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REVIEW PAPER

Nitrate signaling and early responses in Arabidopsis roots

Soledad F. Undurraga, Catalina Ibarra-Henríquez, Isabel Fredes, José Miguel Álvarez, and Rodrigo A. Gutiérrez*

FONDAP Center for Genome Regulation. Millennium Nucleus Center for Plant Systems and Synthetic Biology. Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avenida Libertador Bernardo O'Higgins 340, Santiago, Chile 8331150

* Correspondence: rgutierrez@bio.puc.cl

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Abstract

Nitrogen (N) is an essential macronutrient that impacts many aspects of plant physiology, growth, and development. Besides its nutritional role, N nutrient and metabolites act as signaling molecules that regulate the expression of a wide range of genes and biological processes. In this review, we describe recent advances in the understanding of components of the nitrate signaling pathway. Recent evidence posits that in one nitrate signaling pathway, nitrate sensed by NRT1.1 activates a phospholipase C activity that is necessary for increased cytosolic calcium levels. The nitrate-elicited calcium increase presumably activates calcium sensors, kinases, or phosphatases, resulting in changes in expression of primary nitrate response genes. Consistent with this model, nitrate treatments elicit proteome-wide changes in phosphorylation patterns in a wide range of proteins, including transporters, metabolic enzymes, kinases, phosphatases, and other regulatory proteins. Identifying and characterizing the function of the different players involved in this and other nitrate signaling pathways and their functional relationships is the next step to understand N responses in plants.

Key words: Gene expression, nitrate, nitrogen, phospholipase C, phosphorylation, primary response, signaling.

Introduction

Nitrogen (N) is an essential mineral nutrient that plants require the most in quantitative terms (Frink *et al.*, 1999). It is a critical constituent of proteins and nucleic acids and is therefore indispensable for life. In order to sustain the high crop productivity demanded by modern agriculture, fields are subjected to massive applications of N fertilizers, which contribute 30–50% of their total yield (Crawford and Glass, 1998; Stewart *et al.*, 2005). Global demand for N fertilizers in 2014 was 113 147 000 tonnes. This amount has been projected to grow at approximately 1.4% per year, reaching 119 418 000 tonnes by 2018 (FAO, 2015). From these large amounts of N fertilizer added to crops every year, only 25–50% is taken up by plants. Excess N leaches into water streams, becoming an important promoter of hypoxic zones and eutrophication (Hirel *et al.*, 2011; Robertson and Vitousek, 2009). A significant fraction of N is also converted to N oxide gases that contribute to global warming (Crutzen *et al.*, 2008; Davidson, 2009). Improving N use efficiency in plants is critical for efficient sustainable agriculture.

The main source of N in well-aerated soils is nitrate (NO_3^-) (Crawford and Forde, 2002). Nitrate is also the most abundant N resource available in agricultural lands (Owen and Jones, 2001). Nitrate concentrations in agricultural soils typically range between 1 and 5 mM (Owen and Jones, 2001). This supply is not steady because the predominantly negative charge of ground particles makes the nitrate ion highly mobile in the

soil solution, varying its concentration and potentially causing its depletion by run-off (Miller and Cramer, 2004). Biotic factors such as plant absorption and microbial denitrification also contribute to soil nitrate depletion (Crawford and Glass, 1998).

Plants evolved sophisticated mechanisms to cope with variable N concentrations in the soil. The root architecture adjusts to this fluctuating environment: lateral root elongation is stimulated by exogenous nitrate application, favoring root colonization of nitrate-rich soil patches (Gojon *et al.*, 2009; Zhang and Forde, 1998). However, plants subjected to long-term N treatments have a different behavior: plants grown under N-sufficient conditions develop fewer lateral roots than plants grown under low N conditions, a strategy that allows N foraging only when this nutrient is scarce (Gifford *et al.*, 2008). This local root nitrate acquisition needs to be coordinated with systemic signals in order to coordinate N supply and demand within the plant (Ruffel *et al.*, 2011). Besides its role as a nutrient, nitrate is a local and systemic signal that coordinates its uptake with plant growth and development (Alvarez *et al.*, 2012; Ruffel *et al.*, 2014; Ruffel *et al.*, 2011). Nitrate induces changes in the transcription of genes involved in N acquisition, nitrate assimilation, production of reducing equivalents needed for N metabolism, C metabolism, and an array of other functions (Redinbaugh and Campbell, 1991; Scheible *et al.*, 2004; Vidal and Gutiérrez, 2008; Wang *et al.*, 2000; Wang *et al.*, 2003). Consequently, nitrate has a myriad of effects on plant development: it induces seed germination, regulates root growth and architecture, controls shoot growth, and delays flowering (Alboresi *et al.*, 2005; Castro Marín *et al.*, 2011; Drew and Saker, 1975; Liu *et al.*, 2010; Rahayu *et al.*, 2005; Remans *et al.*, 2006; Vidal *et al.*, 2014; Walch-Liu *et al.*, 2000; Walch-Liu *et al.*, 2006; Yuan *et al.*, 2016).

Inside the cell, nitrate is reduced by Nitrate Reductase (NR) and the nitrite product is further reduced to ammonia by Nitrite Reductase (NiR). The ammonia generated is incorporated into the amino acid glutamate to generate glutamine by the GS/GOGAT cycle (Krapp, 2015; Marschner, 2012; Xu *et al.*, 2012). Subsequent reactions transfer the assimilated N into other amino acids or biomolecules (Krapp, 2015). Since incorporation of inorganic N into amino acids requires a carbon (C) skeleton, a fine balance between N and C must be attained by the plant (Alvarez *et al.*, 2012; Ruffel *et al.*, 2014). Early reports established that nitrate acquisition varies according to the availability of photosynthates, imposing diurnal oscillations over nitrate uptake (Clement *et al.*, 1978; Delhon *et al.*, 1996). Molecular and computational studies proved that the expression of many nitrate-controlled genes is also subjected to regulation by C metabolites (Gutiérrez *et al.*, 2007*b*; Palenchar *et al.*, 2004). Girin *et al.* (2007) identified the N- and-C-dependent transcriptional control of a nitrate transporter, which was localized to a 150 bp sequence that is sensitive to nitrate, N metabolites, and sucrose (Girin *et al.*, 2007). Furthermore, N metabolites control transcription of the circadian clock master regulator *CCA1*, which, in turn, regulates the transcription of genes involved in N assimilation, establishing a connection between the circadian clock and N nutrition (Gutiérrez *et al.*, 2008).

Nitrate signaling has been actively investigated in recent years. In this review, we will discuss recent advances, with an emphasis on early nitrate-elicited changes in calcium levels and protein modification by phosphorylation. For discussion of other aspects of N responses in plants, we recommend a number of recent reviews (Alvarez *et al.*, 2012; Gutiérrez, 2012; Krapp, 2015; Krouk, 2016; Krouk *et al.*, 2011; Medici and Krouk, 2014; O'Brien *et al.*, 2016; Ruffel *et al.*, 2014; Vidal *et al.*, 2014; Vidal *et al.*, 2015).

Nitrate sensing and primary response

The dual-affinity transporter and sensor (transceptor) Nitrate Transporter 1.1 (NRT1.1/NPF6.3) triggers nitrate-dependent changes in gene expression. Besides its nitrate uptake function, NRT1.1 regulates the expression of key nitrate assimilatory genes. Its affinity changes according to the phosphorylation status of residue T101 (Ho *et al.*, 2009; Hu *et al.*, 2009). Under low nitrate conditions, CBL-Interacting Protein Kinase 23 (CIPK23) phosphorylates this residue, shifting NRT1.1 into a high-affinity nitrate carrier (Liu and Tsay, 2003). This change also triggers a weak up-regulation of the *Nitrate Transporter 2.1* (*NRT2.1*) high-affinity nitrate transporter (Ho *et al.*, 2009) (Fig. 1).

The primary transcriptional response to a signal corresponds to changes in gene expression that occur independent of *de novo* protein translation (Herschman, 1991). The nitrate response is characterized by rapid and often transient changes in gene expression that can be detected as early as minutes after nitrate treatments (Gowri *et al.*, 1992; Krouk *et al.*, 2010; Medici and Krouk, 2014). Nitrate response genes are characterized by biological functions such as metabolism, energy, N and sulfur (S) metabolism, amino acid metabolism, ammonium assimilation, the pentose phosphate pathway, glycolysis, and gluconeogenesis (Gutiérrez *et al.*, 2007*a*). The first studies on the primary nitrate response reported a cycloheximide-independent transcript accumulation of the nitrate assimilation enzymes NR, glutamine synthetase (GS2) and ferredoxin-dependent glutamate synthase (Fd-GOGAT) (Gowri *et al.*, 1992; Redinbaugh and Campbell, 1993). With the advent of technologies to evaluate the transcriptome on a routine basis, a more detailed characterization of both primary and secondary nitrate responses has been obtained. An early transcriptomic study using the Affymetrix ATH1 chip showed large effects on gene expression 20 minutes after nitrate treatments. This change was larger in roots, with 1176 affected transcripts, compared with shoots, with only 183 affected transcripts (Wang *et al.*, 2003). Another study reported induction of the nitrate transporters *NRT1.1*, *NRT2.1*, *Nitrate Transporter 2.2* (*NRT2.2*) and *Nitrate Transporter 2.4* (*NRT2.4*) in N-starved seedlings after nitrate addition. All nitrate assimilation genes were also up-regulated (Scheible *et al.*, 2004). To assess the effects of nitrate without the confounding presence of downstream N metabolites, a subsequent transcriptomic study was performed in NR-null double mutant plants. Using this genetic background, 595 nitrate-responsive genes were identified, with over-representation of the following functional categories:

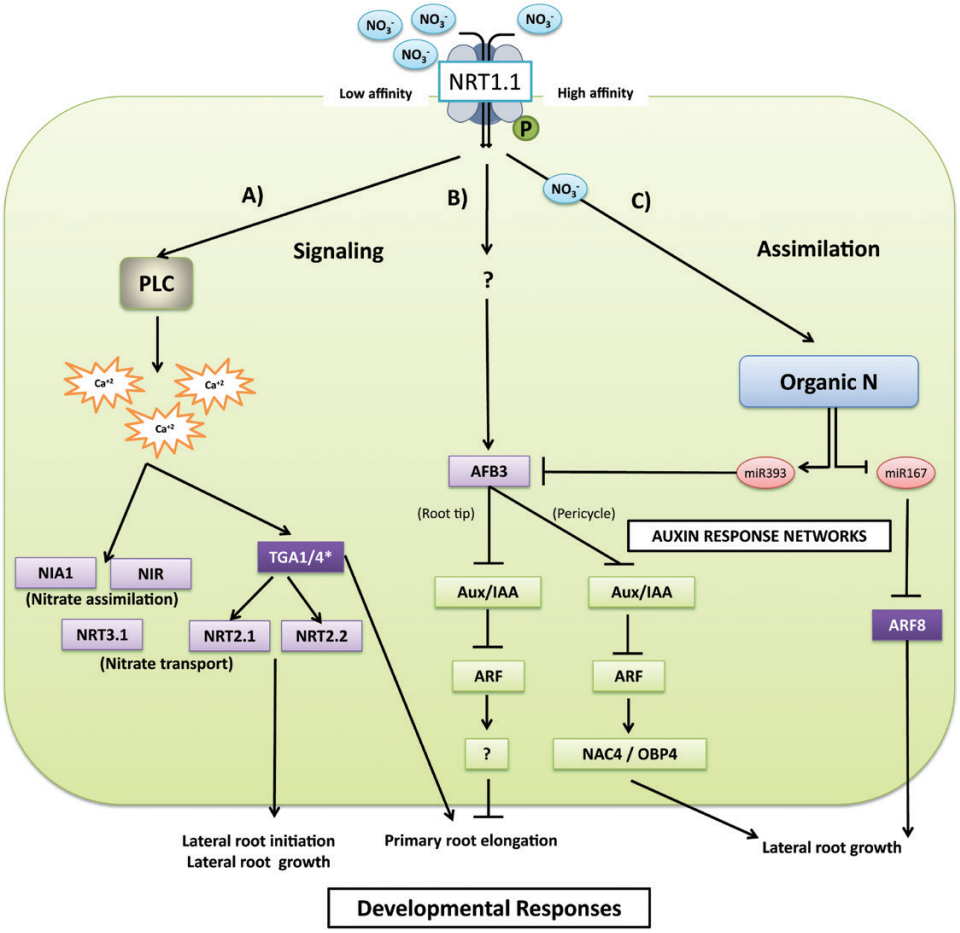

Fig. 1. Summary of nitrate signaling and assimilation. Nitrate is sensed and transported by the NRT1.1 transceptor, changing its affinity by modifications of phosphorylation status and triggering a signaling pathway. Under low nitrate conditions, CIPK23 phosphorylates NRT1.1, changing it into a high-affinity transporter. Nitrate sensing elicits changes in the phosphorylation status of NRT1.1, and activates, through PLC, calcium influx, which acts as a second messenger (A). This cascade mediates changes in the expression of transcription factors (*TGA1/4**) and genes involved in nitrate transport (*NRT2.1*, *NRT2.2* and *NRT3.1*) and nitrate assimilation (*NIA1* and *NiR*). On the other hand, AFB3 is regulated by nitrate in a PLC- and calcium-independent pathway (B). ABF3 modulates the expression of NAC4 and OBP4, with subsequent effects on root remodeling. Finally, nitrate assimilation (C) produces organic N, which induces miR393 and represses miR167, regulating the abundance of AFB3 and ARF8, respectively. Nitrate-responsive genes are depicted in lilac, transcription factors in purple, and microRNAs in pink. For clarity purposes, the cell nucleus is not shown. *TGA1 and TGA4 are redundant regulatory factors that mediate nitrate responses in Arabidopsis roots. However, the connection between TGA4 and the PLC–calcium pathway has not been experimentally validated. Other relevant transcription factors, such as HRS1 and NLP7, were not included in this figure because their connection with calcium signaling is currently unknown. (This figure is available in colour at *JXB* online.)

energy, metabolism, glycolysis, and gluconeogenesis (Wang *et al.*, 2004). A subsequent meta-analysis of transcriptomic data obtained under various nitrate treatments found genes and pathways that were affected in all studies (C and carbohydrate metabolism, N and S metabolism, pentose phosphate pathway, and others), as well as transcripts and functions that were affected only under certain conditions (protein synthesis and others). Comparison of the transcriptomic data of the NR-null double mutants with these datasets suggested that N metabolites regulated changes in expression that were context-dependent (i.e. that responded to nitrate only under certain conditions) (Gutiérrez *et al.*, 2007*a*).

N availability alters hormonal signaling pathways in order to control plant growth (Krouk, 2016). Ristova *et al.* (2016) used combinatorial treatments of hormones (auxin, cytokinin, and abscisic acid) and N (nitrate and ammonium) to dissect N–hormone interactions. Individual treatments had distinct effects upon root growth, whereas combinations of treatments showed an array of interactions that ranged from pronounced (auxin and nitrate) to non-significant (nitrate and ammonium). A strong interaction between auxin and both nitrate and ammonium was found. Another strong and complex interaction between cytokinin, abscisic acid, nitrate, and ammonium suggested the existence of integration mechanisms for these signals. Measurements of genome-wide expression changes and subsequent GeneCloud analysis (Krouk *et al.*, 2015) showed that specific signals or their combinations target specific regulatory modules. Certain genes showed 'logic gate' expression changes, with up-regulation occurring only under combined treatments. From all the ATH1 probes tested in this work, approximately 42% responded to composite signals, indicating that most gene expression changes are regulated by multiple inputs (Ristova *et al.*, 2016).

The importance of NRT1.1 in nitrate responses was first established by a Serial Analysis of Gene Expression (SAGE) approach in *Arabidopsis thaliana* (Muños *et al.*, 2004). In this

study, transcript abundance in roots of the loss-of-function *chl1-5* mutant differed by roughly 400 transcripts compared with wild-type plants when grown in the presence of ammonium nitrate. Transcripts of the *Nitrate Transporter 1.5* (*NRT1.5*/*NPF7.3*) gene as well as the amino acid transporter At4g38250 were overexpressed. Interestingly, this mutant also showed a strong up-regulation of the high-affinity nitrate transporter NRT2.1. These results suggested that NRT1.1 is required for normal regulation of expression of nitrate transporters and other genes (Muños *et al.*, 2004). Additional evidence supporting a role for NRT1.1 in nitrate sensing came from experiments with the loss-of-function mutants *chl1-5* and *nrg1*: both alleles altered the nitrate-dependent induction of *Nitrate reductase 1* (*NIA1*), *Nitrite Reductase 1* (*NiR*), and *NRT2.1* gene expression (Wang *et al.*, 2009). The function of NRT1.1 as a sensor was further supported by analysis of the *chl1-9* mutant, which retains NRT2.1 biphasic gene expression response but is deficient in nitrate transport (Ho *et al.*, 2009). Interestingly, recent evidence indicates that nitrate-dependent gene induction and developmental responses are controlled by independent signaling pathways that are triggered downstream of NRT1.1 (Bouguyon *et al.*, 2015).

Nitrate signaling and calcium

One model for nitrate signaling proposes that nitrate is sensed by NRT1.1, eliciting the immediate production of second messengers, which would consequently trigger changes in gene expression (Medici and Krouk, 2014; Wang *et al.*, 2009). An attractive second messenger in this pathway is calcium (Ca^{2+}). Support for the role of Ca^{2+} in nitrate signaling comes from early experiments in barley and corn, in which the expression of nitrate-responsive genes was shown to be altered by either EGTA or $LaCl_3$ pre-treatment, pointing to a potential role of Ca^{2+} as a second messenger (Sakakibara *et al.*, 1997; Sueyoshi *et al.*, 1999).

Ca^{2+} is a key second messenger required for signal transduction in plants and other organisms (Dodd *et al.*, 2010; Steinhorst and Kudla, 2014). Intracellular Ca^{2+} levels are in the micromolar range and are subject to tight regulation. In contrast, the cell exterior and intracellular compartments, such as the vacuole and endoplasmic reticulum (ER), have higher Ca^{2+} concentrations, typically in the millimolar range. Consequently, these different Ca^{2+} concentrations generate an electrochemical gradient across these compartments (Hashimoto and Kudla, 2011). This gradient allows a fast influx of Ca^{2+}, facilitated by Ca^{2+} channels, into the cytoplasm. Ca^{2+} channels are located in the plasma membrane and in internal reserve compartments such as the vacuole and ER. Three types of Ca^{2+} channels have been identified in animals: voltage-dependent calcium channels (VDCCs), receptor-operated calcium channels (ROCCs), and mechanical-stimulation-gated channels (Hamilton *et al.*, 2015; Sukharev and Sachs, 2012; Tsien and Tsien, 1990). VDCCs and ROCCs have been found in plants (Nagata *et al.*, 2004; Sanders *et al.*, 2002). Electrophysiological and genetic approaches have been used to demonstrate that mechanosensitive calcium channels are also present in plants (Hamilton *et al.*, 2015).

Different biotic and environmental perturbations can cause specific spatiotemporal changes in cytosolic Ca^{2+} concentration (Dodd *et al.*, 2010). Different types of stimuli trigger unique changes in free cytosolic Ca^{2+} that differ in frequency, amplitude, and localization. The 'Ca^{2+} signature' differs according to the identity of the Ca^{2+} elicitor and its intensity. Ca^{2+} sensor proteins, such as calmodulin kinases (CaM), CaM-related proteins, Ca^{2+}-dependent protein kinases (CDPK), and calcineurin-like proteins (CBL), perceive cytosolic Ca^{2+} changes and transduce the signal to downstream signaling cascades that trigger changes in enzyme activity, cytoskeleton orientation, phosphorylation, and gene expression (Dodd *et al.*, 2010; Hashimoto and Kudla, 2011).

It was recently shown that Ca^{2+} has a role in plant nitrate signal transduction and is important for nitrate-dependent regulation of gene expression in *A. thaliana* plants. Using aequorin reporter lines, it was shown that nitrate treatment causes a rapid increase in cytoplasmic Ca^{2+} levels in roots as well as in whole seedlings (Riveras *et al.*, 2015). This response was inhibited when plants were pre-treated with either the Ca^{2+} channel blocker $LaCl_3$ or the chelating agent EGTA. In addition, this Ca^{2+} increase was abolished in the *NRT1.1* mutants *chl1-5* and *chl1-9*, demonstrating that this response requires a functional transceptor. A concomitant increase in inositol 1, 4, 5-triphosphate (IP3) in response to nitrate treatments suggested that activity of a PLC is also involved in this pathway (Riveras *et al.*, 2015).

PLCs are membrane-associated enzymes that break phospholipids, causing lipid membrane remodeling and generating multiple second messengers (Tuteja and Sopory, 2008). In plants, there are two classes of PLCs, which differ according to their substrate specificity: phosphatidylinositol-specific (PI-PLC) and non-specific (NPC). Plant NPCs share homology with bacterial PLCs. NPCs can have a preference for either acetylcholine (PC-PLC), phosphatidylethanolamine (PE-PLC), or phosphatidylserine (PS-PLC) (Rupwate and Rajasekharan, 2012).

PI-PLCs are the most extensively studied PLC class. They hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) from the plasma membrane, producing IP3 and diacylglycerol (DAG) (Rupwate and Rajasekharan, 2012; Singh *et al.*, 2015). The mechanism of action of PI-PLCs has been widely studied in animals and is based on activation of PI-PLCs by the α subunit of G protein (Gαq), which is dissociated in response to a stimulus (Munnik and Testerink, 2009). Once PI-PLC is activated, it splits a membrane phospholipid into IP3 and DAG. Subsequently, IP3 is released into the cytoplasm, where it can bind to IP3 receptors, promoting Ca^{2+} influx from intracellular reserves. The released Ca^{2+} can act as a second messenger (Hashimoto and Kudla, 2011; van Leeuwen *et al.*, 2007). DAG remains membrane bound and can activate protein kinase C (PKC), which transduces the signal to effector molecules (Munnik and Testerink, 2009). DAG can also be converted to diacylglycerolpyrophosphate (DGPP), through the action of diacylglycerol kinase (DGK). DGPP can be used as a substrate to generate phosphatidic acid (PA) through phospholipase D (PLD) activity.

This mode of action of PI-PLCs appears to be present in plants but with some inconsistencies in comparison to animal systems: biochemical studies have shown that the plasma membrane PIP2 levels of plants are much lower than in animals (Delage *et al.*, 2013). This was recently confirmed by experiments using radioactivity and biosensors: only small quantities of PIP2 were found in plasma membrane of plants by FRAP (fluorescence recovery after photobleaching) analysis and co-labeling experiments (Delage *et al.*, 2013; Munnik and Testerink, 2009; van Leeuwen *et al.*, 2007). Furthermore, plant cells lack homologs of animal IP3 receptors and protein kinase C signaling pathway components (Chen *et al.*, 2011; Munnik and Testerink, 2009). Despite these differences from the animal model, products generated from IP3 and DAG, in particular inositol hexaphosphate (IP6) and phosphatidic acid (PA), respectively, can act as second messengers in plants (Arisz *et al.*, 2009; Chen *et al.*, 2011).

PI-PLC proteins have three minimal domains: the EF-hand in the N-terminal; the XY catalytic domains required for phosphotesterase activity; and a C2 domain on the C-terminal, which is responsible for Ca^{2+}-dependent phospholipid binding (Singh *et al.*, 2015; Tasma *et al.*, 2008). In addition, a PH (pleckstrin homology) domain is found only in animals, and is responsible for the recognition of PIP2, which facilitates PLC membrane attachment (Singh *et al.*, 2015). This domain has not been found in plants, and it is not known whether and how PIP2 binding occurs in plants (Pokotylo *et al.*, 2014). PI-PLCs are also subclassified according to their subcellular localization as membrane-bound or cytosolic (Singh *et al.*, 2015). Enzymatic activity for both groups is dependent on Ca^{2+} availability, which can also regulate substrate availability (Chen *et al.*, 2011; Singh *et al.*, 2015). Despite the absence of the PIP2 binding domain, it has been reported that the activity of PI-PLCs requires cell membrane association, either transiently or permanently, in order to associate with PIP2 (Helling *et al.*, 2006; Lomasney *et al.*, 1996).

Nine PI-PLC genes (*AtPLC1–AtPLC9*) have been identified in the *A. thaliana* genome (Mueller-Roeber and Pical, 2002; Tasma *et al.*, 2008). Many of these have been characterized as multifunctional enzymes because of their role in modulating plant physiology under different biotic and abiotic stresses, nutritional deficiencies, or environmental conditions (Rupwate and Rajasekharan, 2012; Singh *et al.*, 2015). In addition, it has been shown that three PLCs and most NPCs are implicated in growth and development processes in *A. thaliana* (Singh *et al.*, 2015). A significant role for *Nt*PLC3 in pollen tube growth has been reported in *Nicotiana tabacum*: *Nt*PLC3 accumulates in the pollen tube plasma membrane during elongation. Growth of tobacco pollen tubes was blocked by treatment with U73122, a PI-PLC inhibitor; it was also reduced by *Nt*PLC3 overexpression (Helling *et al.*, 2006).

Expression analysis for various PI-PLC genes in *A. thaliana* showed that PLC isoforms are differentially expressed in diverse plant organs, as well as in response to biotic and abiotic stresses (Arisz *et al.*, 2009; Helling *et al.*, 2006; Hunt *et al.*, 2004; Liu *et al.*, 2006; Munnik and Testerink, 2009; Pokotylo *et al.*, 2014; Tasma *et al.*, 2008). *AtPLC1*, *AtPLC2*, *AtPLC3*, *AtPLC4*, *AtPLC5*, and *AtPLC9* are specifically expressed in roots (Pokotylo *et al.*, 2014; Tasma *et al.*, 2008). Sequence homology, predicted phylogenetic relationships, and expression patterns suggest that some *AtPLC* pairs (*AtPLC1–ATPLC3*, *AtPLC4–AtPLC5*, and *AtPLC8–AtPLC9*) might be related to each other. *AtPLC8* and *AtPLC9* are proposed to lack enzymatic activity because they have a large deletion in the Y catalytic domain (Tasma *et al.*, 2008). However, it has been shown that *AtPLC9* has a role in stress responses. *plc9* loss-of-function mutant plants have a heat-resistant phenotype (Zheng *et al.*, 2012). Interestingly, it has also been shown that *AtPLC4* and *AtPLC5* expression is induced by nitrate: several previous studies showed that their mRNA levels were up-regulated after KNO_3 treatments (Alvarez *et al.*, 2014; Canales *et al.*, 2014; Riveras *et al.*, 2015; Vidal *et al.*, 2013*b*; Wang *et al.*, 2003; Wang *et al.*, 2004).

A direct link between nitrate signaling and PI-PLCs has been established in Arabidopsis. Nitrate treatment triggered an increase in IP3 and cytoplasmatic Ca^{2+}, which was not observed in plants pre-treated with the PLC inhibitor U73122. This suppression of nitrate-dependent increases in cytoplasmatic Ca^{2+} and IP3 was also observed in two different *NRT1.1* mutants, *chl1-5* and *chl1-9*, demonstrating that this response is NRT1.1-dependent. Furthermore, U73122 pre-treatment also affected the induction of canonical nitrate-responsive genes [*NRT2.1*, *TGACG Sequence-specific Binding Protein 1* (*TGA1*), *NiR*, *Nitrate Transporter 3.1* (*NRT3.1*), and *NIA1*], which resembled the expression levels observed for *nrt1.1* mutants under the same conditions. These responses were not observed when plants were pre-treated with the non-functional analog U73343 (Riveras *et al.*, 2015; Thompson *et al.*, 1991).

These results suggest that NRT1.1 as well as phospholipase activity are required for nitrate-dependent increases in cytoplasmic Ca^{2+} levels and IP3 (Fig. 1A). However, it was also observed that expression of the nitrate-sensitive gene *Auxin Signaling F-Box 3* (*AFB3*) was not affected (Fig 1B). This indicates that as well as the Ca^{2+} and PI-PLC-dependent pathway downstream of NRT1.1, there is another PI-PLC-independent pathway that controls the expression of nitrate-responsive genes (Riveras *et al.*, 2015).

The role of phosphorylation in nitrate signaling

Phosphorylation is arguably one of the most important and well-documented post-translational protein modifications. Activation or inactivation of protein function, including components in signaling pathways, are mediated by phosphorylation/autophosphorylation cycles. Protein phosphorylation can impact the ability of proteins to interact to form hetero- or homodimers, protein stabilization or degradation, and change in localization, among other functions (Huber, 2007; Hunter, 1995; Olsen *et al.*, 2006).

The role of protein phosphorylation in nitrate signaling was initially suggested through experiments with protein kinase and phosphatase inhibitors (Sakakibara *et al.*, 1997;

Sueyoshi *et al*., 1999). Kinase and phosphatase inhibition had distinct effects on the expression of genes coding for nitrogen-assimilatory enzymes, such as NR, NiR, and GS2, in maize leaves (Sakakibara *et al*., 1997). Inhibition of Ca^{2+}/calmodulin-dependent protein kinases by treatment with the antagonists W-7 and trifluoperazine had no effect on *NR* and *NiR* mRNA levels in barley leaves (Sueyoshi *et al*., 1999). However, pre-treatments with W-7 had an inhibitory effect on the nitrate-elicited response of the *NR* (34%), *NiR* (34%), *GS2* (63%), and *Fd-GOGOAT* (59%) genes. This implies that calmodulin-dependent and -independent protein kinases are at least partially involved in the nitrate signaling pathway (Sakakibara *et al*., 1997). Similarly, the importance of other groups of protein kinases has been proposed. It has been reported that the tyrosine kinase inhibitors genistein, quercetin, and curcumin compromise nitrate response in barley leaves (Sueyoshi *et al*., 1999). On the other hand, involvement of type 1 and type 2A serine/threonine protein phosphatases in nitrate signaling has been found using the inhibitors okadaic acid and calyculin A (Sueyoshi *et al*., 1999). Strong down-regulation of nitrate-induced accumulation of *NR* and *NiR* mRNAs in both maize and barley leaves suggests that alteration of protein dephosphorylation also has an impact on nitrate signaling (Sakakibara *et al*., 1997; Sueyoshi *et al*., 1999).

The function of central components involved in nitrate signaling and transport are modulated by phosphorylation/dephosphorylation. Nitrate transport activity across the plasma membrane and the tonoplast is regulated by phosphorylation (Liu and Tsay, 2003; Migocka *et al*., 2013). NRT1.1 can switch its affinity for nitrate depending on the phosphorylation status of a key threonine residue, T101. This residue is located in the intracellular side, between the second and third transmembrane helices of NRT1.1 (Ho *et al*., 2009; Hu *et al*., 2009; Liu and Tsay, 2003). Under low-nitrate conditions, CBL-Interacting Protein Kinase 23 (CIPK23) phosphorylates this residue, shifting NRT1.1 into a high-affinity nitrate carrier (Liu and Tsay, 2003) (Fig. 1). In addition, the nitrate primary response is highly reduced and a weak up-regulation of the NRT2.1 high-affinity transporter also occurs under low-nitrate conditions (Filleur *et al*., 2001; Ho *et al*., 2009). Recently, it was found that the calcium sensor Calcineurin B-Like Protein 1 (CBL1) and the ABA-sensitive Protein Phosphatase 2C (ABI2) are also important players in nitrate signaling in *A. thaliana* (Léran *et al*., 2015). The activity of ABI2 prevents full phosphorylation of CIPK23, inhibiting its kinase activity toward its substrate, NRT1.1. Under abiotic stress conditions, ABA inactivates ABI2, releasing its influence over CIPK23 and enhancing phosphorylation of NRT1.1, resulting in a net decrease in nitrate uptake. This finding suggest a mechanism by which plant stress signaling and nutrient uptake are coordinated by ABI2 (Léran *et al*., 2015).

Mutations mimicking or eliminating T101 phosphorylation abolish the dual-affinity transport activity of NRT1.1 and lock the transporter in either the high-affinity or low-affinity mode, respectively (Liu and Tsay, 2003). Recently, two independent groups unraveled the three-dimensional structure of NRT1.1 by X-ray crystallography (Parker and Newstead, 2014; Sun *et al*., 2014). The structural analysis showed a phosphorylation-dependent dimerization switching mechanism for the dual-affinity transporter. When T101 of NRT1.1 is dephosphorylated, the transporter forms homodimers and works as a low-affinity transporter. On the other hand, phosphorylated T101 turns the protein into a high-affinity transporter by structurally decoupling the dimer (Sun *et al*., 2014; Sun and Zheng, 2015). This finding is consistent with the idea that the non-phosphorylated form of NRT1.1 is the predominantly active form in nitrate signaling (Ho *et al*., 2009). A more recent report showed that phosphorylation of NRT1.1 has different signaling functions: the dephosphorylated form is critical for the primary root response, represented by the short-term induction of *NRT2.1*. The phosphorylated form triggers the induction of auxin transport, repressing lateral root emergence under low-nitrate conditions (Bouguyon *et al*., 2015). Therefore, differential phosphorylation of NRT1.1 not only affects the affinity of the transporter, but can also affect the output targets of the primary nitrate response.

Besides its role in nitrate acquisition and the primary response, phosphorylation also affects N metabolism. The first step of nitrate reduction is catalyzed by NR, an enzyme that is subjected to post-translational control by phosphorylation and a subsequent inhibitory interaction with 14-3-3 proteins (Bachmann *et al*., 1996*b*; Kaiser *et al*., 2002). This regulation was first observed as a change in NR activity during light/dark cycles in spinach leaves. Experiments using ^{32}P labeling and kinase assays established that these changes were dependent on the phosphorylation status of NR (Huber *et al*., 1992; Mackintosh, 1992). Site-specific phosphorylation was assessed with peptide-antibodies raised against serine 543 and phospho-serine 543 of NR, showing that phosphorylation at this site was necessary but not sufficient for 14-3-3 binding (Weiner and Kaiser, 2001). Later studies showed that, in the presence of Mg^{2+} and/or other divalent cations, 14-3-3 proteins bind phospho-NR, inactivating it (Athwal and Huber, 2002; Bachmann *et al*., 1996*a*). This inhibition/activation switch is triggered by several environmental and intrinsic factors, such as photosynthesis, sugars, anoxia, and pH. Nitrate does not have any effect on this post-translational modification. However, it does exert transcriptional control over the *NR* gene (Kaiser *et al*., 2002).

A full-scale proteomic study performed in Arabidopsis seedlings under nitrate deprivation conditions showed that nitrate starvation and resupply affects both protein abundance and phosphorylation modifications. Changes in abundance were observed for 170 proteins, and 36 were reported to modify their phosphorylation status. Nitrate deprivation increased the abundance of stress-responsive proteins and proteins that play roles in catabolism and proteolysis; it also down-regulated biosynthetic proteins. After a 48-hour starvation, a down-regulation of enzymes such as NiR, Carbamoyl Phosphate Synthase (CARB), Arginosuccinate Synthase (ACC), and Carbonic Anhydrase (CA2) was observed (Wang *et al*., 2012). Engelsberger and Schulze (2012) assessed phosphorylation changes triggered by nitrate and ammonia

treatments after N starvation. In general, transient phosphorylation changes were observed predominantly in the AHA1 and 2 subunits from the P-type plasma membrane ATPase and in proteins involved in N assimilation, amino acid biosynthesis, nucleotide metabolism, and tetrapyrrole synthesis. In general, protein phosphorylation status changed upon N resupply, with short-term responses centered in plasma membrane-associated proteins, medium-term responses with cytosolic proteins, and long-term responses with nuclear/cell interior proteins. This phosphorylation response behaved in the manner of 'waves' from the cell membrane to the interior. The most rapid phosphorylation change observed by the authors was in the high-affinity transporter NRT2.1, which was dephosphorylated within 3 minutes of N supply. Changes in the phosphorylation status of the ammonium transporters AMT1.1 and AMT1.3 were also observed (Engelsberger and Schulze, 2012).

A relationship between Ca^{2+} signaling and phosphorylation was previously established in animal models (Gresset *et al.*, 2010). This study showed that the activity of some PI-PLCs was modulated by phosphorylation of the X/Y linker, which connects the two catalytic domains. This post-translational modification regulated PI-PLC activity, establishing a link between phosphorylation and phospholipase activation (Gresset *et al.*, 2010). Phosphorylation sites have already been identified in many plant PI-PLCs by either mass spectrometry or bioinformatics predictions (Durek *et al.*, 2010). A phosphoproteomic study in nitrogen-starved Arabidopsis seedlings showed that proteins from the phosphatidylinositol pathway are phosphorylated upon nitrate resupply. This effect was not observed when the seedlings were provided with ammonium as a N source, establishing that N-dependent phosphorylation of this pathway is nitrate-specific (Engelsberger and Schulze, 2012). Further characterization of plant PI-PLCs involved in nitrate signaling, as well as their modulation by phosphorylation, would provide a better understanding of the underlying mechanism that connects Ca^{2+}, phosphorylation, and nitrate signaling.

Nitrate-elicited changes in gene expression

From the evidence discussed above, it can be concluded that nitrate-triggered changes in phosphorylation status and intracellular Ca^{2+} levels affect the transcription of at least some nitrate-responsive genes. Thousands of N-regulated genes have been identified from transcriptomic studies, generating mounting evidence pointing to either endogenous or exogenous N signals controlling a broad range of responses in different processes, including metabolism, growth, and development (Alvarez *et al.*, 2012; Ruffel *et al.*, 2014; Wang *et al.*, 2004; Wang *et al.*, 2003). Underlying N regulation of metabolism and development are transcription factors, which play important roles in regulating the expression of nitrate-responsive genes, including sentinel genes such as *NRT1.1*/*NPF6.3*, *NRT2.1*, *NRT2.2*, *NIA1*, *NIA2*, and *NIR*. So far, only a handful of transcription factors mediating nitrate responses have been identified: these are Arabidopsis Nitrate Regulated 1 (ANR1) (Zhang and Forde, 1998), NIN-like Protein 6 (NLP6) (Konishi and Yanagisawa, 2013), NIN-like Protein 7 (NLP7) (Marchive *et al.*, 2013), LOB Domain-Containing proteins (LBD37/38/39) (Rubin *et al.*, 2009), Squamosa Promoter Binding Protein-Like 9 (SPL9) (Krouk *et al.*, 2010), Basic Leucine-Zipper 1 (bZIP1) (Para *et al.*, 2014), NAC Domain Containing Protein 80 (NAC4) (Vidal *et al.*, 2013*b*), TGA1/TGA4 (Alvarez *et al.*, 2014), Teosinte Branched1/Cycloidea/Proliferating Cell Factor 20 (TCP20) (Guan *et al.*, 2014), and Nitrate Regulatory Gene (NRG2) (Xu *et al.*, 2016). Interaction with target gene promoters has been experimentally verified for only TGA1, NLP6/7, bZIP1, and TCP20.

NLP7 has been described as an important regulator of early nitrate-dependent gene expression changes. Its subcellular localization is nitrate responsive: in the presence of nitrate, NLP7 moves from the cytoplasm into the nucleus. Different *nlp7* mutant alleles treated with nitrate show a misregulation of up to 58% of nitrate-induced genes. In one study using chromatin immunoprecipitation coupled to a whole-genome tiling array (ChIP-chip), 851 genes were identified as being bound by NLP7 in the presence of nitrate, further supporting a role for NLP7 as a main regulator (Marchive *et al.*, 2013). Recently, a genetic screen led to the identification of NRG2 as a new regulatory factor of the nitrate response: a mutation in *NRG2* disrupted the induction of nitrate-responsive genes after nitrate treatment. Interestingly, NRG2 interacts physically with NLP7 in the nucleus (Xu *et al.*, 2016). TCP20 was identified by yeast one-hybrid screens that used the nitrate enhancer DNA fragments of *NIA1* and *NRT2.1* as baits. Expression of over 100 nitrate-responsive genes is controlled by this transcription factor. Electrophoretic mobility shift experiments demonstrated that TCP20 binds to the promoters of *NIA1*, *NRT2.1*, and *NRT1.1*. Root growth assays of loss-of-function mutants revealed an impairment in nitrate-induced lateral root growth (root foraging) and an impairment in systemic root growth. Therefore, this transcription factor has a critical role in nitrate-induced transcriptional changes, systemic signaling, and root foraging (Guan *et al.*, 2014). The transcription factors TGA1 and TGA4 were identified through an integrative bioinformatics approach. Transcript levels of *TGA1* and *TGA4* vary in response to nitrate treatments of Arabidopsis roots. Experiments with the ATH1 Affymetrix microarray showed that the *tga1*/*tga4* double mutant and wild-type plants had different nitrate responses, demonstrating that these transcription factors regulate genes involved in nitrate transport and metabolic functions. ChIP analysis indicated that these transcription factors control the expression of the high-affinity nitrate transporters *NRT2.1* and *NRT2.2* by binding directly to their promoters. Moreover, mutations of either *tga1*/*tga4* or *nrt2.1*/*nrt2.2* show similar alterations in nitrate-dependent lateral root growth. This suggests that TGA1/TGA4 and NRT2.1/NRT2.2 function in the same nitrate signaling pathway, regulating lateral root density in Arabidopsis (Alvarez *et al.*, 2014).

In recent years, a novel technique named TARGET (Transient Transformation System for Genome-Wide Transcription Factor Target Discovery) was developed for analysis of direct transcriptional control. Briefly, this method

consists of the transfection of protoplasts with a construct containing red fluorescent protein (RFP) and the transcription factor of interest fused to the glucocorticoid receptor (GR). RFP-containing protoplasts are then selected by fluorescence-activated cell sorting (FACS). The effect of the transcription factor on gene induction is subsequently assessed by dexamethasone treatment, which allows entry of the transcription factor into the nucleus. Experiments are performed either with or without the translation repressor cycloheximide, allowing discrimination between direct and indirect transcription factor targets (Bargmann *et al.*, 2013).

This approach enabled the discovery of downstream targets for the transcription factor Hypersensitivity to Low Pi-Elicited Primary Root Shortening 1 (HRS1), which is strongly induced by nitrate. Interestingly, GO and semantic gene enrichment analysis (Krouk *et al.*, 2015) of targets of HRS1 showed that the terms 'phosphate' and 'cell division' are up-regulated by this transcription factor. Further analysis showed that HRS1 is involved in both nitrate and phosphate signaling, integrating both pathways (Medici *et al.*, 2015).

The downstream targets of the master transcription factor bZIP1, which integrates light and N sensing, were also identified using TARGET and ChIP-Seq (Obertello *et al.*, 2010; Para *et al.*, 2014). A total of 1308 bZIP1 primary targets were found by Para *et al.* (2014). They were categorized according to their interaction with bZIP1 and by the downstream regulation exerted by this transcription factor, as type I, poised (bound by bZIP1, but not regulated by it); type II, stable (bound and regulated by bZIP); and type III, transient (not bound, but regulated by bZIP). Interestingly, N-related biological processes were enriched in the type III class. Further analysis revealed that the canonical early N response genes *NRT2.1*, *NIN-like protein 3* (*NLP3*), and *LBD39* belong to this category. These results support the conclusion that the early N response follows a 'hit-and-run' transcription model, in which a transcription factor rapidly and transiently activates a large number of targets, facilitating the spread of the signal (Para *et al.*, 2014).

Post-transcriptional mechanisms have also been found to be involved in nitrate-elicited gene expression changes. A study focusing on cell-specific root nitrogen responses (Birnbaum *et al.*, 2003) showed that exogenous application of nitrate causes an increase in organic N, triggering the down-regulation of miR167, which targets the auxin response factor ARF8. This mechanism has the net effect of an up-regulation of ARF8 in pericycle cells in response to nitrate, controlling N-dependent lateral root initiation (Gifford *et al.*, 2008) (Fig. 1C). The authors also reported that, from the initial ~6000 nitrate-responsive transcripts found, only 771 responded across all five cell types studied, and 87% were differentially regulated in one or a few cell types, indicating that N responses vary within root cell types (Gifford *et al.*, 2008). It has been shown that the miR393/AFB3 regulatory module regulates nitrate-mediated root branching by an incoherent feed-forward loop in which exogenous nitrate induces early induction of AFB3. However, at later time points, miR393 is also induced, targeting the AFB3 mRNA and decreasing its abundance (Vidal *et al.*, 2010). Subsequently, AFB3/miR393 control the expression of the transcription factor NAC4 (Vidal *et al.*, 2013*b*) (Fig 1b).

Many early transcriptomic studies that address plant responses to nitrate relied on the Affymetrics Arabidopsis ATH1 Genome Array. This methodology has inherent limitations, in that it allows researchers to analyze only genes that are represented within the array. Vidal *et al.* (2013*a*) used next-generation sequencing technology to explore mRNA and small RNA components of the transcriptome. Besides the 13411 nitrate-responsive genes that are present in the array, this study identified 3022 additional nitrate-induced genes. Furthermore, not only coding RNAs were induced by nitrate, but also small non-coding RNAs such as miR5640, which targets Phosphoenol Pyruvate Carboxylase 3 (AtPPC3), an enzyme that has a role in carbon balance in plants. Interestingly, it was found that antisense transcripts for *TCP transcription factor 23* (*TCP23*) are induced by nitrate, suggesting that additional post-transcriptional regulatory mechanisms may be important for nitrate responses (Vidal *et al.*, 2013*a*).

Future perspectives

The dual-affinity transceptor NRT1.1 and its modification by phosphorylation at the T101 residue have been well characterized. However, the effect of differential phosphorylation on other factors, and what role it plays in nitrate signaling, needs to be further examined. In addition, many questions about N-dependent changes in phosphorylation remain. Are all nitrate-elicited phosphorylation changes dependent on the NRT1.1 transceptor? How is the sensed nitrate signal relayed to the immediate targets in the signaling pathway? Which kinases/phosphatases are responsible for the phosphorylation status switches of the different components? Given the limitations of current proteomics experiments, additional studies evaluating rapid and transient phosphorylation changes in response to nitrate and other N-nutrient/metabolites are necessary to assess the breath and impact of this particular post-translational modification.

Characterization of the early Ca^{2+}-elicited response described in this review has just begun: the Arabidopsis genome has nine PLC genes and two of them are induced by nitrate. Uncovering which PLCs are relevant for N signaling, and understanding the biochemical mechanisms and post-translational modifications that link them to nitrate sensing, is essential to better understand nitrate signaling in plants. Additional elements of this pathway, such as IP3-derived signals, remain to be characterized in plants. Furthermore, nitrate signaling also has a PLC and Ca^{2+}-independent component, as evidenced by AFB3 expression (Fig. 1B), suggesting the possibility of other second messenger(s) being involved in nitrate responses. The identity of this second messenger (or messengers) is still an open question.

Finally, elucidating the different nitrate-sensing mechanisms and understanding their spatiotemporal cross-talk, at the cell-specific, organ-specific, and organism level, will be essential to provide a holistic understanding of N-nutrient/metabolite sensing and responses in plants.

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