1) to transport auxin in addition to  $\text{NO}_3^-$  (Bougyon et al., 2015; Krouk et al., 2010b). Interestingly, several other NPF proteins have been reported to transport both  $\text{NO}_3^-$  and organic molecules such as amino acids, Gibberellic acid, ABA or glucosinolates (reviewed in (Leran et al., 2014). **NRT2.1 also regulates lateral root development (Little et al., 2005; Remans et al., 2006b), but the underlying mechanism is unknown.**

Another unexpected outcome of the functional characterization of  $\text{NO}_3^-$  transporters is that many of them are expressed in the phloem (*i.e.*, NPF1.1 (NRT1.12), NPF1.2 (NRT1.11), NPF2.9 (NRT1.9), NPF2.13 (NRT1.7), NRT2.4 and NRT2.5 (Fig. 1). This was surprising because phloem transport of  $\text{NO}_3^-$  was always considered to be negligible and of limited physiological significance (Schobert and Komor, 1992). Clearly, this conclusion must now be challenged by the observation that the above transporters play a significant role in  $\text{NO}_3^-$  allocation and redistribution between source and sink tissues, and in regulation of growth either in response to N starvation (Fan et al., 2009; Kiba et al., 2012; Lezhneva et al., 2014) or to ample  $\text{NO}_3^-$  provision (Hsu and Tsay, 2013). It remains to be determined whether this role is associated with a purely nutritional effect, or rather indicate the action of long-distance  $\text{NO}_3^-$  signaling mechanisms.

## **NITRATE SIGNALING**

### **Calcium, one of the missing links in nitrate signaling**

Calcium is probably one of the most studied second messenger in cell signaling, and plants are no exception (Dodd et al., 2010). In plants, calcium function as a key second messenger in a broad array of cellular and plant responses such as stomatal aperture, biotic stress, abiotic stress, nodulation, circadian clock, polar tip growth and self-incompatibility (reviewed by (Dodd et al., 2010). The first association between calcium and  $\text{NO}_3^-$  was established in detached leaves of Maize and Barley (Sakakibara et al.,

1997; Sueyoshi et al., 1999).  $\text{NO}_3^-$  treatments induce expression of *NITRATE REDUCTASE (NR)*, *NITRITE REDUCTASE (NiR)*, *PLASTIDIC GLUTAMINE SYNTHETASE (GS2)* and *GLUTAMATE SYNTHASE (GOGAT)* genes independent of *de novo* protein synthesis (Sakakibara et al., 1997). However, mRNA for these genes does not accumulate to the same extent in response to  $\text{NO}_3^-$  treatments in detached maize leaves pretreated with EGTA or  $\text{La}^{3+}$  (Sakakibara et al., 1997). A comparable result was obtained in a separate study using excised barley leaves. When leaves are pretreated with  $\text{La}^{3+}$ , *NR* and *NiR* gene expression in response to  $\text{NO}_3^-$  treatments was significantly dampened (Sueyoshi et al., 1999). Besides these initial results, the role of calcium in the  $\text{NO}_3^-$ -signaling pathway was not explored in more detail until recently. Using *Arabidopsis* reporter lines that expressed aequorin in the cytosol, Riveras et al (2015) monitored cytoplasmic calcium changes *in-vivo* in response to  $\text{NO}_3^-$  treatments. This study extended previous work by showing that  $\text{NO}_3^-$ -elicited accumulation of cytoplasmic calcium requires NPF6.3 (CHL1/NRT1.1) function. Moreover, the use of U73122 phospholipase C inhibitor and measurements of inositol 1,4,5 triphosphate (IP3) suggested a phospholipase C (PLC) activity downstream of NPF6.3 (CHL1/NRT1.1) and upstream of calcium changes. This work leads to a working model of  $\text{NO}_3^-$ -signaling where  $\text{NO}_3^-$  is sensed by the transceptor NPF6.3 (CHL1/NRT1.1) and activates a PLC activity that triggers an increase in cytoplasmic calcium. This calcium signal is necessary for changes in gene expression for some primary response genes, such as *NRT2.1* and *TGAI*. As expected, not all  $\text{NO}_3^-$  responsive genes depend on this signaling pathway. For instance, up-regulation of the auxin receptor *AFB3* requires the transceptor NPF6.3 (CHL1/NRT1.1) but is independent of *PLC* and calcium (Fig. 2). These results are consistent with multiple signaling pathways branching downstream of the NPF6.3 (CHL1/NRT1.1)  $\text{NO}_3^-$  transceptor (Bougyon et al., 2015).

### **Protein phosphorylation in nitrate signaling**

One of the direct consequences of cytosolic calcium increase is the change in protein phosphorylation status (Sanders et al., 1999). The importance of protein phosphorylation for  $\text{NO}_3^-$  signaling was addressed years ago using protein phosphatase and tyrosine

protein kinase inhibitors (Sueyoshi et al., 1999). In the presence of the inhibitors,  $\text{NO}_3^-$ -dependent induction of *NR* and *NiR* is severely compromised in Barley leaves.

The activity of key proteins of the  $\text{NO}_3^-$  signaling pathway is also regulated by phosphorylation (Kaiser et al., 2002; Liu and Tsay, 2003; Migocka et al., 2013). As mentioned earlier, NPF6.3 (CHL1/NRT1.1) is phosphorylated at threonine 101, which plays a key role in the control of NPF6.3 (CHL1/NRT1.1) signaling and  $\text{NO}_3^-$ -dependent auxin transport (Bougyon et al., 2015). NR is also phosphorylated and its activity is fine-tuned by the phosphorylation of the two consecutive serines -543 and -534 (in *Spinacia oleracea*) and its inactivation by the binding of 14-3-3 proteins (Athwal et al., 1998). Calcineurin B-like protein 1 (CBL1) and CBL9 phosphorylations are also essential for the *in vivo* activation of  $\text{K}^+$  TRANSPORTER 1 by the CBL1-CIPK23 and CBL9-CIPK23 protein complexes (Hashimoto et al., 2012).

More recently, untargeted phosphoproteomic studies revealed that up to 38 proteins change phosphorylation status in  $\text{NO}_3^-$ -deprived whole seedlings (Wang et al., 2012a). Most of the proteins identified in this study were functionally classified as fundamental metabolic pathways. Using a different experimental setup, Engelsberger and Schulze (2012) identified 589 differentially phosphorylated proteins after  $\text{NO}_3^-$  treatments of nitrogen-starved *Arabidopsis* seedlings (Engelsberger and Schulze, 2012). These proteins can be divided into two categories: Fast responsive proteins, such as GPI-anchored proteins, receptor kinases and transcription factors. The second category corresponds to proteins involve in protein synthesis and degradation, as well as central metabolism and hormone metabolism.

How are calcium changes sensed in response to  $\text{NO}_3^-$  and how is this signal transduced to phosphorylate target proteins remains unclear. Interestingly, these phosphoproteomic studies showed an overrepresentation of kinases and phosphatases among the proteins with changes in their phosphorylation pattern, suggesting candidate protein effectors for phosphorylation changes in response to N (Engelsberger and Schulze, 2012; Wang et al., 2012a). Receptor-like kinases, Mitogen-activated protein (MAP) kinases kinases, Sucrose non-fermenting-1 (Snf1)-related protein kinases, calcium-dependent protein kinases and Calcineurin-B like (CBL)-CBL-interacting protein kinase (CIPK) kinases were identified in this group. *CIPK8* and *CIPK23* have

been previously implicated in  $\text{NO}_3^-$  signaling (Ho et al., 2009) and are candidate components of the signaling pathway downstream of calcium. CIPK23 is known to phosphorylate the  $\text{NO}_3^-$  transceptor NPF6.3 (*CHL1/NRT1.1*), negatively regulating the primary  $\text{NO}_3^-$  response under low  $\text{NO}_3^-$  concentrations (Ho et al., 2009). In contrast, the only known CIPK8 target is ABA INSENSITIVE 2 (*ABI2*) (Ohta et al., 2003). However, it has been shown that this kinase plays a role in the low-affinity  $\text{NO}_3^-$  response, acting as a positive regulator (Hu et al., 2009).

### Nitrate transcriptional regulation

Little is known about the transcriptional mechanisms underlying regulation of prototypical  $\text{NO}_3^-$  responsive genes such as *NPF6.3 (CHL1/NRT1.1)*, *NRT2.1*, *NRT2.2*, *NIA1*, *NIA2* or *NiR*. A series of transcription factors have been identified as important regulatory factors in the  $\text{NO}_3^-$  response: *NIN LIKE PROTEIN (NLP7)*, *NLP6*, *TGACG MOTIF-BINDING FACTOR (TGA1)*, *TGA4*, *ARABIDOPSIS NITRATE REGULATED 1 (ANR1)*, *BASIC LEUCINE-ZIPPER 1 (bZIP1)*, *LOB DOMAIN-CONTAINING PROTEIN (LBD37)*, *LBD38*, *PCF (TCP)-DOMAIN FAMILY PROTEIN 20 (TCP20)*, *NAC DOMAIN CONTAINING PROTEIN (NAC4)* and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9)*. The role of these transcription factors has been extensively reviewed therefore we will only highlight here some key aspects for each case (Castaings et al., 2011; Chardin et al., 2014; Medici and Krouk, 2014; Vidal et al., 2015). Changes in the activity or expression levels of these transcription factors in response to  $\text{NO}_3^-$  affect expression of  $\text{NO}_3^-$ -responsive genes. Direct interactions of these transcription factors with target gene promoters have been confirmed for *NLP7*, *TGA1*, *bZIP1* and *TCP20* which we discuss below.

*NLP7* was identified by homology to the NIT2 protein of *Chlamydomonas* (Camargo et al., 2007), an important transcription factor regulating  $\text{NO}_3^-$ -responsive genes in *Chlamydomonas*. *NLP7* is not regulated at the transcriptional level. But in response to  $\text{NO}_3^-$ , *NLP7* protein is rapidly translocated to the nucleus to regulate gene expression (Marchive et al., 2013). Chromatin immunoprecipitation (ChIP) followed by microarray hybridization (ChIP-chip) identified 851 gene promoters where *NLP7* is

bound in response to  $\text{NO}_3^-$  (Marchive et al., 2013). The 851 genes over-represented functions include N-metabolism,  $\text{NO}_3^-$  signaling, and hormone transport and metabolism. Moreover, transcriptomic analysis in *nlp7* mutants allowed identification of NLP7 targets. Interestingly, 101 genes were identified as NLP-bound and NLP7-regulated, from them 91 were also  $\text{NO}_3^-$  regulated. This further supports the key role of NLP7 as an important regulator of the primary nitrate response.

*TGA1* and *TGA4* transcription factors were identified using a systems biology approach to predict novel  $\text{NO}_3^-$  regulatory factors (Alvarez et al., 2014). Both *TGA1* and *TGA4* gene expression are regulated in response to  $\text{NO}_3^-$ , their increase in transcript levels peaks one and two hours after  $\text{NO}_3^-$ -treatment respectively (Alvarez et al., 2014). Both *TGA1* and *TGA4* are regulated by  $\text{NO}_3^-$  in root organs. *tga1/tga4* double mutant have impaired responses to  $\text{NO}_3^-$  treatments such as modulation of primary root length and lateral root density (Alvarez et al., 2014). As discussed above, calcium is a key component of  $\text{NO}_3^-$  signaling and up-regulation of *TGA1* gene expression requires calcium (Riveras et al., 2015). Identification of target genes for *TGA1/TGA4* was done by comparing  $\text{NO}_3^-$  response of wild-type and *tga1/tga4* double mutant plants using Affymetrix microarrays. The large number of genes affected by *tga1/tga4* mutation, including important components of N metabolism supports a role for these transcription factors as important regulators of the primary  $\text{NO}_3^-$  response. Recruitment of *TGA1* to target gene promoters was validated by ChIP for *NRT2.1* and *NRT2.2* (Alvarez et al., 2014).

A more recent study identified *TCP20* as a component of systemic N-signaling (Guan et al., 2014). *TCP20* is a transcription factor from the TCP family and was identified using yeast one-hybrid screens against a 109 bp  $\text{NO}_3^-$  enhancer region of *NIA1* and a 150 bp  $\text{NO}_3^-$  enhancer region of *NRT2.1* promoters (Girin et al., 2007; Wang et al., 2010). *TCP20* is expressed in root tips, root vasculature and young leaves. Interestingly, the primary and lateral roots develop normally under homogeneous nitrate supply, however, their growth is affected in root foraging studies, were plants are grown on heterogeneous media in split-root plates (Guan et al., 2014). Interestingly, *TCP20* binds Type-A Arabidopsis Response Regulators (ARRs) *ARR5/7* promoters and these genes are up-regulated by  $\text{NO}_3^-$  in shoots, so *TCP20* might provide a link between N-signaling and



cytokinin signaling (Ruffel, 2011; Ruffel et al., 2016). TCP20 would also interact with local signaling by inducing NPF6.3 (CHL1/NRT1.1) expression on low nitrate (LN) only (Guan et al., 2014).

Another key transcription factor is *bZIP1*, which integrates light and  $\text{NO}_3^-$  signals in plants (Obertello et al., 2010). Moreover, the TARGET approach (Transient Assay Reporting Genome-wide Effects of Transcription factors, Bargmann et al., 2013) helped to identify *bZIP1* direct target genes, giving this master transcription factor a role in rapid and dynamic N-signal propagation (Para et al., 2014). This same work led to the elucidation of the different modes of actions of *bZIP1* that acts following a hit-and-run transcriptional model. In response to an N-signal such as nitrate or ammonium nitrate, *bZIP1* will transiently bind to its target gene promoters that will then be induced to trigger the  $\text{NO}_3^-$  response (Para et al., 2014).

The transcription factors *LBD37* and *LBD38* are also regulating  $\text{NO}_3^-$  responsive genes (Rubin et al., 2009). Over-expression of these LBDs results in defects in shoot branching and altered anthocyanin levels. Accordingly, *LBD37* and *LBD38* are negative regulators of  $\text{NO}_3^-$  responsive genes such as *NIA1*, *NIA2*, *NPF6.3* (*CHL1/NRT1.1*), *NRT2.1*, *NRT2.2*, and *NRT2.5* (Rubin et al., 2009) and may also act in N free conditions (Medici and Krouk, 2014).

Another transcription factor controlling expression of prototypical  $\text{NO}_3^-$  response genes is *SPL9* (Krouk et al., 2010c). This gene was identified in a high-resolution kinetics transcriptome experiment. Using a systems biology approach, state space modeling of the data generated allowed the authors to identify influential transcription factors. Thus, changes in the transcript levels or activity of these transcription factors alters the transcript levels of *NRT2.1* and other sentinel genes such as *NRT1.1*, *NRT2.2*, *NIA1*, *NIA2* and *NIR*.

Future studies should address how these important regulatory factors interact or cross talk to orchestrate N responses in *Arabidopsis* and other plants (Vidal et al., 2015).

### **Hormonal signaling in response to Nitrate**

$\text{NO}_3^-$  signaling and particularly the downstream physiological responses rely strongly on plants hormones. Because N provision impacts plant growth and

development, and hormones are key molecular players shaping plants at all stages of their life cycle, it is not surprising to see an increasing number of reports describing connections between N transcriptional networks and hormonal responses (reviewed in (Krouk, 2016; Krouk et al., 2011; Rubio et al., 2009)).

The  $\text{NO}_3^-$ -hormonal crosstalk can be simplified into two categories. At a first level, N impact hormone biosynthesis, transport and signaling. Another level of interaction is the control of N related transcriptional networks by hormonal signals. N provision ( $\text{NO}_3^-$  or other alternative source of reduced N) can alter biosynthesis and transport of auxin (Avery et al., 1937; Avery and Pottorf, 1945; Caba et al., 2000; Castaings et al., 2009; Chen et al., 1998; Krouk et al., 2010b; Ma et al., 2014; Walch-Liu et al., 2006), cytokinins (CK) (Rahayu et al., 2005; Sakakibara et al., 1998; Sakakibara et al., 2006; Takei et al., 2001; Takei et al., 2002; Takei et al., 2004; Wang et al., 2004), ethylene (Tian et al., 2009) and abscisic acid (ABA) (Matakiadis et al., 2009; Ondzighi-Assoume et al., 2016; Signora et al., 2001). However, the molecular mechanisms and gene network involved in such crosstalk are still poorly understood.

A well documented connection at the molecular level is the control of CK biosynthesis by  $\text{NO}_3^-$  provision. Indeed, a corpus of work (Hu et al., 2009; Ruffel, 2011; Ruffel et al., 2015; Sakakibara et al., 2006; Takei et al., 2004) demonstrates that N, particularly  $\text{NO}_3^-$ , can transcriptionally activate isopentenyl transferase (*IPT*) genes that catalyze the limiting step of CK biosynthesis. More precisely,  $\text{NO}_3^-$  induced *IPT3* gene expression (Wang et al., 2004) was shown to be a crucial component of the  $\text{NO}_3^-$  regulated CK biosynthesis (Takei et al., 2004). The lack of *IPT3* transcriptional regulation in the *npf6.3* (*chl1*) mutant suggests that *IPT3* regulation of gene expression requires the NPF6.3 (CHL1/NRT1.1)  $\text{NO}_3^-$  transceptor activity (Hu et al., 2009; Medici and Krouk, 2014; Wang et al., 2004).

The cross-talk between  $\text{NO}_3^-$  and auxin was first suggested in the 1940s by George S. Avery and Louise Pottorf, showing that there is a direct correlation between auxin levels and  $\text{NO}_3^-$  supply (Avery et al., 1937; Avery and Pottorf, 1945). Recent work by Ma et al. identified TAR2 (part of the main component of the auxin biosynthesis pathway (Zhao, 2012)) as important to maintain auxin biosynthesis under low N conditions (Ma et al., 2014). Interestingly, TAR2 is expressed in pericycle cells and root

vasculature (Ma et al., 2014). However, this is not compatible with the main route of auxin translocation from shoot to roots (Robert and Friml, 2009). Thus, the main actors of N controlled shoot auxin synthesis are still rather elusive. Concerning auxin transport, transcriptome analysis in response to N provision showed that *PINs* (*ARABIDOPSIS THALIANA PIN-FORMED*, a family of auxin efflux carriers, reviewed by (Adamowski and Friml, 2015)) are regulated in response to N provision (Gutierrez, 2007). Moreover, it has been shown that the  $\text{NO}_3^-$  transceptor itself NPF6.3 (*CHL1/NRT1.1*) (Tsay et al., 1993), can behave as an auxin transporter under low  $\text{NO}_3^-$  conditions (Krouk et al., 2010b). This auxin/ $\text{NO}_3^-$  transporter is transcriptionally responsive to  $\text{NO}_3^-$  and has been shown to be bound by NLP7 (Marchive et al., 2013). This potential regulatory module constitutes an important link between the defined  $\text{NO}_3^-$  related regulatory networks and the control of auxin transport and homeostasis.

Vidal et al. (2010) demonstrated auxin-  $\text{NO}_3^-$  crosstalk by identifying a negative feed forward mechanism composed of the auxin receptor *AUXIN SIGNALING F-BOX 3* (*AFB3*) and *microRNA 393* (*miRNA393*) (Vidal et al., 2010). *AFB3* transcript level increases in response to  $\text{NO}_3^-$  treatments, which in turn results in induction of *NAC4* gene expression. This signaling pathway is negatively modulated by *miRNA393*. This microRNA is induced by reduced products of  $\text{NO}_3^-$  and post-transcriptionally represses *AFB3* gene expression (Fig. 2). This module integrates external inorganic nitrogen sources and the internal nitrogen status of the plant to modulate auxin signaling and downstream developmental responses (Gutierrez, 2012).

The connection between ethylene and N has also been reported (Tian et al., 2009; Zheng et al., 2013). In their work, Tian and colleagues showed an increase in  $\text{NO}_3^-$  provision triggers ethylene biosynthesis through transcriptional activation of 1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS*) and ACC oxidase (*ACO*) genes (Tian et al., 2009). In the *Arabidopsis etr1* and *ein2* mutants plants treated with N, root transcript levels for *NRT2.1* decreases and *NPF6.3* (*CHL1/NRT1.1*) increases. These results suggest ethylene signaling can also crosstalk or regulate N-signaling and N-responsive genes.

The molecular mechanisms controlling ABA biosynthesis in response to N provision are more scarce and implicates ABA DEFICIENT 1 (*ABA1*), *ABA2* and *ABA3*

genes in the control of lateral root repression in response to very high  $\text{NO}_3^-$  provision (>50mM) (Signora et al., 2001). During seed germination, which is controlled by  $\text{NO}_3^-$  (Alboresi et al., 2005; Matakias et al., 2009), the Cytochrome P450 protein CYP707A2, which has ABA 8'-hydroxylase activity, has been shown to control ABA degradation in response to  $\text{NO}_3^-$  and thus alleviate seed dormancy (Matakias et al., 2009). The transcriptional control of these genes by the major components of N response still needs to be investigated in these particular contexts (ie: very high  $\text{NO}_3^-$  provision, and during germination).

Very recently, another level of complexity between ABA and  $\text{NO}_3^-$  has been reported. Immunodetection of ABA accumulation in Arabidopsis root demonstrated that this hormone tends to accumulate in a very cell-specific manner that resembles the pattern of expression of the SCARECROW gene (Ondzighi-Assoume et al., 2016). This signal is enhanced when the  $\text{NO}_3^-$  provision is increased. This increase is likely due to ABA release from a conjugated form (ABA-glucose) by the action of a beta-glucosidase (BG1) (Ondzighi-Assoume et al., 2016). Furthermore, at the transport level ABA and gibberellic acid (GA) are also regulated by a nitrate transporter in Arabidopsis (Tal et al., 2016). The nitrate transporter NPF3, an influx carrier expressed in root endodermis, can transport both ABA and GA, suggesting an ABA-GA interaction.

As mentioned above, the role of hormonal signaling in the control of N response is also an important feature of N/hormone entanglement. It is important to report that hormonal signaling pathways are able to control genes involved in N response. As a striking example, the central  $\text{NO}_3^-$  transceptor NPF6.3 (CHL1/NRT1.1) is under the regulation of many hormonal signals (Guo et al., 2002; Leran et al., 2015a; Tian et al., 2009). In Arabidopsis seedlings treated with auxin (Guo et al., 2002) or ethylene synthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) NPF6.3 (CHL1/NRT1.1) is upregulated. Moreover, ABA signaling also modulates NPF6.3 (CHL1/NRT1.1) through the phosphatase ABA INSENSITIVE 2 (ABI2), which is a component of the protein complex regulating its activity (Leran et al., 2015a). The central  $\text{NO}_3^-$  assimilation enzyme, NR is also under a post-traductional control by auxin and cytokinins (Vuylsteker et al., 1997). These are 2 key examples among many (fully

described in (Krouk et al., 2011)) showing that hormones are also important feedback regulators of the  $\text{NO}_3^-$  and Nitrogen sensing and assimilatory systems.

## **ACT LOCALLY, THINK GLOBALLY: THE IMPACT OF LOCAL AND SYSTEMIC SIGNALS ON ROOT N-FORAGING**

Plant developmental processes are strongly regulated by hormones (Vanstraelen and Benkova, 2012). For instance, root length and lateral root formation are modulated by the crosstalk of auxin, cytokinin and ethylene (Ruzicka et al., 2009), which are key for N signaling. However, N controls hormone biosynthesis and also transport throughout the plant (described above). This tight N-dependent hormonal control leads to profound changes of plant development, locally and systemically (Krouk et al., 2011; Ruffel et al., 2014).

### **The history of root nutrient foraging studies.**

When nutrient-deficient plants are supplied with a heterogeneous nutrient environment, they activate a set of morphological and physiological responses called foraging (De Kroon et al., 2009; Gojon et al., 2009; Gruber et al., 2013; Hodge, 2004; Nacry et al., 2013). The most dramatic aspect of this response is an enhanced root growth and proliferation particularly in the nutrient-rich zone. Specifically, root foraging enables plants to compensate for the non-uniform distribution of nutrients and may determine plants fitness (competitive success and productivity) (Giehl and Wirén, 2014; Hodge, 2004). Therefore, root foraging has been considered to be an interesting agronomic trait and has historically been studied in cereals since 1917 (Gile and Carrero, 1917). Studies on root foraging include responses to various heterogeneous nutrient treatments (nitrogen, phosphorus, potassium) (Hodge, 2004), and using diverse culture systems (Robinson, 1994). However, these early studies mostly focused on physiological and morphological parameters of foraging (plant growth, root growth, nutrient content and uptake) (Hodge, 2004; Robinson, 1994). More recently, the use of the model plant *Arabidopsis* allowed us to gain insights into the genomics of nutrient foraging (cf. below). Many studies address responses to  $\text{NO}_3^-$  treatments. But it has not always been established whether the responses observed are due to nitrate as signal or are due to

products of nitrate reduction or N assimilation. We specify  $\text{NO}_3^-$  when the role of this nutrient has been demonstrated and N otherwise.

Foraging results from the integration by the plant of many signals. Some signals come from roots such as nutrient availability (e.g. concentration, distribution, diffusion). However, root foraging is also affected by signals coming from shoots such as plant nutritional and energy status (e.g. nutrients and carbon reserves, nutrients remobilization) (Giehl and Wirén, 2014). As such, nutrient root foraging is a good model for systems biology to study systems-wide signal integration.

### **N-signaling and root responses to a homogeneous N-supply.**

The effect of N-deprivation and N-supply on root system architecture has been largely investigated under homogeneous treatment conditions (e.g. +N or – N), reviewed by Nacry et al. (2013) and Zhang et al. (2007).

In such studies, N-supply was shown to have a dual effect on lateral roots (LRs) depending on the concentration: (1) a stimulation of LR growth at limiting to sufficient N-supply ( $[\text{NO}_3^-] < 10\text{mM}$ ) and (2) an inhibition of LR meristem activation at supra-optimal N-supply ( $[\text{NO}_3^-] \geq 10\text{mM}$ ) (Gruber et al., 2013; Zhang and Forde, 1998; Zhang et al., 1999). Gene responses to a homogeneous N-supply at the whole genome level have also been studied extensively in both time and space since the 2000's, reviewed in (Krouk et al., 2010c; Tsay et al., 2011; Vidal and Gutierrez, 2008). These studies significantly contributed to the identification of genes involved in the control of LR development in response to N, reviewed in (Alvarez et al., 2012; Gutierrez, 2012; Krapp et al., 2014; Krouk et al., 2010a; Vidal et al., 2015; Vidal et al., 2010). Table 1 lists the genes involved in LR development in response to such homogeneous supplies of N (cf. Table 1) reviewed in (Vidal et al., 2015). These include: the  $\text{NO}_3^-$  transceptor NPF6.3 (CHL1/NRT1.1) (Krouk et al., 2010b); the  $\text{NO}_3^-$  transporter NRT2.1 (Little et al., 2005; Remans et al., 2006b); the BTB AND TAZ DOMAIN PROTEIN 1 (BT1) and BT2 (Araus et al., 2016); master TFs involved in N-signaling (NLP7 (Castaings et al., 2009; Marchive et al., 2013), SPL9 (Krouk et al., 2010c), NAC4 (Vidal et al., 2013) and TGA1/TGA4 (Alvarez et al., 2014); the auxin-related modules AFB3/miR393 and

ARF8/miR167 (Gifford et al., 2008; Vidal et al., 2010); the auxin biosynthetic enzyme TAR2 (Ma et al., 2014); the CLE peptides and their receptor CLV1 (Araya et al., 2014). While many connections between these different regulatory genes are unknown, some have been made (Canales et al., 2014; Gutierrez, 2012). For example, NPF6.3 (CHL1/NRT1.1), AFB3/miR393 and NAC4 were shown to belong to a single  $\text{NO}_3^-$  specific signaling pathway that controls LR initiation (Vidal et al., 2014a).

Following are five lessons learned from these studies. (1) PR and LR are regulated independently by N. Indeed some mutants (like *nac4* and *npf6.3*) have altered LR growth but normal PR growth in response to N (Krouk et al., 2010b; Vidal et al., 2014a). (2) Different steps of LR development can be modulated by N depending on the experimental conditions. For instance, very low  $\text{NO}_3^-$  concentrations (<0.5mM) control LR emergence (Krouk et al., 2010a), whereas supra-optimal concentrations (>10mM) act on LR meristem activation (Zhang et al., 2007). (3) Root traits can exhibit dose-dependent sensitivity to the imposed N-deficiency (Gruber et al., 2013). (4) N-signaling pathways overlap with hormones signaling pathways as shown by the role of AFB3 and ARF8 in LR response to N (Gifford et al., 2008; Vidal et al., 2010). (5) N-signaling implements negative feedback loops, notably involving miRNAs (Canales et al., 2014; Gifford et al., 2008; Gutierrez, 2012; Vidal et al., 2010).

### **N-signaling and root responses to a heterogeneous N-supply**

The effect of heterogeneous N-supply on systemic N-signaling has also received much attention (reviewed in (Nacry et al., 2013; Zhang et al., 2007). Two types of *in vitro* systems have been set up for *Arabidopsis*. The first system was made of vertical plates containing 3 segments of solid medium, separated by trenches to prevent diffusion between them (cf. Fig. 3-A left panel) (Zhang and Forde, 1998). Low N (LN) concentration is applied to the top and bottom segments and high N (HN) concentration to the middle one (LN/HN/LN). The controls are LN/LN/LN or HN/HN/HN or contain a total N (TN) quantity equivalent to that available in the heterogeneous medium but homogeneously distributed ( $\text{TN}_{1/3}/\text{TN}_{1/3}/\text{TN}_{1/3}$ ). In literature, the concentration range frequently used for LN was 0-1 mM  $\text{NO}_3^-$  and 0.05-50 mM  $\text{NO}_3^-$  for HN, but foraging was actually observed for  $\text{LN} < 1 \text{ mM}$  and  $\text{HN} > 0.1 \text{ mM}$ . In this vertical system, the PR

grows progressively on the successive segments. Later, a second system – the “split root” system – was made of two solid medium segments, side by side and separated by a trench, containing either low or high N concentration (LN-HN) (cf. Fig. 3-A right panel) (Remans et al., 2006a; Ruffel, 2011). The controls are LN-LN or HN-HN. In split root, the root system is split into two equivalent parts generated from two lateral roots that are “PR-like”. The split root system allowed to compensate for some drawbacks of the vertical system and provided important insights to understand foraging signaling. Morphological and physiological studies showed that: heterogeneous N-supply (HN vs. LN) induces (1) a stimulation of LR growth and N-uptake on HN and (2) a repression of LR meristem activation on LN (Linkohr et al., 2002a; Remans et al., 2006a; Ruffel, 2011; Ruffel et al., 2016; Zhang and Forde, 1998; Zhang et al., 1999). For both responses, nitrate reductase mutants have been used to show that the  $\text{NO}_3^-$  molecule itself is the signal - but not some down-stream products of  $\text{NO}_3^-$  assimilation (Ruffel, 2011; Zhang et al., 1999).

Early studies suggested the existence of a local  $\text{NO}_3^-$  signal that stimulates LR growth on HN as compared to LN under heterogeneous  $\text{NO}_3^-$  supply (cf. Fig. 3-A) (Zhang and Forde, 1998; Zhang et al., 1999). This was later confirmed by demonstrating that NPF6.3 (CHL1/NRT1.1) controls a local  $\text{NO}_3^-$ -specific signaling pathway within LR primordia (cf. Fig. 4-bottom part) (Mounier et al., 2014; Remans et al., 2006a). Zhang’s studies also suggested that a systemic signal inhibits LR development on LN. More recently, Ruffel et al. showed that there are actually two different systemic N-signals managing the “N-economics” of root foraging (cf. Fig. 3-B) (Ruffel, 2011). (1) The systemic N-demand signal stimulates LR growth on split HN compared to control HN whereas (2) the systemic N-supply signal inhibits LR growth on split LN as compared to control LN (Ruffel, 2011). These two latter signals are *systemic* since roots are locally submitted to the same treatment (presence or absence of N respectively), thus any difference observed on the split root compared to the control must result from a systemic signal coming from the other side of the split root (Araya et al., 2014; Ruffel et al., 2011). Therefore, we propose that root foraging results from the integration of both local and systemic signals.



Some of the genes involved in root foraging have been identified (reviewed in (Alvarez et al., 2012; Bouguyon et al., 2012; Nacry et al., 2013) (Fig. 4). The first genes identified were the MADS-box TF *ANR1* (Gan et al., 2012; Zhang and Forde, 1998) and the  $\text{NO}_3^-$  transporter/sensor NPF6.3 (CHL1/NRT1.1) (Krouk et al., 2010b; Mounier et al., 2014; Remans et al., 2006a). Both were shown to be involved in local signaling. On HN, NPF6.3 (CHL1/NRT1.1) would sense  $\text{NO}_3^-$  and stimulate *ANR1* expression (Mounier et al., 2014; Remans et al., 2006a). *ANR1* would then induce cell proliferation in LR tips and thus LR growth. On LN, NPF6.3 (CHL1/NRT1.1) would work as an auxin transporter removing auxin from LR primordia therefore blocking their development (Krouk et al., 2010b; Mounier et al., 2014). The link between auxin and local signaling has also been proposed through *AXR4* (Auxin resistant 4), but contradictory data question its actual implication (Linkohr et al., 2002a; Zhang et al., 1999).

An important milestone in studying systemic N-signaling came from the Ruffel et al. study (2011). The authors investigated the transcriptomic response associated to root foraging in *Arabidopsis*. Clustering analysis of the time-series transcriptome data from split root plants showed that the root N-response is mirrored by the gene expression: initially, gene expression responds to local N-signals, whereas at later time-points genes respond to systemic N-signals (N-demand or N-supply patterns) (Ruffel, 2011). Importantly, the authors demonstrated that shoots are necessary for systemic N-signaling in roots, and that cytokinins are involved in N-demand but not N-supply signaling (Ruffel, 2011). As cited above ((Ruffel, 2011), there is an interesting parallel between root growth regulation on heterogeneous (HN-LN) conditions and gene regulation suggesting that both could share - at least partly - the same signaling pathways. Therefore, studying the N-signaling pathways that regulate gene expression in split root could also give information on the control of root development. *NRT2.1* (encoding a  $\text{NO}_3^-$  transporter) is a good model gene, since it is submitted to both local and systemic regulations just like LR development (Gansel et al., 2001; Li et al., 2006). Recently, Tabata et al. (2014) studied *NRT2.1* regulation in split root and identified small peptides – C-TERMINALLY ENCODED PEPTIDES (CEPs) - and their receptors as part of N-demand signaling. They showed that CEPs are produced on the root half exposed to LN, and translocate to the shoots where they bind their receptors. Next, an as yet unknown

signal circulates from shoots to roots to up-regulate NRT2.1 expression in the root half exposed to HN. Unfortunately, the authors did not report the role of CEPs and their receptors in root morphology on split root, so this is still an open question.

Besides cytokinin (Ruffel, 2011) and CEPs (Tabata et al., 2014), a few more molecules have been proposed to act as root-shoot-root circulating signals involved in systemic N-signaling:  $\text{NO}_3^-$  itself or other N-metabolites, auxin and miRNAs (reviewed in (Alvarez et al., 2012; Li et al., 2014). The role of TCP20 in redirecting root growth to nitrate-rich zones suggests that this protein is a key component of the systemic nitrate-signaling pathway (Guan et al., 2014).

### **Integration of N-signaling pathways under heterogeneous and homogeneous N-supply**

Interestingly, the  $\text{NO}_3^-$  transceptor NPF6.3 (CHL1/NRT1.1) controls root development under both homogeneous and heterogeneous  $\text{NO}_3^-$ -supply, suggesting it is central in plants adaptation to  $\text{NO}_3^-$ -availability. Recently, a meta-analysis comparing transcriptomic data obtained on homogeneous and heterogeneous N-supply revealed that genes response to homogeneous  $\text{NO}_3^-$ -supply combines both local and systemic genes responses (as defined in split root) (Li et al., 2014). Thus, homogeneous and heterogeneous N-signaling largely overlap. Consequently all genes known to control root development in response to homogeneous N should also be tested in split root heterogeneous conditions. For instance, since miRNAs are considered good root-shoot-root circulating candidate signal, it would be interesting to investigate in split root the role of miR393/AFB3 (Vidal et al., 2010) and miR167/ARF8 (Gifford et al., 2008) that both control LR development under homogeneous N-supply.

To our knowledge only one gene was shown to act in both local and systemic regulations of N-foraging: TCP20 (cf. above, (Guan et al., 2014) However, CEP peptides (cf. above) have been suggested to act locally - in addition to their systemic effect - by reducing LR growth at the site where they are produced (Bisseling and Scheres, 2014). Bisseling and Scheres (2014) argued that this dual effect of CEPs would perfectly make sense from an engineering perspective. Indeed the plants could thus decide centrally (in the shoot) if the overall nutrient status is satisfying and then send systemic signals to

stimulate root growth. But growth would be targeted to HN owing to a parallel inhibition on the LN side.

There are also some interesting clues about the time-space relationship between local and systemic N-signaling. Ruffel et al. showed that genes respond to a local N-signal within a couple of hours of exposure to heterogeneous N-conditions in split root, whereas the response to systemic N-signaling appears at later time-points (8h) (Ruffel, 2011). A meta-analysis (Alvarez et al., 2012) comparing time-series (Krouk et al., 2010c) and tissue-specific transcriptomes (Gifford et al., 2008) on homogeneous N-medium, showed that the early N-response genes are mostly specific to lateral-root cap, stele and pericycle, whereas late response genes are found in all cell types. Another meta-analysis (Li et al., 2014) also showed that the early N-response largely overlap with local N-signal, whereas the late N-response genes overlap with the systemic N-response. Taken together, these results suggest that the local N-response is induced early in specific tissues (LRC, stele, pericycle), whereas the systemic N-response propagates late and in all cell types. One particular interest is the root tip, which seems to have a very special role in N signaling. Root tips are at the forefront of the growing roots, and thus they are pioneers exploring new soil areas. Root tips are major sites of early – and so local – response (Alvarez et al., 2012). Root tips are also necessary and sufficient to induce major changes in RSA in response to N-treatment (Walch-Liu and Forde, 2008). In addition, several important components of N-signaling are expressed in root tips (e.g. NPF6.3 (CHL1/NRT1.1), *ANRI*, *NLP7*) (Castaings et al., 2009; Marchive et al., 2013; Remans et al., 2006a). Considering, all these data, it is tempting to postulate that root tips could be central in N-perception: they would precociously sense N-signals and send a signal to the shoots via the stele. A systemic signal would then come back to the roots, enabling a root-shoot-root interplay, as described in Ruffel et al., 2011.

## **EXPLOITING GENETIC DIVERSITY IN NATURAL POPULATIONS: GENOME-WIDE ASSOCIATION APPROACHES TO UNCOVER GENES IMPLICATED IN N MODULATION OF ROOT SYSTEM ARCHITECTURE**

**GWAS: principle, advantages and limitations.**

Genome wide association studies (GWAS) exploit the natural polymorphism (SNP) present in different individuals from a single species to identify loci associated to a phenotype (Atwell et al., 2010; Gupta et al., 2014; Hindorff et al., 2009; Hirshhorn and Mark, 2005). Concretely, GWAS try to find correlations between genotypes and phenotypes by testing each SNP individually across various individuals. GWAS output is visualized by a “Manhattan plot” showing the SNPs position along the ordered chromosomes on X-axis and the  $-\log(\text{P-value})$  resulting from the correlation test on Y-axis. Each dot represents a SNP. Therefore a SNP very significantly associated to a phenotype will appear as a “SNP hit” on the Manhattan plot. However, because of linkage disequilibrium (LD), one cannot conclude that this particular SNP is causative of the phenotype. Instead, the SNP indicates a region where a causative gene might be located. In other words, all the genes present in that region are candidates. To restrict the candidate gene list, complementary information might be used (Gene Ontology terms, gene expression data, among others).

GWAS has been extensively used in human health research and, for a few years now, in plants thanks to the “1001 Genomes Project” (<http://1001genomes.org/>). This project - started by the Max Planck Institute in 2008 – has now completed the genome sequencing of over 1100 *Arabidopsis* accessions and keep extending the list (<http://1001genomes.org/>). Many good reviews have been written about advantages and limits of GWAS (Bush and Moore, 2012), and its particularities in plants (Bergelson and Roux, 2010; Korte and Farlow, 2013; Weigel, 2012). Notably, one major asset of GWAS is that even for complex integrated traits - like quantitative traits - causative genes can potentially be identified, whereas this is very difficult by classic genetics.

### **GWAS applied to Root System Architecture (RSA) N-response in *Arabidopsis*.**

Recently, the RSA response to N availability has been studied across various *Arabidopsis* ecotypes, but without investigating the underlying genetics basis (Chardon et al., 2010; De Pessemier et al., 2013; Ikram et al., 2012; North et al., 2009; Ristova and Busch, 2014). GWAS is a method of choice to fill this gap. However, to our knowledge, only two studies have used GWAS to identify the molecular basis of the N-signaling that controls RSA (Gifford et al., 2013; Rosas et al., 2013).

Rosas et al. (2013) studied the natural variation of RSA in 69 *Arabidopsis thaliana* accessions grown on high  $\text{NO}_3^-$  (5mM). The authors used a semi-automatic method (*RootScape*), to quantify root system architecture holistically (Ristova et al., 2013). They showed that about 20% of the variability in root morphometrics across accessions could be explained by variation in allometry (i.e. the modification of the proportions in RSA independently of size). A GWA analysis performed on the “allometry” trait successfully identified two loci associated to this trait (Rosas et al., 2013). One of the loci included PHO1, which was already known to control RSA. The second locus contained a good candidate gene - RSA1 (Root Systems Architecture 1) – that was identified and validated. Therefore, this GWAS successfully unraveled the genetic underpinnings of a very complex trait (allometry).

In a parallel study, Gifford et al. (2013) also looked at natural variation in root plasticity, but here in response to various N environments. They quantified various root traits in response to two different N-environments across 96 *Arabidopsis thaliana* accessions, and they performed a GWAS on the data. They identified 53 highly significant SNP hits corresponding to 17 different loci. Then gene expression data were used to assist the selection of candidate genes. Eventually, two genes specifically controlling RSA on low N were identified: *JASMONATE RESPONSIVE 1 (JRI)* and *D-AMINO ACID RACEMASE2 (DAAR2)* (Gifford et al., 2013) (Table 1). In that study, it was suggested that crossing GWAS data with gene expression data highly increased the precision of the prediction. These two examples show that standard GWAS is a powerful tool to study the complex genetic control of RSA in response to N-sensing, even using a relatively limited number of accessions.

## FUTURE PROSPECTS

Careful in-depth characterization of  $\text{NO}_3^-$  transporters in *Arabidopsis* and other plant species provided us with a detailed view of how  $\text{NO}_3^-$  is uptaken, mobilized and used in the plant. Study of transporters also provided key insights to understand sensing of  $\text{NO}_3^-$  as well as other N nutrients (Giehl and von Wirén, 2015). The ability of NPF6.3 (CHL1/NRT1.1) to transport as well as sense  $\text{NO}_3^-$ , places this protein at the top of a  $\text{NO}_3^-$ -signaling pathway. Despite its importance, NPF6.3 (CHL1/NRT1.1) does not

explain the full extent of  $\text{NO}_3^-$  responses observed in plants. For example, transcriptional responses to  $\text{NO}_3^-$  in *nrt1.1* mutants can be restored by a 24h nitrogen starvation (Wang et al., 2009). On the other hand, transcriptome analysis of *nrg1* (mutant allele of NPF6.3 (CHL1/NRT1.1)) reveals 111 genes with lower induction to  $\text{NO}_3^-$  treatment compared to wild type and up to 300 miss-regulated genes (Wang et al., 2009). Furthermore, a meta-analysis combining *npf6.3* and *nlp7* transcriptomes demonstrated that these two key genes for PNR: i) control different set of genes ii) explain only about 50% of the nitrate response (Medici and Krouk, 2014). What explain the full extent of nitrate responses? Future efforts should be directed to better understand the role of NRT2.1 in signaling as well as identifying additional components involved in  $\text{NO}_3^-$  sensing and signal transduction in plants.

Certainly, the crosstalk of  $\text{NO}_3^-$  and auxin and other plant hormones is intricate. Biosynthesis and transport of hormones such as auxin, cytokinin, ethylene and ABA are regulated in response to  $\text{NO}_3^-$ . This is likely only one of the mechanisms of N-dependent modulation of plant development. Many questions remain. One interesting avenue for future research is to dissect  $\text{NO}_3^-$  hormonal crosstalk in the context of known interactions between the hormones themselves at a tissue specific level (Vanstraelen and Benkova, 2012).

There has been remarkable progress in our understanding of  $\text{NO}_3^-$  signaling pathways, particularly the characterization of several master transcription factors downstream of NPF6.3 (CHL1/NRT1.1). These are involved in transcriptional control of key  $\text{NO}_3^-$  responsive genes, such as *NIA1*, *NIA2*, *NiR*, *NR* and *NRT2.1*. However, the fact that different transcription factors (e.g. NLP7, TGA1, TGA4 and TCP20) can regulate expression of the same target genes (e.g. *NRT2.1*) poses the question of how these transcription factors are interacting in the nitrate response. Are they part of a transcription factor complex that mediates transcriptional control in response to  $\text{NO}_3^-$  treatments? Do they work additively? On the other hand, we still do not know how these transcription factors are activated. Phosphoproteomics work is helping characterize many changes in protein phosphorylation that may contribute to address this question. Work on NLP7 also highlights the relevance of subcellular localization. These questions are important to understand how transcription factor cascades unfold downstream of  $\text{NO}_3^-$  sensing.

Responses to  $\text{NO}_3^-$  availability occur at the cellular level and are tissue-specific. This localized response triggers a signaling pathway that will then modulate plant root architecture and also plant physiological responses systemically. During the last 15 years, there have been major advances in our understanding of root foraging and N-signaling (local and systemic). However, some key regulators are still missing to have a more comprehensive view of how N-signaling operates as an integrated system. We need to understand how different N-signals are generated, transmitted and integrated. In that context, "omics" studies (e.g. transcriptomics, metabolomic, proteomic) combined with systems biology tools will be methods of choice to gain insights into the regulatory networks involved in root nutrient foraging. Once root nutrient foraging can be dissected in simple systems (like *in vitro* split root), more complex conditions could be considered to replace foraging in a more realistic and ecological context. In particular, understanding interactions between nitrogen and other nutrients (carbon, phosphate, potassium) that control plant growth will be a major challenge for systems biology in the future. Previous studies investigated foraging using heterogeneous N-systems in a very restricted number of *Arabidopsis* accessions (Remans et al., 2006b; Zhang and Forde, 1998). Expanding the research to various ecotypes would help to disentangle foraging signaling by exploiting natural genetic diversity (cf. below).

Root system architecture is a complex trait that has been successfully assessed in two dimensions (i.e. on plates with agar media), unraveling  $\text{NO}_3^-$ -dependent physiological and developmental responses. The current progress in advanced imaging, combined with new computational tools now allows to add another dimension to this complex analyses (Bao et al., 2014; Dinneny et al., 2008; Rellan-Alvarez et al., 2015; Smith and De Smet, 2012; Topp et al., 2013). Moreover, such studies can also take advantage of GWAS to decode the underlying genetic complexity of the modulation of the root system architecture (Pace et al., 2014). Besides, GWA alternative methods have been developed recently to overcome the classical limitations of standard GWAS (Bergelson and Roux, 2010; Korte and Farlow, 2013; Weigel, 2012). For example, we can now test the association between a phenotype and several SNPs (instead of a single ones) (Qiao et al., 2013; Ray et al., 2015; Segura et al., 2012; Zhang et al., 2013), knowledge from networks datasets can be integrated in GWAS (Huang, 2015; Jia and

Zhao, 2014; Leiserson et al., 2013) and associations can be made using several phenotypes at once (Korte et al., 2011; O'Reilly et al., 2015). These alternative methods should help to gain new insights into the complex genetic networks controlling RSA in response to N.

During the past decade, technological advances helped us produce large amounts of data that catalyzed advances in the characterization of  $\text{NO}_3^-$  responses in plants. However, a major challenge still stands, which is integration of all the available information to generate a holistic model of plant  $\text{NO}_3^-$  signaling. Understanding how  $\text{NO}_3^-$  signaling works and integrates with other plant processes such as other nutrients or stress responses is key to support generation of novel biotechnological solutions for enhanced N-use efficiency for sustainable agriculture.

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## Figure legends

### Figure 1. Localization and function of the *Arabidopsis* nitrate transporters of the NRT2 and NPF families.

The functions depicted are: root uptake (influx/efflux), loading/unloading of the xylem, loading/unloading of the phloem, accumulation in seed vacuoles, and transport into the embryo. At the cellular level, all proteins are localized at the plasma membrane, except NRT2.7 localized at the tonoplast. All NRT2 proteins are assumed to interact with NAR2.1 to be functional, with the possible exception of NRT2.7 (see text). cHATS: constitutive High-Affinity Transport System, iHATS: inducible High-Affinity Transport System.

### Figure 2. Simplified model of the auxin- and calcium-dependent nitrate signaling pathways in roots

Nitrate is sensed by NPF6.3 (CHL1/NRT1.1), that activates a PLC to trigger an increase in cytoplasmic calcium. This increase in calcium activates gene expression of nitrate responsive genes via TGA1/TGA4. The auxin sensor *AFB3* is also transcriptionally regulated in response to nitrate in a calcium-independent manner. Via auxin signaling, *AFB3* activates gene expression of *NAC4* and *OBP4*. After nitrate induction, *AFB3* expression level is regulated post-transcriptionally by miR393.

### Figure 3. Root N-foraging : experimental set-ups, root system architecture (RSA) response and the three underlying signals.

A) The two types of experimental set-ups used to study root N-foraging. +N /-N: medium segment that contains N/no N respectively. Dark blue : PR or « PR-like »; Light blue : LRs. B) RSA response to homogeneous and heterogeneous N-supply in split-root system and the three signals that can be deduced. The bottom of the figure shows how the three signals can be deduced by comparing RSA in different conditions. Loc. : local signal, Syst. : systemic signal. Vertical system (Linkohr et al., 2002b; Zhang and Forde, 1998;

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**Figure 4. Molecular basis of local and systemic N-signaling in split root in *Arabidopsis*.** A) Overview of systemic signals at the whole plant level. B) Local signaling pathway in root cells. IAA : auxin, CK : cytokinin, LR : lateral root.

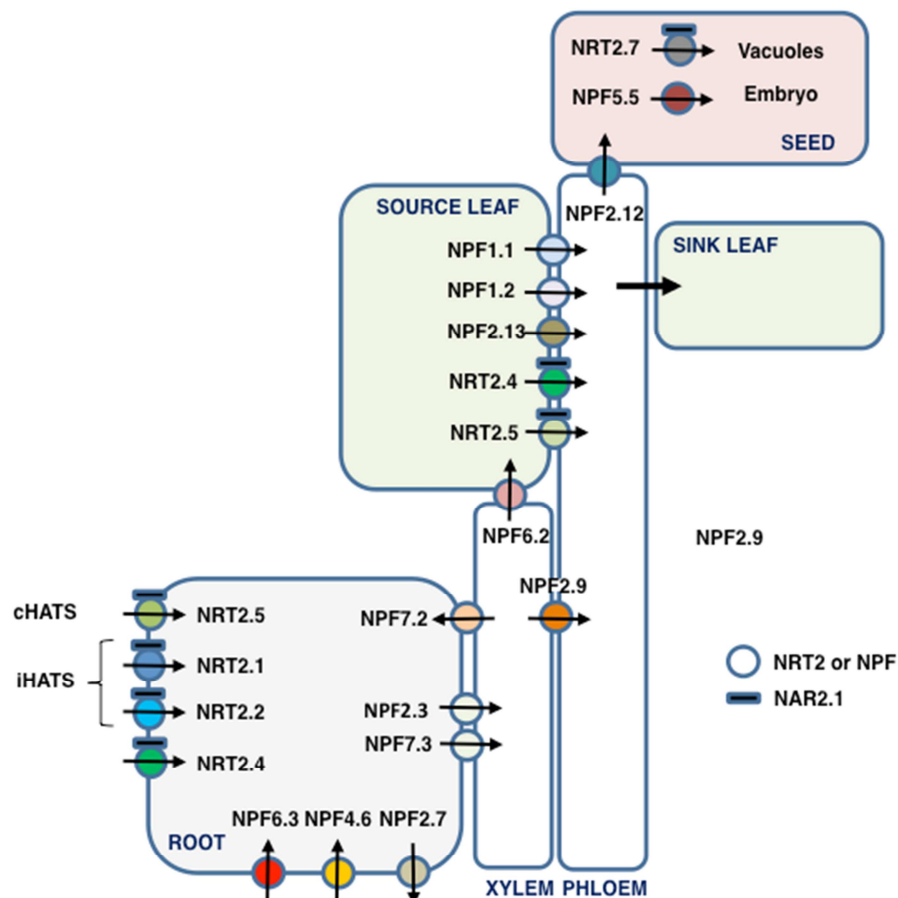
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**Table 1: Genes involved in lateral root development in response to homogeneous N-supply in *Arabidopsis*.**

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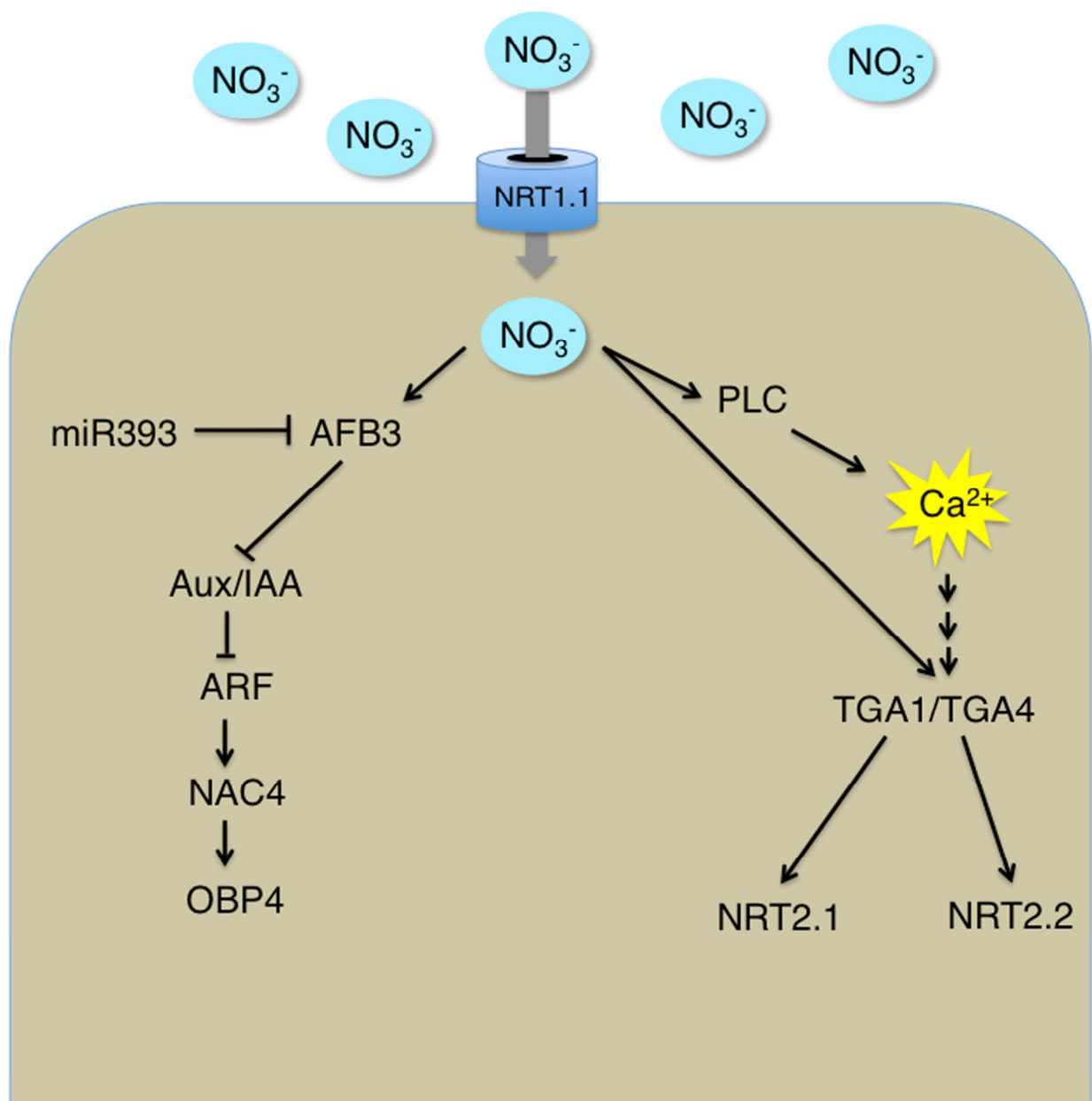
AGI	Gene	Function	Treatment*	Effect on LR development	Transcriptional regulation by N	References
AT1G12110	<i>NRT1.1</i>	Nitrate transceptor	N deprivation	LR repression (emergence)	Induced (in roots) + Post-translational	(Krouk et al., 2010b)
AT1G08090	<i>NRT2.1</i>	Nitrate transporter	C/N availability	LR repression/induction (initiation)	Induced (in roots) + Post-transcriptional and post-translational	(Little et al., 2005; Remans et al., 2006b)
AT2G14210	<i>ANR1</i>	TF MADS box family	Low/medium N	LR induction (elongation)	Induced (in roots)	(Zhang, 1998)
AT4G24020	<i>NLP7</i>	TF RWPPK family	High N	LR (and PR) repression	No +Post-translational (nuclear retention)	(Castaings et al., 2009; Guan et al., 2014; Marchive et al., 2013)
AT5G07680	<i>NAC4</i>	TF NAC family	High N-supply	LR induction	Induced (in roots)	(Vidal et al., 2014a)
AT5G65210/ AT5G10030	<i>TGA1/TGA4</i>	TF bZIP family	High N-supply	LR induction (initiation)	Induced (in roots)	(Alvarez et al., 2014)
AT5G37020	<i>ARF8/mir167</i>	TF ARF family	High N-supply	LR induction (initiation)	Induced (in pericycle)	(Gifford et al., 2008)
AT2G42200	<i>SPL9</i>	TF SBP-box family	Medium N	LR (and PR) induction	Induced (in roots)	(Krouk et al., 2010c)
AT1G12820	<i>AFB3/mir393</i>	Auxin receptor	High N-supply	LR induction (PR repression)	Induced (in roots)	(Vidal et al., 2010)
AT4G24670	<i>TAR2</i>	Auxin biosynthesis- related	Low N	LR induction (emergence)	Repressed (in roots)	(Ma et al., 2014)
AT4G28410	<i>RSA1</i>	Tyrosine transaminase	High N	Root allometry	–	(Rosas et al., 2013)
AT3G23430	<i>PHO1</i>	Phosphate transporter	High N	Root allometry	–	(Rosas et al., 2013)
AT3G16470	<i>JR1</i>	JA responsive gene	Low N	LR induction (elongation)	–	(Gifford et al., 2013)
AT4G02860	<i>DAAR2</i>	Phenazine biosynthesis PhzC/PhzF protein	Low N	LR induction	–	(Gifford et al., 2013)
AT1G75820	<i>CLE-CLV1</i>	Peptides/receptor	N deprivation	LR repression (development and emergence)	CLE repressed (in pericycle)	(Araya et al., 2014)

\*N-deprivation : 0mM N, Low N : [0-0.5]mM, Medium N : [0.5-1]mM, High N : >1mM; N-supply : transitory treatment (3-4 days)



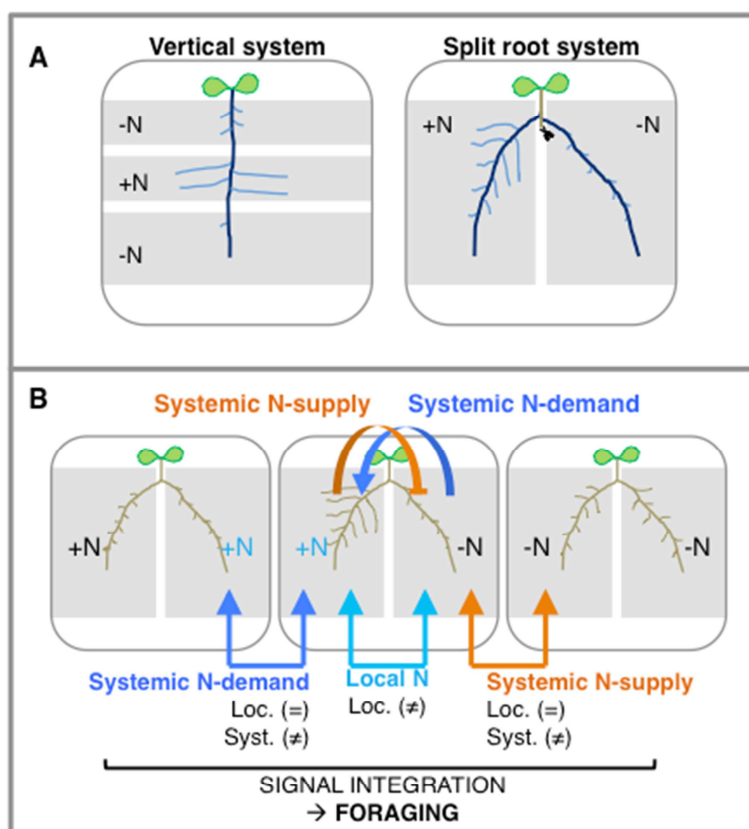
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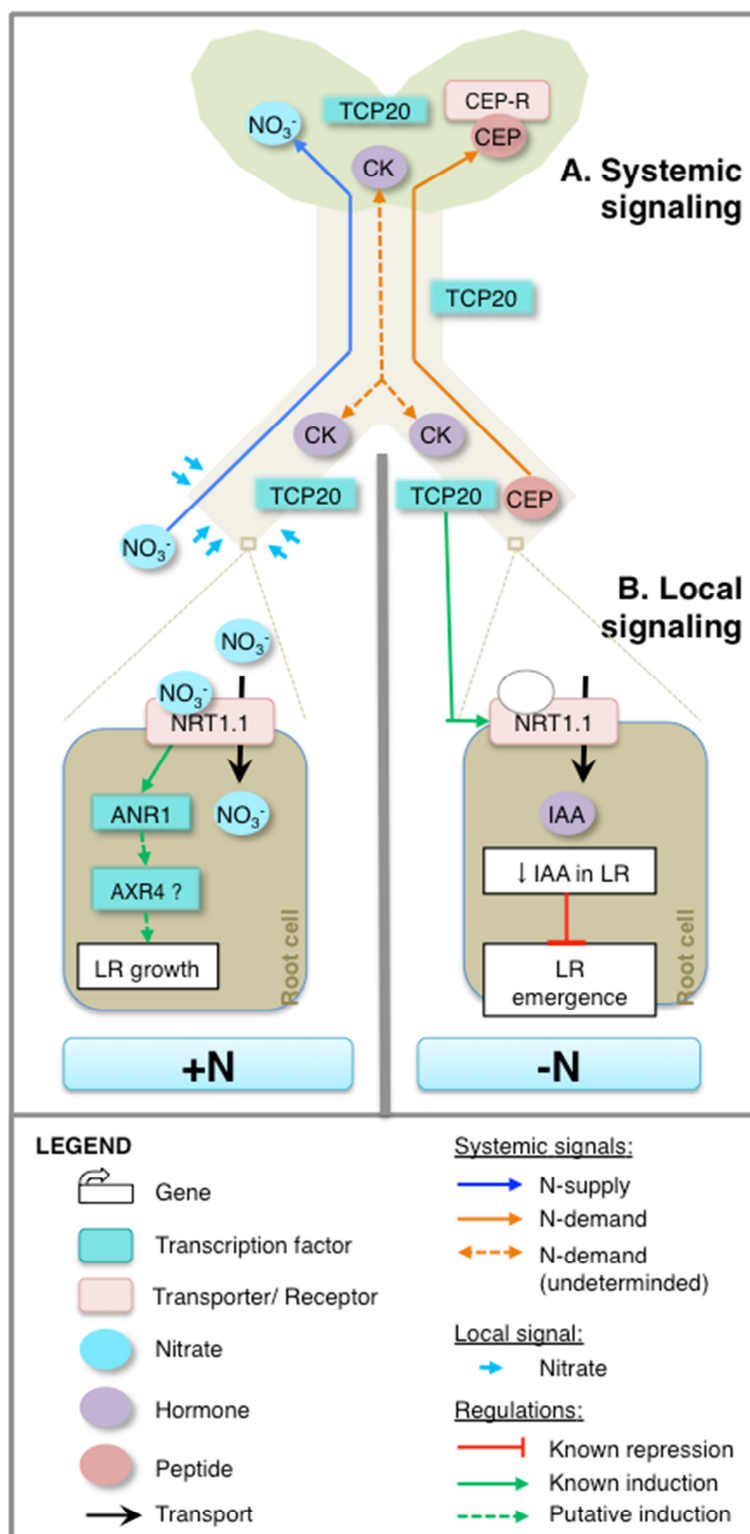
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