REVIEW PAPER

Primary nitrate responses mediated by calcium signalling and diverse protein phosphorylation

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Abstract

Nitrate, the major source of inorganic nitrogen for plants, is a critical signal controlling nutrient transport and assimilation and adaptive growth responses throughout the plant. Understanding how plants perceive nitrate and how this perception is transduced into responses that optimize growth are important for the rational improvement of crop productivity and for mitigating pollution from the use of fertilizers. This review highlights recent findings that reveal key roles of cytosolic–nuclear calcium signalling and dynamic protein phosphorylation via diverse mechanisms in the primary nitrate response (PNR). Nitrate-triggered calcium signatures as well as the critical functions of subgroup III calcium-sensor protein kinases, a specific protein phosphatase 2C, and RNA polymerase II C-terminal domain phosphatase-like 3 are discussed. Moreover, genome-wide meta-analysis of nitrate-regulated genes encoding candidate protein kinases and phosphatases for modulating critical phosphorylation events in the PNR are elaborated. We also consider how phosphoproteomics approaches can contribute to the identification of putative regulatory protein kinases in the PNR. Exploring and integrating experimental strategies, new methodologies, and comprehensive datasets will further advance our understanding of the molecular and cellular mechanisms underlying the complex regulatory processes in the PNR.

Keywords: Calcium signalling, nitrate signalling, primary nitrate response, protein kinase, protein phosphatase, transcription factor.

Introduction

Plant growth requires a source of nitrogen for the biosynthesis of amino acids, nucleic acids, and other nitrogen-containing biomolecules, and insufficient nitrogen reduces the productivity and quality of crops (Xu et al., 2012; Bloom, 2015; Kiba and Krapp, 2016; Wang et al., 2018; Fredes et al., 2019). While nitrate and ammonium are major sources of inorganic nitrogen in soil (Wang et al., 2018), most photosynthetic plants favour the assimilation of nitrate (Stitt, 1999; Crawford and Forde, 2002).

In addition to being a key nutrient, nitrate acts as a signalling molecule and modulates nutrient uptake, assimilation, and metabolism. Nitrate signalling also controls morphological and physiological responses throughout the plant. Adaptation to fluctuations in available nitrate involves multifaceted responses in distinct developmental programmes controlling root system architecture, shoot morphology, seed germination, stomatal closure, and flowering time (Guo et al., 2003; Dechorgnat et al., 2019).
Canales candidate protein kinases (PKs) and protein phosphatases the potential functions of nitrate-responsive genes encoding PHOSHATASE-LIKE 3 (CPL3) (Liu, 2013; Vidal et al., 2015; Liu et al., 2017). Global transcriptional response to nitrate in Arabidopsis has been extensively investigated over the past few decades. These studies provide comprehensive information on the identity and role of selected transcription factors (TFs), including NIN-like proteins (NLPs), that can partially account for the changes in gene expression in the primary nitrate response (PNR) (Konishi and Yanagisawa, 2013; Marchive et al., 2017; Liu et al., 2015; Gaudinier et al., 2018; Varala et al., 2018; Brooks et al., 2019). However, the primary signalling mechanisms that connect nitrate transporters/sensors to nitrate-mediated transcription and other biological processes remain mostly elusive.

Recent findings have provided new insights into how nitrate triggers dynamic changes in intracellular calcium signalling and protein phosphorylation to generate rapid control over nitrate uptake and the transcriptional PNR (Ho et al., 2009; Hu et al., 2009; Engelsberger and Schulze, 2012; Leran et al., 2015; Riveras et al., 2015; Menz et al., 2016; Liu et al., 2017). Although calcium signals with distinct amplitudes, locations (in the cytosol or other organelles), and durations are common features of plant responses to intrinsic or environmental cues (Dodd et al., 2010), it has been challenging to demonstrate the precise calcium signatures triggered by nitrate due to technical limitations. In this review, we discuss the new evidence for nitrate-associated calcium signalling dynamics and regulatory mechanisms in the Arabidopsis PNR (Ho et al., 2009; Hu et al., 2009; Leran et al., 2015; Riveras et al., 2015; Liu et al., 2017).

In addition to transcriptional regulation, nitrate signalling involves post-translational modifications such as protein phosphorylation, protein ubiquitination, and chromatin modification (Widiez et al., 2011; Sato et al., 2009; Liu et al., 2017; Alvarez et al., 2019; Hu et al., 2019; Poza-Carrion and Paz-Ares, 2019). In particular, protein phosphorylation can lead to rapid, versatile, and reversible modifications that directly regulate the localization, stability, interaction, function, and enzymatic activity of target proteins (Yip Delormel and Boudsocq, 2019). Here, we highlight the significance of nitrate-triggered calcium signalling, nitrate-activated calcium-sensor protein kinases (CPKs), and the nitrate–CPK–NLP regulatory network (Fig. 1) (Ho et al., 2009; Hu et al., 2009; Leran et al., 2015; Riveras et al., 2015; Liu et al., 2017). In addition, we review the recently defined roles for ABA-INSSENSITIVE 2 (ABI2) (Leran et al., 2015) and C-TERMINAL DOMAIN (CTD) PHOSHATASE-LIKE 3 (CPL3) (Liu et al., 2012), as well as the potential functions of nitrate-responsive genes encoding candidate protein kinases (PKs) and protein phosphatases (PPs) in the Arabidopsis PNR. Finally, we consider the use of phosphoproteomics approaches to identify nitrate-modulated protein phosphorylation targeting putative PKs (Fig. 2). These emerging discoveries clearly indicate the enormous complexity of the signalling network involving multifaceted calcium and protein phosphorylation regulations in the PNR.

**Nitrate-triggered calcium signalling in the PNR**

The involvement of calcium and protein phosphorylation in nitrate signalling was first suggested over 20 years ago (Sakakibara et al., 1997). Experiments in which detached maize and barley leaves were treated with chemical inhibitors of PKs or PPs, calcium chelators, or calcium channel blockers have suggested that calcium, PKs, and PPs are critical to nitrate-responsive...
gene transcription (Sakakibara et al., 1997; Sueyoshi et al., 1999). Using Arabidopsis seedlings, it has recently been shown that pre-treatment with gadolinium ions and lanthanide ions (which act as calcium channel blockers) or W7, an inhibitor of intracellular calmodulin and CPK, significantly reduces the nitrate-stimulated expression of marker genes. However, the findings also suggest the existence of calcium-dependent and calcium-independent pathways in nitrate-responsive gene regulation (Riveras et al., 2015; Liu et al., 2017). Despite the biochemical and molecular evidence for the involvement of calcium in nitrate-regulated gene expression, there has been no report on cellular observation of a defined calcium signature triggered by nitrate until very recent studies using various calcium biosensors in plants (Riveras et al., 2015; Liu et al., 2017).

**Nitrate-associated calcium signalling dynamics**

The nitrate-triggered changes in intracellular calcium were first reported in studies using plants expressing the aequorin reporter gene, which showed that nitrate stimulates a rapid calcium spike within 10 s in excised roots. This rapid cytoplasmic calcium signal is abolished in the roots of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY 6.3 (NPF6.3)/CHLORATE-RESISTANT 1 (CHL1)/NITRATE TRANSPORTER 1.1 (NRT1.1) mutants chl1-5 and chl1-9, indicating that the nitrate transceptor is responsible for this very transient calcium spike (Riveras et al., 2015). However,
in intact Arabidopsis seedlings, the nitrate-stimulated calcium dynamics appear dissimilar when using the same aequorin reporter (Liu et al., 2017). In the whole seedling, nitrate and potassium chloride (as a control for signal specificity) do not trigger detectable changes in intracellular calcium before 10 s. Nitrate specifically stimulates a subtle and gradual increase in calcium, peaking at around 100 s (Liu et al., 2017).

Because the nitrate-activated calcium signal in aequorin-expressing plants is much weaker than that stimulated by the bacterial flagellin peptide flg22 (Liu et al., 2017), a new generation of genetically encoded calcium biosensors, such as the ultrasensitive GCaMP6s (Chen et al., 2013), are needed to more clearly visualize the calcium dynamics triggered by nitrate. Furthermore, the use of GCaMP6s enables the quantification of calcium dynamics at subcellular resolution in single cells, which cannot be achieved with the aequorin reporter. In isolated leaf cells co-expressing GCaMP6s and a nuclear mCherry marker, time-lapse recordings in single cells reveal that nitrate specifically stimulates a unique calcium signature in the nucleus and cytosol. Nitrate triggers a similarly gradual increase in calcium signals, peaking at 2–3 min, in the mesophyll cells of intact transgenic cotyledons. In the root cap and root stele of intact GCaMP6s-expressing plants, the nitrate-induced calcium signal peaks at around 50–90 s (Liu et al., 2017). The calcium-binding dissociation constant (Kd) for GCaMP6s is 144 nM, just above the resting calcium level of ~100 nM in plant cells, whereas the calcium-binding Kd for aequorin is around 7.2–13 μM (Chen et al., 2013; Costa et al., 2018). Because the increase in intracellular calcium triggered by nitrate is relatively modest, the use of a calcium biosensor of appropriate sensitivity is critical for detecting a physiologically relevant link to calcium (Liu et al., 2017).

Dynamic fluorescent imaging using GCaMP6s reveals that the relatively weak calcium signals induced by nitrate are clearly distinct from the strong but very transient (lasting less than 60 s) cytoplasmic calcium spike triggered by cold or osmotic stress (Knight et al., 1996; Yuan et al., 2014), or the strong and sustained calcium flood induced by microbial signals or effectors, which persists for 20–120 min (Grant et al., 2000; Ranf et al., 2015; Liu et al., 2017). Visualization of more modest calcium signals is now possible with high reproductibility using GCaMP6 and GCaMP7, which surpass the sensitivity of other protein-based and commonly used synthetic calcium indicators (Chen et al., 2013; Dana et al., 2019). Such calcium biosensors have lower limits of detection and higher resolution than previously used biosensors, and enable the monitoring of physiological and dynamic calcium signalling triggered by diverse nutrients, peptides, and hormones at subcellular resolution in plants and animals. Future research will determine the precise sources and subcellular localization of nitrate-stimulated calcium signals using sensitive calcium biosensors targeted to specific organelles or subcellular compartments, which should also facilitate the identification of physiologically relevant calcium channels as well as additional nitrate and calcium sensors in the PNR (Fig. 1).

Mechanisms of calcium regulation

Inositol 1,4,5-trisphosphate (IP3) plays a critical role in triggering calcium signals by regulating calcium-selective intracellular channels in eukaryotic cells (Berridge, 2016). Although IP3 receptors have not been identified in plants, a positive correlation between increasing IP3 levels in the cell and elevated calcium signals has been suggested (Singh et al., 2015). Because phospholipase C (PLC) releases IP3 and diacylglycerol from phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] in plants (Kanera et al., 2015), the effect of the PLC inhibitor U73122 on nitrate signalling has been examined. As U73122 inhibits nitrate-induced marker gene expression and IP3 accumulates at 10 s of nitrate induction, it has been suggested that PLC–IP3 plays a critical role in nitrate signalling. However, the IP3 accumulation detected at 10 s is not followed by a further increase of calcium signalling in excited Arabidopsis roots (Riveras et al., 2015). Further biochemical, cellular, and genetic studies are needed to connect these promising findings of a link between nitrate regulation of PLC–IP3 and calcium signalling.

Interestingly, nitrate induces the expression of a gene encoding myo-inositol polyphosphate 5-phosphohexasate (5-PTase2), which has the ability to remove 5’ phosphate from IP3 and inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P5) in vitro (Berdy et al., 2001; Canales et al., 2014). This suggests that nitrate may induce 5-PTase2 and negatively regulate IP3 accumulation in the cell. Prior studies have shown that IP3 accumulates in both 5ptase1 and 5ptase2 mutants (Gunesekera et al., 2007), and plants constitutively expressing the human type I 5-PTase have a reduced basal IP3 level (Perera et al., 2006). Nitrate-induced 5-PTase2 gene expression may contribute to the control of IP3 homeostasis by a negative feedback mechanism. Elucidating the connections between nitrate, the nitrate transceptor, PLC, IP3, and calcium signalling may open up a new direction for studying the PNR (Fig. 1).

The nitrate–CPK–NLP signalling network

As both calcium and protein phosphorylation were implicated in early nitrate signalling, an in–gel kinase assay was conducted to determine whether putative calcium-regulated PKs could be activated by nitrate (Liu et al., 2017). The estimated molecular weight of nitrate-activated PKs was approximately 60 kDa, which is similar to the predicted molecular size of most CKPs but larger than the calcineurin B-like protein (CBL)-interacting PKs (CIPKs) previously reported to regulate nitrate transport and signalling in Arabidopsis (Ho et al., 2009; Hu et al., 2009; Léran et al., 2015; Liu et al., 2017). A targeted functional screen for Arabidopsis CIPKs that enhance the expression of a nitrate-responsive reporter gene in the presence of very low nitrate to sensitize the response led to the identification of the functionally redundant subgroup III CIPK10/30/32 as new regulators in the PNR. Because single cipk mutants lack overt growth phenotypes and the cipk10,30 double mutant exhibits embryonic lethality, a chemical genetic approach was developed to generate a so-called ‘inducible cipk10,30,32’ (ipck) triple mutant (Liu et al., 2017). In the ipck mutant, a transgene
was introduced to express the CPK10(M141G) variant protein that can be reversibly inhibited by a selective and potent PK inhibitor analogue, 3MB-PP1-Isop (Liu et al., 2017). Thus, chemical genetics circumvents lethality and redundancy, and offers a new strategy to further elucidate the dynamic and physiological functions of higher-order cpk mutants.

Integrated transcriptomic and phenotypic analyses of wild-type and ipk plants indicate that CPK10/30/32 control nitrate-specific gene expression and growth. CPK10/30/32 act as functionally redundant master regulators, modulating a broad spectrum of genes involved in diverse cellular and metabolic pathways immediately activated by nitrate. For example, target genes of CPK10/30/32 are enriched for functions in: nitrate/ammonia transport and assimilation; two routes of glucose-6-phosphate metabolism via the oxidative pentose phosphate pathway and glycolysis; amino acid transport and metabolism; cell wall remodelling; other transporters; carbon/nitrogen metabolism; cytokinin, auxin, and abscisic acid (ABA) metabolism and signalling; protein degradation; stress; signalling; and transcription. Notably, the expression of genes encoding functionally important TFs such as basic region/leucine zipper motif (bZIP), MYB, basic helix-loop-helix (bHLH), LATERAL ORGAN BOUNDARIES (LBD), SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL), WRKY, HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (HRS1), and TGACG MOTIF-BINDING FACTOR (TGA) is induced by nitrate-activated CPK10/30/32 to support potential amplification of the downstream nitrate transcriptional network (Liu et al., 2017; Vidal et al., 2015; Wang et al., 2018). Moreover, nitrate–CPK signalling activates an Arabidopsis gene encoding the cytochrome P450 enzyme CYP735A2, which directly enhances trans-zeatin synthesis to promote shoot deterioration; stress; tolerance, and pathogen resistance (Boudsocq and Sheen, 2013; Marchive et al., 2013; Yan et al., 2016; Liu et al., 2017). It is possible that more TFs are substrates of CPKs to mediate gene regulation in the PNR, or CPKs may also phosphorylate transporters, channels, and enzymes to exert broader controls in the response to nitrate. The manipulation of specific CPKs may facilitate targeted plant modification and improvement for multiple traits beneficial in terms of nutrient utilization, stress tolerance, and pathogen resistance (Boudsocq and Sheen, 2013; Simeunovic et al., 2016; Liu et al., 2017; Yip Delormel and Boudsocq, 2019). Further exploration of the nitrate–CPK–NLP signalling network will provide new insight into the nutrient–growth signalling pathways and the importance of post-translational modifications in nitrate signalling (Fig. 1).

**ABI2–CBL1/9–CIPK23 signalling in NPF6.3-dependent nitrate responses**

NPF6.3 is a dual-affinity nitrate transporter and a nitrate sensor (Liu and Tsay, 2003; Ho et al., 2009). The high-affinity mode of NPF6.3 function in nitrate transport is switched on by CIPK23 phosphorylation of Thr101 in the presence of low external nitrate, which also inhibits the PNR mediated by NPF6.3 based on *Nrt1 transporter 2.1 (NRT2.1) activation* (Ho et al., 2009; Liu et al., 2017). The putative calcium sensors CBL1 and CBL9 interact with CIPK23 to activate kinase activity (Ho et al., 2009; Léran et al., 2015; Bender et al., 2018). To identify PPs that counteract the inhibitory effect of CBL1–CIPK23 on NPF6.3-dependent nitrate transport activity, co-expression of PP2Cs belonging to clade A was examined in *Xenopus* oocytes. ABI2 was identified as a specific PP2C that can antagonize CBL1–CIPK23 inhibition of NPF6.3-mediated nitrate uptake in the oocyte assay, which was supported by the characterization of

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nitrate influx in different PP2C mutant plants (Léran et al., 2015).
AB12 regulates nitrate-mediated NRT2-1 expression and root growth by dephosphorylating CBL1 and CIPK23 and enhancing NPF6.3 activity in nitrate signalling (Léran et al., 2015). It will be interesting to determine whether different concentrations of nitrate trigger differential calcium signals to modulate CPK10/30/32 as positive regulators (Fig. 1) and CBL1/9–CIPK23 as negative regulators (Fig. 2) in the PNR. Besides inhibiting NPF6.3, CBL1/9–CIPK23 has been shown to activate the potassium channel AKT1, although a role in calcium signalling remains unclear (Xu et al., 2006; Bender et al., 2018). It has been proposed that drought and osmotic stress induce ABA accumulation and then inactivate AB12, thereby enhancing the uptake of potassium ions as cellular osmolites and reducing the uptake of nitrate to shut down energy-consuming processes of nitrate reduction (Léran et al., 2015). The ABI2–CBL1/9–CIPK23 link shows how stresses affect nutrient uptake and utilization at the level of post-translational regulation. Whether subcellular compartmentalization or substrate specificity distinguishes ABI2 from other related clade A PP2Cs in exerting its unique regulation of different ion channels/transporters deserves further research.

CPL3 as a regulator in nitrate responses

A forward genetic screen based on two consecutive assays, the first of nitrate-responsive reporter gene expression and the second of plant growth phenotypes, has led to the isolation of nis (nitrate insensitive) and ncr (nitrate constitutive response) mutants. The ncr1 mutant exhibits elevated expression of the nitrite reductase gene (NR) in the absence of nitrate and lacks the normal inhibitory effect of high nitrate on lateral root elongation (Liu et al., 2012). NCR1 encodes CPL3, which has functions in multiple regulatory pathways, for example, as a negative regulator of immune gene expression, stress-responsive gene transcription, or plant development (Bang et al., 2006; Li et al., 2014).

The RNA polymerase II complex is composed of multiple evolutionarily conserved subunits and is responsible for the transcription of protein-coding genes in all eukaryotes. The CTD of the largest subunit consists of conserved heptapeptide repeats with the consensus sequence Y1S2P3T4S5P6S7 (Buratowski, 2009). It has been demonstrated that CPL3 dephosphorylates Ser-2 to suppress CTD phosphorylation by mitogen-activated PK (MAPK) and cyclin–dependent kinase C (CDKC). Based on the transcriptome analysis of plant immune responses in wild type and cpl3, CPL3 does not control general gene transcription but participates in the regulation of specific flg22-responsive gene expression (Li et al., 2014). The molecular mechanism of high-nitrate inhibition of lateral root elongation is unclear. It is possible that CPL3 may mediate high-nitrate suppression of genes involved in regulating lateral root elongation. Whether NCR1 specifically or broadly regulates gene expression in the PNR or affects the expression of genes involved in modulating lateral root elongation through the modulation of RNA polymerase II activity will require further molecular and genomic dissection.

Nitrate-regulated genes encoding candidate PKs and PPs in the PNR

Over the past two decades, independent studies have investigated nitrate-regulated global transcription using various microarray platforms in Arabidopsis with different nitrate concentrations, at different response time points, in different plant organs, and at different developmental stages using various growth conditions (Wang et al., 2000, 2003, 2004, 2007; Gutiérrez et al., 2007; Gifford et al., 2008; Hu et al., 2009; Krouk et al., 2010b; Patterson et al., 2010; Ruffel et al., 2011; Canales et al., 2014; Vidal et al., 2015). Recent studies using RNA sequencing (RNA-seq) capture the expression of more genes and better resolve differential expression in the PNR (Li et al., 2017; Liu et al., 2017). These comprehensive transcriptome datasets are rich information resources for constructing integrated nitrate signalling and gene regulatory networks based on TF–target gene relationships and for identifying candidate signalling components (Canales et al., 2014; Vidal et al., 2015).

To evaluate the hypothesis that additional nitrate-responsive genes encoding PKs and PPs are involved in nitrate signalling, we surveyed the differentially expressed genes identified in a published meta-analysis of transcriptomic data derived from Affymetrix microarrays and two datasets from more recent RNA-seq analyses (Canales et al., 2014; Li et al., 2017; Liu et al., 2017). We ranked nitrate-induced or nitrate-repressed genes encoding PKs or PPs by the number of experiments in which they were detected as being nitrate responsive (Table 1). Interestingly, some of the most robustly enriched nitrate-regulated genes encode different CIPKs, putative MAPK cascade components, R.Lks, and PP2Cs (Table 1) (Fig. 2). Although there is limited information on their physiological roles in nitrate signalling, we will discuss potentially relevant functions of these four major groups of PKs and PPs encoded by nitrate-responsive genes.

Distinct signalling by different CIPKs

The CBL–CIPK complexes play essential roles in regulating the homeostasis of intracellular potassium, sodium, and magnesium in stressed and unstressed plants (Zhu, 2003; Luan, 2009; Mao et al., 2016; Bender et al., 2018). Both CIPK23 and CIPK8 genes are activated by nitrate, although two CBL–CIPK modules, CBL1/9–CIPK23 and CBL1/9–CIPK8, have been shown to play differential roles in nitrate signalling. CBL1/9–CIPK8 positively regulates the low-affinity phase [with a Michaelis constant ($K_m$) of $\sim 0.9$ mM nitrate] of the PNR, whereas CBL1/9–CIPK23 can respond to low-nitrate conditions and phosphorylate Thr101 of NPF6.3, which negatively regulates the high-affinity phase (with a $K_m$ of $\sim 30$ μM nitrate) of nitrate responses (Ho et al., 2009; Hu et al., 2009). Whether different nitrate levels could trigger differential calcium dynamics, which are decoded by CIPK8 and CIPK23 associated with the
### Table 1. Genome-wide meta-analysis of nitrate-responsive genes encoding protein kinases and protein phosphatases

| Gene ID   | Description                                                                 | Total | Induced | Repressed |
|-----------|-----------------------------------------------------------------------------|-------|---------|-----------|
| AT2G26980 | CBL-interacting protein kinase 3 (CIPK3)                                    | 20    | 20      | 0         |
| AT3G16560 | Protein phosphatase 2C family protein                                       | 16    | 16      | 0         |
| AT4G32950 | Protein phosphatase 2C family protein                                       | 15    | 15      | 0         |
| AT4G38470 | ACT-like protein tyrosine kinase family protein (STY46)                     | 14    | 14      | 0         |
| AT2G30040 | Mitogen-activated protein kinase kinase 14 (MAPKKK14)                       | 14    | 14      | 0         |
| AT1G49160 | Protein kinase superfamily protein (VINK7)                                  | 14    | 14      | 0         |
| AT3G17510 | CBL-interacting protein kinase 1 (CIPK1)                                   | 12    | 12      | 0         |
| AT5G26010 | Protein phosphatase 2C family protein                                       | 11    | 2       | 9         |
| AT5G54130 | Calcium-binding endonuclease/exonuclease/phosphatase family                 | 10    | 10      | 0         |
| AT2G17820 | Histidine kinase 1 (HKT1)                                                   | 9     | 9       | 0         |
| AT1G07150 | Mitogen-activated protein kinase kinase 13 (MAPKK13)                       | 8     | 8       | 0         |
| AT2G17700 | ACT-like protein tyrosine kinase family protein (STY8)                      | 7     | 0       | 7         |
| AT1G30270 | CBL-interacting protein kinase 23 (CIPK23)                                 | 6     | 6       | 0         |
| AT2G20303 | SNF1-related protein kinase 2.9 (SNRK2.9)                                  | 6     | 6       | 0         |
| AT1G73030 | Leucine-rich receptor-like protein kinase family protein (PSY1R)            | 5     | 5       | 0         |
| AT3G11410 | Protein phosphatase 2CA (PP2CA)                                             | 5     | 5       | 0         |
| AT1G3500  | MAP kinase kinase 9 (MPK9)                                                  | 5     | 0       | 5         |
| AT4G29990 | Leucine-rich repeat transmembrane protein kinase protein                     | 4     | 4       | 0         |
| AT1G53510 | Mitogen-activated protein kinase 18 (MPK18)                                 | 4     | 4       | 0         |
| AT5G67080 | Mitogen-activated protein kinase kinase 19 (MAPKK19)                       | 4     | 4       | 0         |
| AT3G22750 | Protein kinase superfamily protein                                          | 4     | 4       | 0         |
| AT2G30020 | Protein phosphatase 2C family protein (AP2C1)                              | 4     | 2       | 2         |
| AT5G38240 | Protein kinase family protein                                                | 4     | 0       | 4         |
| AT1G33260 | Protein kinase superfamily protein                                          | 4     | 0       | 4         |
| AT2G04180 | CBL-interacting protein kinase 13 (CIPK13)                                 | 3     | 3       | 0         |
| AT2G25090 | CBL-interacting protein kinase 16 (CIPK16)                                 | 3     | 3       | 0         |
| AT4G21410 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 29 (CRK29)                | 3     | 3       | 0         |
| AT4G11460 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 30 (CRK30)                | 3     | 3       | 0         |
| AT3G08760 | Integrin-linked protein kinase family (ILK)                                 | 3     | 3       | 0         |
| AT5G01820 | CBL-interacting protein kinase 16 (CIPK14)                                 | 3     | 0       | 3         |
| AT1G76040 | Calcium-dependent protein kinase 29 (CPK29)                                 | 2     | 2       | 0         |
| AT2G38490 | CBL-interacting protein kinase 22 (CIPK22)                                 | 2     | 2       | 0         |
| AT1G49100 | Leucine-rich repeat protein kinase family protein                           | 2     | 2       | 0         |
| AT1G51810 | Leucine-rich repeat protein kinase family protein                           | 2     | 2       | 0         |
| AT5G62710 | Leucine-rich repeat protein kinase family protein                           | 2     | 2       | 0         |
| AT2G28250 | Protein kinase superfamily protein (NCRK)                                  | 2     | 2       | 0         |
| AT3G07070 | Protein kinase superfamily protein                                          | 2     | 2       | 0         |
| AT3G28690 | Protein kinase superfamily protein                                          | 2     | 2       | 0         |
| AT1G63580 | Receptor-like protein kinase-related family protein                         | 2     | 2       | 0         |
| AT1G05000 | Phosphotyrosine protein phosphatases superfamily protein (PFA-DSP1)        | 2     | 2       | 0         |
| AT1G16220 | Protein phosphatase 2C family protein                                       | 2     | 2       | 0         |
| AT3G16800 | Protein phosphatase 2C family protein (EGR3)                               | 2     | 2       | 0         |
| AT5G25110 | CBL-interacting protein kinase 25 (CPK25)                                 | 2     | 1       | 1         |
| AT5G59650 | Leucine-rich repeat protein kinase family protein                           | 2     | 1       | 1         |
| AT1G07880 | Protein kinase superfamily protein (ATMPK13)                               | 2     | 1       | 1         |
| AT3G09350 | Protein kinase superfamily protein                                          | 2     | 1       | 1         |
| AT3G14370 | Protein kinase superfamily protein (WAG2)                                  | 2     | 1       | 1         |
| AT2G04180 | Phosphatase 2CS (PP2CS)                                                     | 2     | 1       | 1         |
| AT1G51805 | Leucine-rich repeat protein kinase family protein (SIF3)                    | 2     | 0       | 2         |
| AT2G23300 | Leucine-rich repeat protein kinase family protein                           | 2     | 0       | 2         |
| AT4G33950 | Protein kinase superfamily protein (OST1)                                  | 2     | 0       | 2         |
| AT1G66930 | Protein kinase superfamily protein (LRK10L2)                               | 2     | 0       | 2         |
| AT5G66210 | Calcium-dependent protein kinase 28 (CPK28)                                | 1     | 1       | 0         |
| AT4G57630 | CBL-interacting protein kinase 21 (CIPK21)                                 | 1     | 1       | 0         |
| AT4G24400 | CBL-interacting protein kinase 8 (CIPK8)                                   | 1     | 1       | 0         |
| AT2G29220 | Concanavalin A-like lectin protein kinase family protein (LECRK-III.1)     | 1     | 1       | 0         |
| AT4G23230 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 15 (CRK15)                | 1     | 1       | 0         |
| AT4G21400 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 28 (CPK28)                | 1     | 1       | 0         |
| AT4G04490 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 36 (CPK36)                | 1     | 1       | 0         |
| AT4G04540 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 39 (CPK39)                | 1     | 1       | 0         |
Nitrate may mediate the nitrate promotion of germination that is antagonized by ABA. Further analysis using the cipk1,3 double mutant or even higher-order cipk mutants may be necessary to elucidate the functions of CIPK1, CIPK3, or other CIPKs in nitrate signalling (Fig. 2) (Kolukisaoglu et al., 2004).

**Potential functions of MAPK cascades**

The evolutionarily conserved MAPK cascades consist of upstream MAPK kinase kinases (MAPKKKs) that phosphorylate MAPK kinases (MAPKKs), which in turn phosphorylate MAPKs. Hundreds of protein substrates can be potentially phosphorylated by MAPKs, which serve as the central regulatory hubs in diverse plant signalling networks (Popescu et al., 2009). A strikingly large number of putative MAPK

### Table 1. Continued

| Gene ID         | Description                                                                 | Total | Induced | Repressed |
|-----------------|-----------------------------------------------------------------------------|-------|---------|-----------|
| AT4G03140       | Cysteine-rich RLK (RECEPTOR-like protein kinase) 6 (CRK6)                    |       | 1       | 0         |
| AT2G19090       | FLG22-receptor-like kinase 1 (SIRK)                                        |       | 1       | 0         |
| AT1G75820       | Leucine-rich receptor-like protein kinase family protein (CLV1)             |       | 1       | 0         |
| AT2G29000       | Leucine-rich repeat protein kinase family protein                           |       | 1       | 0         |
| AT3G46370       | Leucine-rich repeat protein kinase family protein                           |       | 1       | 0         |
| AT5G47800       | Leucine-rich repeat protein kinase family protein                           |       | 1       | 0         |
| AT2G19210       | Leucine-rich repeat transmembrane protein kinase protein                    |       | 1       | 0         |
| AT2G01450       | MAP kinase 17 (MPK17)                                                       |       | 1       | 0         |
| AT5G54380       | Protein kinase family protein (THE1)                                        |       | 1       | 0         |
| AT3G32600       | Protein kinase superfamily protein (ATMRK1)                                 |       | 1       | 0         |
| AT1G68690       | Protein kinase superfamily protein (PERK9)                                  |       | 1       | 0         |
| AT1G66460       | Protein kinase superfamily protein                                          |       | 1       | 0         |
| AT5G41680       | Protein kinase superfamily protein                                          |       | 1       | 0         |
| AT4G18900       | Protein kinase superfamily protein (ARCK1)                                 |       | 1       | 0         |
| AT1G63550       | Receptor-like protein kinase-related family protein                          |       | 1       | 0         |
| AT4G00100       | SNF1-related protein kinase 2.7 (SNRK2.7)                                   |       | 1       | 0         |
| AT3G27920       | Protein phosphatase 2C family protein (EGR2)                                |       | 1       | 0         |
| AT1G07160       | Protein phosphatase 2C family protein                                        |       | 1       | 0         |
| AT2G33700       | Protein phosphatase 2C family protein                                        |       | 1       | 0         |
| AT1G48260       | CBL-interacting protein kinase 17 (CIPK17)                                 |       | 1       | 0         |
| AT5G45820       | CBL-interacting protein kinase 20 (CIPK20)                                 |       | 1       | 0         |
| AT3G45330       | Concanavalin A-like lectin kinase family protein (LECRK-I.1)               |       | 1       | 0         |
| AT5G01550       | Lectin receptor kinase a4.1 (LECRK-VI.3)                                   |       | 1       | 0         |
| AT5G01560       | Lectin receptor kinase a4.3 (LECRK-IV.3)                                   |       | 1       | 0         |
| AT1G51890       | Leucine-rich repeat protein kinase family protein                           |       | 1       | 0         |
| AT5G49770       | Leucine-rich repeat protein kinase family protein                           |       | 1       | 0         |
| AT1G05700       | Leucine-rich repeat transmembrane protein kinase protein                    |       | 1       | 0         |
| AT1G26150       | Prolin-rich extensin-like receptor kinase 10 (PERK10)                       |       | 1       | 0         |
| AT1G61590       | Protein kinase superfamily protein                                          |       | 1       | 0         |
| AT3G09010       | Protein kinase superfamily protein                                          |       | 1       | 0         |
| AT4G09760       | Protein kinase superfamily protein (CEK3)                                   |       | 1       | 0         |
| AT3G35920       | Protein kinase superfamily protein                                          |       | 1       | 0         |
| AT3G46270       | Receptor protein kinase-related                                             |       | 1       | 0         |
| AT5G10520       | ROP binding protein kinases 1 (RBK1)                                        |       | 1       | 0         |
| AT3G23150       | Signal transduction histidine kinase, hybrid-type, ethylene sensor (ETR2)  |       | 1       | 0         |
| AT1G61360       | S-fucos lectin protein kinase family protein                               |       | 1       | 0         |
| AT1G61500       | S-fucos lectin protein kinase family protein                               |       | 1       | 0         |
| AT4G27300       | S-fucos lectin protein kinase family protein                               |       | 1       | 0         |
| AT1G21230       | Wall associated kinase 5 (WAK5)                                             |       | 1       | 0         |
| AT2G32960       | Phosphotyrosine protein phosphatases superfamily protein (PFA-DSP2)        |       | 1       | 0         |

Differential gene expression (Canales et al., 2014; Li et al., 2017; Liu et al., 2017). In our analysis, we applied a ≥2-fold cut-off.

\* Total number of experiments in which expression of a gene is either induced or repressed by nitrate. Genes that are induced or repressed by nitrate in three or more experiments are in bold text.

NPF6.3 transceptor, to mediate their distinct regulatory roles in the PNR remains to be resolved (Fig. 2).

In the Arabidopsis genome, 26 CIPK and 10 CBL genes encode members of the CIPK and CBL protein families, respectively (Kolukisaoglu et al., 2004; Mao et al., 2016). Depending on the experimental conditions, nitrate may induce the expression of CIPK1, 3, 8, 13, 14, 16, 21, 22, 23, and 25 or repress the expression of CIPK17, 20, and 25. CIPK1 and CIPK3 have been consistently induced by nitrate in numerous experiments (Table 1), but the characterization of cipk1 and cipk3 mutants has not yielded any clue as to their physiological roles in the PNR (Hu et al., 2009). As cipk3 is hypersensitive to ABA during germination (Pandey et al., 2008), CBL9–CIPK3 may mediate the nitrate promotion of germination that is
cascade-encoding genes have been predicted in Arabidopsis (Ichimura et al., 2002). Numerous intrinsic and extracellular stimuli can activate MAPK cascades to regulate gene expression and integrate developmental processes (Xu and Zhang, 2015; Komis et al., 2018). However, current knowledge on the physiological functions of MAPK cascades in nutrient signalling is relatively limited (Chardin et al., 2017).

The key TF NLP7 binds directly to the promoters of MAPKKK13, 14, and 19, which are the top nitrate-activated genes in many transcriptome datasets (Table 1). It will be interesting to define their downstream MAPKKs and MAPKs, which may promote nitrate signalling. Nitrate also activates other genes encoding putative RAF-like MAPKKKs, including INTEGRIN-LINKED KINASE 4 (ILK4), which interacts with RLKs and a putative calcium channel (Popescu et al., 2017), as well as STY8 and STY46, which phosphorylate nuclear-encoded proteins targeted to the chloroplast (Lamberti et al., 2011). STY8/46 may be important for nitrate-induced leaf greening and chloroplast development because mutations of STY8, STY46, and another homologous gene, STY17, in the sty8,17,46 triple mutant result in reduced growth and chlorophyll accumulation.

As nitrate consistently activates STY46 but represses STY8 (Table 1) and the sty46 mutant displays severe retardation, STY46 likely plays the predominant role in chloroplast and leaf development promoted by nitrate. The ambiguous effect of nitrate on the expression of STY8/46 suggests that other stimuli or tissue-specific expression may contribute to the regulation of STY8/46.

Intriguingly, the expression of MKK9 is repressed by nitrate in five experimental conditions (Table 1) and is induced by relatively low (0.1 mM) nitrate concentrations in 2–3 days, which probably corresponds to nitrate starvation conditions (Luo et al., 2017). The expression of constitutively active MKK9 kinase in low-nitrate concentrations reduces the expression of anthocyanin biosynthesis genes as well as the accumulation of anthocyanin (Luo et al., 2017). It was suggested that MKK9 negatively regulates the production of anthocyanins but promotes the acquisition of nitrogen through the activation of NRT2.1. This finding is inconsistent with the role of MKK9 in promoting MPK6 activation in ethylene signalling and plant senescence promoted by nitrate starvation (Yoo et al., 2009; Zhou et al., 2009). A recent study indicated that MPK3 and MPK6 are required for controlling the proper progression of lateral root primordia (Zhu et al., 2019). Whether nitrate modulates lateral root formation through MKK9–MPK3/6 signalling is unclear and requires further study. Finally, MPK18 is activated by nitrate (Table 1), and phosphorylated MPK18 can interact with and be dephosphorylated by the dual-specific MAPK phosphatase PROPYZAMIDE HYPERSENSITIVE 1 (PHS1), which is involved in the stabilization of cortical microtubules (Walía et al., 2009). As mpk18 exhibits enhanced microtubule stability, future experiments may determine whether the MPK18–PHS1 signalling module governs the morphological response of the root system to nitrate (Fig. 2).

Connections to RLKs

Although nitrate regulates a large number of genes encoding different subfamilies of RLKs, their responses to nitrate are highly variable in different transcriptome datasets (Table 1), which suggests that they have specialized roles in nitrate signalling in different cell types, organs, developmental stages, and growth conditions. More than 200 genes in the Arabidopsis genome encode homologs of leucine-rich repeat RLKs (LRR-RKs), the largest family of putative receptor kinases (Shiu and Bleecker, 2001; Chakraborty et al., 2019). The extracellular LRR domains of some LRR-RKs mediate responses to endogenous peptides that modulate cell proliferation, cell differentiation, immunity, symbiosis, and the response to wounding. For instance, the perception of PLANT PEPTIDE CONTAINING SULFATED TYROSINE (PSY1) by the PSY1 receptor (PSY1R) is reported to promote cell proliferation in roots and cell expansion in the elongation zone of roots (Amano et al., 2007). The up-regulation of the PSY1R gene in multiple microarray datasets (Table 1) may suggest its positive role in enhancing the growth of roots supplied with nitrate. Moreover, many genes encoding cysteine-rich RLKs (CRKs) are also induced by nitrate (Chen, 2001). CRK29 and CRK30 stand out as the most consistently up-regulated nitrate-responsive genes (Table 1). Although CRK29 and CRK30 were reported to be involved in plant immunity and cell-to-cell communication, the possible functions of CRK29/30 in nitrate signalling could be further explored (Amari et al., 2010; Lee et al., 2011; Caillaud et al., 2014).

Multiple roles of PP2Cs

Protein dephosphorylation is a crucial process for reversing protein phosphorylation and controlling intracellular signalling events. In Arabidopsis, around 112 PP genes have been predicted. Among these, there are 76 PP2C gene members that can be clustered into 10 groups (A to J) with the exception of six unclustered genes (Schweighofer et al., 2004). There are 12 nitrate-induced PP2C genes (Table 1). PP2CA activation was detected in five microarray datasets (Table 1) and has been shown to negatively regulate ABA-mediated responses in seeds and at the vegetative stage, the kinase activity of the SNF1-RELATED PROTEIN KINASE 1 (SnRK1) energy sensor, and the guard cell outward-rectifying potassium efflux channel (Rodrigues et al., 2013; Lefoulon et al., 2016). SnRK1 is the central metabolic regulator of energy homeostasis in plants. PP2CA dephosphorylates SnRK1 and inhibits energy signalling, modulating growth, stress tolerance, and senescence (Rodrigues et al., 2013). Investigation of the PP2CA–SnRK1 connection in nitrate signalling may uncover new roles for the energy sensor complexes in the PNR (Fig. 2).

The genes encoding PP2C-type phosphatases AP2C1 and AP2C5 are also activated by nitrate (Table 1). AP2C1 and
PP2C5 share overlapping functions and can directly interact with and dephosphorylate MPK3/4/6 predominantly in the nucleus (Schweighofer et al., 2007; Brock et al., 2010). The ap2c1 mutant produces more jasmonate upon wounding and is more resistant to phytophagous mites, whereas plants with increased AP2C1 levels reduce ethylene production and are more susceptible to a necrotrophic fungal pathogen (Schweighofer et al., 2007). Unexpectedly, the analyses of ap2c1, pp2c5, and ap2c1 pp2c5 mutants suggest that they have positive roles in seed germination, stomatal closure, and gene regulation in ABA responses (Brock et al., 2010). Besides interacting with MPK3/4/6, AP2C1 dephosphorylates CIPK9 to regulate root growth and seedling development under low-potassium conditions (Singh et al., 2018). Nitrate may induce PP2C-type phosphatases to modulate these important regulators to control or fine-tune plant growth. Further studies may clarify the functions of AP2C1 and PP2C5 in nitrate signalling as well as the complex nutrient–hormone crosstalk mediating growth, stress, and immune responses (Fig. 2).

Nitratedependent phosphorylation of plant proteins

Complementary approaches to identify the relevant substrates of diverse PKs or PPs that are regulated by nitrate at the transcriptional or post-transcriptional level provide further molecular and biochemical insight into the PNR. Advances in phosphoproteomics analyses have enabled site-specific quantification of in vivo phosphorylation of a broad range of proteins (Engelsberger and Schulze, 2012; Li et al., 2015). Two studies have generated quantitative data focusing on protein phosphorylation dynamics and phosphopeptide identification in response to nitrate or ammonium supply (Engelsberger and Schulze, 2012) or deprivation (Menz et al., 2016). Within 5 min of nitrate resupply, the high-affinity nitrate transporter NRT2;1, ammonium transporter AMT1;1, and nitrate reductase NIA2 are rapidly dephosphorylated, while diverse PKs show increased phosphorylation (Engelsberger and Schulze, 2012). These findings are consistent with NIA2 phosphorylation at Ser–534 and NRT2;1 phosphorylation at Ser–28 under nitrate-deprivation conditions (Engelsberger and Schulze, 2012; Menz et al., 2016). The highly conserved Ser–534 in NIA2 has been reported to exert an inhibitory function when it is phosphorylated (Menz et al., 2016). Future research will identify more nitrate-regulated phosphorylation sites and their cognate PKs or PPs that are relevant to the regulation of transporter, sensor, channel, enzyme, or TF activities and the control of nitrate-dependent plant development.

Nitrate resupply stimulates the rapid phosphorylation of many interesting PKs, including the LRR–RKs IMPAIRED OOMYCETE SUSCEPTIBILITY 1 (IOS1) (Hok et al., 2014) and KINASE7 (KIN7) (Isner et al., 2018), the membrane-associated PKs CAST AWAY (CST) (Burr et al., 2011; Groner et al., 2016), CIPK2 (Linn et al., 2017), osmotic stress regulator SnRK2.4 (McLoughlin et al., 2012), BR-SIGNALING KINASE 1 (BSK1) (Tang et al., 2008; Sreeramulu et al., 2013), and the kinesin-associated PK NEVER IN MITOSIS GENE A-RELATED KINASE 6 (NEK6) (Takatani et al., 2017) (Fig. 2). IOS1 and KIN7 could be involved in nitrate–ABA signalling crosstalk (Ondzighi-Assoume et al., 2016; Ristova et al., 2016). The iot51 mutant plant shows hypersensitivity to ABA-induced inhibition of seed germination and primary root elongation (Hok et al., 2014), and KIN7 participates in ABA-induced stomatal closure by modulating tonoplast potassium channel activity (Isner et al., 2018). CST activates the cell separation process that may be required for the promotion by nitrate of lateral root primordia emergence (Burr et al., 2011; Groner et al., 2016; Zhu et al., 2019).

SnRK2.4 links the perception of salt stress to the modulation of root growth and development (McLoughlin et al., 2012). However, it is unclear how nitrate triggers SnRK2.4 phosphorylation and regulates root growth. brassinosteroid (BR) promotes plant growth through the BR receptor (BRJ)–BKS1 kinase relay to regulate BR signalling, and BRII1 phosphorylates BSK1 at Ser–230 (Tang et al., 2008). As nitrate also induces Ser–230 phosphorylation of BSK1, future studies will elucidate the molecular mechanism integrating nitrate and BR signalling in the control of plant growth. NEK6 is a mitotic kinase that destabilizes cortical microtubules and functions in promoting longitudinal cell elongation and suppressing radial ectopic growth (Takatani et al., 2017). Although nitrate could trigger the phosphorylation of diverse PKs, the molecular and physiological functions underlying these phosphorylation events are largely unknown. Elucidating the precise roles and regulations of nitrate-activated phosphorylation of these PKs via calcium-dependent and calcium-independent mechanisms will significantly expand the current scope of nitrate signalling in plants (Fig. 2).

Perspectives

Here we consider the new and essential roles of calcium signalling and dynamic protein phosphorylation via diverse mechanisms in the PNR. Although most nitrate responses have been characterized in root systems, recent findings have demonstrated in both roots and shoots that nitrate triggers unique calcium signatures and acts synergistically with calcium-activated, nuclear-localized CPK10/30/32 to phosphorylate NLP7. The activated NLP7 is retained in the nucleus and specifies a global reprogramming of nitrate-responsive genes (Marchive et al., 2013; Liu et al., 2017). Besides NLPs, other TFs, such as ARABIDOOPSIS NITRATE REGULATED 1, LBD37/38/39, TGA1/4, TCP20, HRS1/HRS1 Homolog 1, Bric-a-Brac/Tramtrack/Broad gene family 1/2, bZIP1, SPL9, and NAM/ATAF/CUC4, have been shown to control transcriptional responses to nitrate (Vidal et al., 2015; Wang et al., 2018; Brooks et al., 2019). Future investigations could examine whether the regulation of these TFs is also dependent on CPKs and define the precise and extensive connections between specific CPKs and TFs in nitrate signalling. It is possible that other NLPs and TFs may also participate in the nitrate–calcium–CPK–TF signalling network that integrates the transcriptome, nutrient transport, cellular metabolism, hormone signalling, and cell proliferation, which mediate the shoot–root coordination and developmental plasticity that shape plant growth.
and development. Further studies may determine novel target genes and cis-regulatory elements for NLP7 and other NLPs and TFs in response to nitrate–calcium–CPK signalling or other upstream regulators in native biological contexts and cell types (Walker et al., 2017).

The nitrate transceptor NPF6.3 and PLC–IP3 have been suggested to play key roles in nitrate-triggered calcium signalling (Fig. 1) (Riveras et al., 2015). However, it cannot be ruled out that other nitrate transporter-related and novel nitrate-binding proteins may serve as nitrate sensors in the PNR. The examination of specific subcellular sites for nitrate-stimulated calcium signals based on targeted GCaMP6s may facilitate screening for and identification of new signalling molecules and calcium channels that activate nitrate responses (Yuan et al., 2014; Jiang et al., 2019). Although the nitrate-responsive expression of most genes associated with metabolism and transport depends on CPK10/30/32, CIPK8/CIPK23, in complex with putative CBL1/CBL9 calcium sensors, can regulate the transport activity of NPF6.3 and signalling downstream of both NPF6.3 and calcium (Fig. 2) (Ho et al., 2009; Hu et al., 2009). Integrated molecular, cellular, biochemical, and genetic analyses may identify additional nitrate sensors, calcium channels, calcium sensors, PKs, and PPs contributing to the PNR (Léran et al., 2015; Liu et al., 2017).

Global changes in gene expression were first reported for the PNR nearly two decades ago, and nitrate-induced genes encoding TFs have been shown to play key roles in regulating nitrate signalling (Vidal et al., 2015; Wang et al., 2018; Brooks et al., 2019). However, the roles of only a few nitrate-regulated genes encoding putative PKs or PPs have been defined. In future investigations, functional screens might focus on these PK and PP genes as candidates to discover their roles in nitrate signalling and establish new nitrate-based molecular wiring in the gene regulatory network. Technical advances in the preparation and enrichment of samples and quantification strategies for phosphoproteomics could improve the scope and resolution of early protein phosphorylation events in the PNR. The identification of new targets of nitrate-activated CPKs, CIPKs, or other PKs could reveal additional components or connections in the PNR.

Nitrogen is the fourth most abundant element in plant biomass after hydrogen, carbon, and oxygen, and nitrate, the preferred source of inorganic nitrogen for most plants, is acquired from soil. The Green Revolution, which profoundly increased agricultural productivity worldwide, depends on the application of nitrogenous fertilizers to soil to ensure that the photosynthetic potential of crops is not limited by nitrogen assimilation and nitrate signalling. However, the production and use of fertilizer requires substantial energy consumption and represents a large economic burden. Moreover, the leaching of nitrate from the use of fertilizer pollutes waterways and promotes eutrophication. A better understanding of nitrate signalling and downstream responses could suggest rational ways to improve nitrogen utilization and sustain agriculture with reduced energy input and pollution.

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