

# Primary nitrate responses via calcium signalling and diverse protein phosphorylation

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## Abstract

Nitrate, the major source of inorganic nitrogen to plants, is a critical signal controlling nutrient transport, 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 mitigation of pollution from 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 responses (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.

**Key words:** 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 N-containing biomolecules, and insufficient nitrogen reduces the productivity and quality of crops (Bloom, 2015; Fredes *et al.*, 2019; Kiba and Krapp, 2016; Wang *et al.*, 2018; Xu *et al.*, 2012). While nitrate and ammonium are major sources of inorganic nitrogen in soil (Wang *et al.*, 2018), most photosynthetic plants favour the assimilation of nitrate (Crawford and Forde, 2002; Stitt, 1999). 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 programs controlling root system architecture, shoot morphology, seed germination, stomata closure, and flowering time (Dechornat *et al.*, 2011; Fredes *et al.*, 2019; Guo *et al.*, 2003; Lin and Tsay, 2017; Liu *et al.*, 2017). For example, the root system architecture of *Arabidopsis* is highly plastic in response to external nitrate levels. While nitrate starvation arrests root growth, moderate level of nitrate in the environment promotes the elongation of lateral roots whose growth would be attenuated in high nitrate conditions (Linkohr *et al.*, 2002; Liu *et al.*, 2017; Zhang and Forde, 2000).

Nitrate signalling orchestrates the adaptive responses of diverse biological processes through reprogramming of the transcriptome (Canales *et al.*, 2014; Liu *et al.*, 2017; Medici and Krouk, 2014; Scheible *et al.*, 2004; Vidal *et al.*, 2015; Wang *et al.*, 2000; Wang *et al.*, 2003; Wang *et al.*, 2018). The global transcriptional response to nitrate in *Arabidopsis* has been extensively investigated over the past 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 changes in gene expression in the PNR (Brooks *et al.*, 2019; Gaudinier *et*

*al.*, 2018; Konishi and Yanagisawa, 2013; Liu *et al.*, 2017; Marchive *et al.*, 2013; Varala *et al.*, 2018; Vidal *et al.*, 2015). However, the primary signalling mechanisms that connect nitrate transporters/sensors to nitrate-mediated transcription and other biological processes remains 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 (Engelsberger and Schulze, 2012; Ho *et al.*, 2009; Hu *et al.*, 2009; Léran *et al.*, 2015; Liu *et al.*, 2017; Menz *et al.*, 2016; Riveras *et al.*, 2015). Although calcium signals with distinct amplitudes, locations (in the cytosol or other organelles) and durations are common features in 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 the past. 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; Léran *et al.*, 2015; Liu *et al.*, 2017; Riveras *et al.*, 2015).

In addition to transcriptional regulation, nitrate signalling involves post-translational modifications such as protein phosphorylation, protein ubiquitination, and chromatin modification (Alvarez *et al.*, 2019; Hu *et al.*, 2019; Liu *et al.*, 2017; Poza-Carrion and Paz-Ares, 2019; Sato *et al.*, 2009; Widiez *et al.*, 2011). In particular, protein phosphorylation can lead to rapid, versatile and reversible modifications to directly regulate the localization, stability, interaction, function, and enzymatic activity of target proteins (Yip Delormel and Boudsocq, 2019). We highlight the significance of nitrate-triggered calcium signalling, nitrate-activated calcium-sensor protein kinase (CPK), and the nitrate-CPK-NLP regulatory network (Fig. 1) (Ho *et al.*, 2009; Hu *et al.*, 2009; Léran *et al.*, 2015; Liu *et al.*, 2017; Riveras *et al.*, 2015). In addition, we review the recently defined

roles for ABA In-sensitive 2 (ABI2) (Léran *et al.*, 2015) and C-terminal domain (CTD) phosphatase-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 PNR**

The involvement of calcium and protein phosphorylation in nitrate signalling was first implicated 20 years ago (Sakakibara *et al.*, 1997). Treating detached maize and barley leaves with chemical inhibitors of PK or PP, calcium chelator or calcium channel blocker 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 the calcium channel blocker, gadolinium ion and lanthanide ion, or W7 inhibitor of intracellular calmodulin (CaM) and CPK significantly reduces marker gene expression stimulated by nitrate. However, the findings also suggest calcium-dependent and calcium-independent pathways in nitrate responsive gene regulation (Liu *et al.*, 2017; Riveras *et al.*, 2015). 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 (Liu *et al.*, 2017; Riveras *et al.*, 2015).

## Nitrate-associated calcium signalling dynamics

The nitrate-triggered changes in intracellular calcium were first reported using plants expressing the aequorin reporter gene, which show that nitrate stimulates a rapid calcium spike within 10 sec 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) (hereafter NPF6.3) 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 appeared dissimilar 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 sec. Nitrate specifically stimulates a subtle and gradual increase in calcium peaking at around 100 sec (Liu *et al.*, 2017).

Because the nitrate-activated calcium signal is much weaker than that stimulated by a bacterial flagellin peptide flg22 in the aequorin plants (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 calcium dynamics triggered by nitrate. Furthermore, the use of GCaMP6s enables the quantification of calcium dynamics at subcellular resolution in single cells, which is unachievable 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 plants, the nitrate-induced calcium signal peaks at around 50-90 sec (Liu *et al.*, 2017). The calcium binding dissociation constant ( $K_d$ ) for GCaMP6s is 144 nM just above the resting calcium level of  $\sim 100$  nM in plant cells, whereas the calcium binding  $K_d$  for aequorin is around 7.2-13  $\mu$ M (Chen *et al.*, 2013; Costa *et al.*, 2018). Because the intracellular calcium increase triggered by nitrate is relatively modest, 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 the relatively weak calcium signals induced by nitrate to be clearly distinct from the strong but very transient cytoplasmic calcium spike triggered by cold or osmotic stress lasting less than 60 sec (Knight *et al.*, 1996; Yuan *et al.*, 2014), or the strong and sustained calcium flood for 20-120 min induced by microbial signals or effectors (Grant *et al.*, 2000; Liu *et al.*, 2017; Ranf *et al.*, 2015). Visualization of more modest calcium signals is now possible with high reproducibility 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 reduce the previous limits of detection and increase the resolution for monitoring physiological and dynamic calcium signalling triggered by diverse nutrients, peptides and hormones at subcellular resolution beyond previous detection and recognition 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 (IP<sub>3</sub>) plays a critical role in triggering calcium signals by regulating calcium-selective intracellular channels in eukaryotic cells (Berridge, 2016). Although IP<sub>3</sub> receptors have not been identified in plants, the positive correlation between increasing IP<sub>3</sub> level in the cell and elevated calcium signals has been suggested (Singh *et al.*, 2015). Because phospholipase C (PLC) releases IP<sub>3</sub> and diacylglycerol from phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] in plants (Kanehara *et al.*, 2015), the effect of PLC inhibitor (U73122) on nitrate signalling has been examined. As U73122 inhibits nitrate-induced marker gene expression and IP<sub>3</sub> accumulates at 10 sec of nitrate induction, it has been suggested that PLC-IP<sub>3</sub> plays a critical role in nitrate signalling. However, the IP<sub>3</sub> accumulation detected at 10 sec is not followed by a further increase of calcium signal in the excised Arabidopsis roots (Riveras *et al.*, 2015). Further biochemical, cellular and genetic studies are necessary to connect these promising findings of a link between nitrate regulation of PLC-IP<sub>3</sub> and calcium signalling.

Interestingly, nitrate induces the expression of a gene encoding myo-inositol polyphosphate 5-phosphatase2 (5-PTase2), which has the ability to remove 5' phosphate from IP<sub>3</sub> and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P<sub>4</sub>) in vitro (Berdy *et al.*, 2001; Canales *et al.*, 2014). This suggests that nitrate may induce 5-PTase2 and negatively regulate IP<sub>3</sub> accumulation in the cell. Prior studies have showed that IP<sub>3</sub> accumulates in both *5ptase1* and *5ptase2* mutants (Gunesekera *et al.*, 2007), and plants constitutively expressing the human type I 5-PTase have reduced basal IP<sub>3</sub> level (Perera *et al.*, 2006). Nitrate-induced 5-PTase2 gene expression may contribute to controlling IP<sub>3</sub> homeostasis by a negative feedback. Elucidating the connections between nitrate, transceptor, PLC, IP<sub>3</sub>, and calcium signalling may open a new direction for studying 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 CPKs but larger than 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 CPKs 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 functionally redundant subgroup III CPK10/30/32 as new regulators in PNR. Because single *cpk* mutants lack overt growth phenotypes and the *cpk10,30* double mutant exhibits embryonic lethality, a chemical genetic approach was developed to generate a so-called “inducible *cpk10,30,32*” (*icpk*) triple mutant (Liu *et al.*, 2017). In the *icpk* 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 transcriptome and phenotypical analyses of wild type and *icpk* plants indicate that CPK10/30/32 control nitrate-specific gene expression and growth. CPK10/30/32 act as the functionally redundant master regulators modulating a broad spectrum of genes involved in diverse cellular and metabolic pathways immediately activated by nitrate. For example, CPK10/30/32 target genes 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, 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 development and provides an interconnection between local and systemic nitrate signalling via the action of a mobile growth hormone (Fig. 1) (Kiba *et al.*, 2013; Liu *et al.*, 2017).

Importantly, CPK10/30/32 can directly phosphorylate Ser205 of NLP7 and activate this master TF in the PNR. It is demonstrated that CPK10/30/32 phosphorylation of NLP7 is calcium dependent and leads to NLP7 nuclear retention and activation (Liu *et al.*, 2017; Marchive *et al.*, 2013). A previous study suggested that subgroup III CPKs are relatively insensitive to calcium in an in vitro assay for kinase activity. However, the kinase activity assay was conducted using a washing buffer containing ~600 nM calcium, which may be sufficient to activate subgroup III CPKs (Boudsocq *et al.*, 2012). Consistently, the calcium-binding K<sub>d</sub> of GCaMP6s required for the clear detection of specific calcium increase stimulated by nitrate appears to match the calcium sensitivity of nitrate-activated CPKs (Chen *et al.*, 2013; Costa *et al.*, 2018).

Transient expression of ectopic NLP7, but not NLP7(S205A), in mesophyll protoplasts is sufficient to activate a wide range of putative NLP7 target genes beyond the well-known nitrate responsive marker genes (Liu *et al.*, 2017). These novel CPK-NLP7 target genes include S phase genes involved in cell cycle initiation as well as genes involved in auxin synthesis, transport and signalling. These new CPK-NLP7-auxin pathways can be mechanistically separated from the NPF6.3-dependent auxin transport function (Krouk *et al.*, 2010a) or the nitrate stimulated expression of *AFB3* encoding an auxin receptor (O'Brien *et al.*, 2016; Vidal *et al.*, 2010). Notably, auxin biosynthesis mediated by Tryptophan Aminotransferase Related2 in root meristem cells and auxin signalling mediated by a receptor-like kinase (RLK) Transmembrane kinase 1 are likely crucial for nitrate promotion of lateral root development (Dai *et al.*, 2013; Ma *et al.*, 2014). These unique NLP7 target genes may partially explain the specific growth defects found in *nlp7*, but not other *nlp* single mutant plants, which can be fully rescued by NLP7 but not NLP(S205A) (Liu *et al.*, 2017). Further studies of these novel NLP7 target genes in response to nitrate-CPK signalling may expand our understanding of nitrate-modulated development in specific cell types of different plant organs.

CPKs are the emerging master regulator targeting a broad range of cellular substrates in diverse signalling pathways (Boudsocq and Sheen, 2013; Curran *et al.*, 2011; Simeunovic *et al.*, 2016; Yip Delormel and Boudsocq, 2019). As the CPK-phosphorylated Ser defined in NLP7 is conserved in Arabidopsis NLP1-9 and in NLP orthologs of different plant species, CPK10/30/32 may regulate other NLPs for common or distinct target genes in diverse cell types for different developmental processes in the PNR (Konishi and Yanagisawa, 2013; Liu *et al.*, 2017; Marchive *et al.*, 2013; Yan *et al.*, 2016). 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 nutrient utilization, stress tolerance, and pathogen resistance in plants (Boudsocq and Sheen, 2013; Liu *et al.*, 2017; Simeunovic *et al.*, 2016; 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 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 (Ho *et al.*, 2009; Liu and Tsay, 2003). The high affinity mode of NPF6.3 function in nitrate transport is switched on by CIPK23 phosphorylation of Thr101 at low external nitrate, which also inhibits the primary nitrate response mediated by NPF6.3 based on *Nitrate 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 (Bender *et al.*, 2018; Ho *et al.*, 2009; L eran *et al.*, 2015). 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 (ABA Insensitive 2) 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 characterizing nitrate influx in different PP2C mutant plants (L eran *et al.*, 2015).

ABI2 regulates nitrate-mediated *NRT2-1* expression and root growth by dephosphorylating CBL1 and CIPK23 and enhancing NPF6.3 activity in nitrate signalling (L eran *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 (Bender *et al.*, 2018; Xu *et al.*, 2006). It has been proposed that drought and osmotic stress induces ABA accumulation and then inactivate ABI2, thereby enhancing the uptake of potassium ions as cellular osmolytes 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 to exert 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, first by nitrate-responsive reporter gene expression and second by 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 nitrite reductase gene (*NIR*) in the absence of nitrate and lacks the normal inhibitory effect of high nitrate on lateral root elongation (Liu *et al.*, 2012). *NCR1* encodes C-terminal domain (CTD) phosphatase-like 3 (CPL3) with functions in multiple regulatory pathways, such 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 responsible for transcription of protein-coding genes in all eukaryotes. The CTD of the largest subunit consists of conserved heptapeptide repeats with the consensus sequence Y<sub>1</sub>S<sub>2</sub>P<sub>3</sub>T<sub>4</sub>S<sub>5</sub>P<sub>6</sub>S<sub>7</sub> (Buratowski, 2009). It has been demonstrated that CPL3 dephosphorylates Ser2 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 the *cpl3* mutant, CPL3 does not control general gene transcription but participates in regulating specific flg22-responsive gene expression (Li *et al.*, 2014). The molecular mechanism for 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 PNR or affects expression of genes involved in modulating lateral root elongation through modulating 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 thaliana* with different concentrations, at different response time points, in different plant organs, and at different developmental stages using various growth conditions (Canales *et al.*, 2014; Gifford *et al.*, 2008; Gutierrez *et al.*, 2007; Hu *et al.*, 2009; Krouk *et al.*, 2010b; Patterson *et al.*, 2010; Ruffel *et al.*, 2011; Vidal *et al.*, 2015; Wang *et al.*, 2000; Wang *et al.*, 2003; Wang *et al.*, 2004; Wang *et al.*, 2007). 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 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 nitrate responsive (Table 1). Interestingly, some of the most robustly enriched nitrate regulated genes encode different CIPKs, putative MAPK cascade components, RLKs, and PP2Cs (Table 1) (Fig. 2). Despite limited information on their physiological roles in nitrate signalling, we 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 (Bender *et al.*, 2018; Luan, 2009; Mao *et al.*, 2016; Zhu, 2003). 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  $K_m$  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  $\sim 30$   $\mu$ M 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 NPF6.3 transceptor, to mediate their distinct regulations in 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). Nitrate induces the expression of *CIPK1,3,8,13,14,16,21,22,23,25* but represses the expressions of *CIPK17,20,25*. *CIPK1* and *CIPK3* are 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 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 cascade-encoding genes have been predicted in *Arabidopsis thaliana* (Ichimura et al., 2002). Numerous intrinsic and extracellular stimuli can activate MAPK cascades to regulate gene expression and integrate developmental processes (Komis et al., 2018; Xu and Zhang, 2015). 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 *MAPKKK13,14,19* genes that are the top nitrate-activated genes in many transcriptome datasets (Table 1). It will be interesting to define their downstream MAPKKs and MAPKs that may promote nitrate signalling. Nitrate also activates other genes encoding putative RAF-like MAPKKs, including Integrin-linked kinase 4, which interacts with RLKs and a putative calcium channel (Popescu et al., 2017) as well as *STY8* and *STY46* that 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 growth retardation, *STY46* likely plays the predominant role in chloroplast and leaf development promoted by nitrate. The ambiguous effect of nitrate on expression of *STY8/46* suggests that other stimuli or tissue-specific expression may contribute to the regulation of *STY8/46*.

Intriguingly, the expression of *MAPKK9* is repressed by nitrate in five experimental conditions (Table 1) and is induced by a relatively low (0.1 mM) nitrate condition in 2-3 days, which probably corresponds to nitrate starvation condition (Luo et al., 2017). Expression of constitutively active *MAPKK9* kinase in low nitrate concentrations reduces the expression of anthocyanin biosynthetic genes as well as the accumulation of anthocyanin (Luo et al., 2017). It

was suggested that *MAPKK9* negatively regulates the production of anthocyanins but promotes acquisition of nitrogen through *NRT2.1* activation. The 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 *MAPK3* and *MAPK6* are required for controlling proper progression of lateral root primordia (Zhu *et al.*, 2019). Whether nitrate modulates lateral root formation through *MAPKK9*-*MAPK3/6* signalling is unclear and requires further studies. 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)* involved in the stabilization of cortical microtubules (Walia *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 their 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 PK family protein (*LRR-RKs*), the largest family of putative receptor kinases (Chakraborty *et al.*, 2019; Shiu and Bleecker, 2001). The extracellular *LRR* domains of some *LRR-RKs* mediate responses to endogenous peptides that modulate cell proliferation, cell differentiation, immunity, symbiosis and response to wounding. For instance, 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 upregulation of *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 upregulated nitrate responsive genes (Table 1). Although *CRK29* and *CRK30* were reported to be involved in plant immunity and plant cell-to-cell communication, the possible functions for *CRK29/30* in nitrate signalling could be further explored (Amari *et al.*, 2010; Caillaud *et al.*, 2014; Lee *et al.*, 2011).

### 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 belonging to group A, B, C, and E (Table 1). *PP2CA* activation was detected in five microarray datasets (Table 1) and has been shown to negatively regulate ABA-mediated responses in seed and at the vegetative stage, the SNF1-related protein kinase 1 (SnRK1) energy sensor kinase activity, and the guard cell outward-rectifying potassium efflux channel (Lefoulon *et al.*, 2016; Rodrigues *et al.*, 2013). 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 phosphatase AP2C1 (AP2C1) and PP2C5 are also activated by nitrate (Table 1). AP2C1 and PP2C5 share overlapping functions and can directly interact with and dephosphorylate MAPK3/4/6 predominantly in the nucleus (Brock *et al.*, 2010; Schweighofer *et al.*, 2007). 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 their positive roles in seed germination, stomatal closure and gene regulation in ABA responses (Brock *et al.*, 2010). Beside interacting with MAPK3/4/6, AP2C1 dephosphorylates CIPK9 to regulate root growth and seedling development under low-potassium conditions (Singh *et al.*, 2018). Nitrate may induce PP2C 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).

### **Nitrate-dependent 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 levels provide further molecular and biochemical insight into the PNR. Advances in phosphoproteomics analyses has 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 five minutes 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). The findings are consistent with NIA2 phosphorylation at Ser-534 and NRT2.1 phosphorylation at Ser-28 under the nitrate deprivation condition (Engelsberger and Schulze, 2012; Menz *et al.*, 2016). The highly conserved Ser-543 in NIA2 has been reported to exert 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 control of nitrate-dependent plant development.

Nitrate resupply stimulates rapid phosphorylation of many interesting PKs, including leucine-rich repeat RLK (LRR-RK) Impaired oomycete susceptibility 1 (IOS1) (Hok *et al.*, 2014) and Kinase 7 (KIN7) (Isner *et al.*, 2018), membrane-associated PK 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) (Sreeramulu *et al.*, 2013; Tang *et al.*, 2008), and 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 (Ondizhi-Assoume *et al.*, 2016; Ristova *et al.*, 2016). The *ios1* 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 stomata closure by modulating tonoplast potassium channel activity (Isner *et al.*, 2018). CST activates the cell separation process that may

be required for the nitrate promotion 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, how nitrate triggers SnRK2.4 phosphorylation and regulates root growth are unclear. Brassinosteroid (BR) promotes plant growth through the BR receptor (BRI)-BKS1 kinase relay to regulate BR signalling and BRI1 phosphorylates BSK1 at Ser-230 (Tang *et al.*, 2008). As nitrate also induces BSK1 Ser-230 phosphorylation, future studies will elucidate the molecular mechanism integrating nitrate and BR signalling in controlling 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 physiology 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).

## **Perspective**

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 demonstrate that nitrate, in both roots and shoots, 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 global reprogramming of nitrate-responsive genes (Liu *et al.*, 2017; Marchive *et al.*, 2013). Besides NLPs, other TFs such as ARABIDOPSIS 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 (Brooks *et al.*, 2019; Vidal *et al.*, 2015; Wang *et al.*, 2018). 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 transcriptome, nutrient transport, cellular metabolism, hormone signalling, and cell proliferation, which mediate the shoot-root coordination and developmental plasticity that shapes 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-IP<sub>3</sub> 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 the screening for and identification of new signalling molecules and calcium channels that activate nitrate responses (Jiang *et al.*, 2019; Yuan *et al.*, 2014). 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 showed to play key roles in regulating nitrate signalling (Brooks *et al.*, 2019; Vidal *et al.*, 2015; Wang *et al.*, 2018). 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 H, C and O, 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 represents substantial energy consumption and economic burden. Moreover, the leaching of nitrate from 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.

### **Acknowledgements**

K.H.L., Z.L and C.L are supported by start-up fund from NWFU, NSFC-31670246 and NSFC-31870227. A.D. and J.S. are supported by the National Institute of General Medical Sciences (R01 GM060493 and GM129093). Deposited in PMC for release after 12 months.

Accepted Manuscript

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## Figure Legends

**Fig. 1.** Calcium-CPK-NLP in nitrate signalling. Nitrate triggers a gradually rising calcium transient in the cytosol and nucleus. The involvement of PLC-IP<sub>3</sub> and the external or internal sources responsible for nitrate-triggered calcium increase are unclear. Increased calcium activates calcium-sensor protein kinases (CPKs), including CPK10, CPK30 and CPK32, which then phosphorylate NLP7 or other NLPs and transcription factors (TFs). This calcium-CPK-NLP signalling cooperates with the unknown nitrate-responsive signal (grey dotted arrow line) to control gene expression in the PNR as well as the NLP7-specific target genes mediating cell cycle and auxin regulation. Dashed arrow lines are proposed connections. PNR: primary nitrate responses; NRT: Nitrate transporter/sensor; PLC: Phospholipase C; IP<sub>3</sub>: Inositol 1,4,5-trisphosphate.

**Fig. 2.** Diverse mechanisms modulate PKs and PPs in the PNR. At the transcriptional level (green), nitrate up- or down-regulates expression of genes encoding CIPKs, MAPKKs, PP2Cs, MAPKK9, MPK18, and PSY1R. Their downstream target proteins or partners are shown (grey). At the post-transcriptional level, nitrate activates CPK10/CPK30/CPK32, which then phosphorylate and activate NLP7, and possibly NLP6/8 and other NLPs (yellow). CPL3 might dephosphorylate the CTD domain of RNA Polymerase II to negatively regulate its activity for genes involved in lateral root suppression. ABI2 interacts with and dephosphorylates the CIPK23-CBL1/CBL9 complex to regulate NPF6.3-mediated nitrate transport and signalling. The PNR induces phosphorylation of PKs (white ovals) and reduces phosphorylation of NRT2.1. The dashed arrow lines are proposed connections. PSY1R: Plant peptide containing sulfated receptor; ABI2: ABA In-sensitive 2; PP2C: protein phosphatase C; CST: Cast away; NEK6: Never in mitosis gene A-related kinase 6; BSK1: BR-signalling kinase 1; CBL: calcineurin B-like protein; CIPK: calcineurin B-like protein (CBL)-interacting protein kinase; CPK: calcium-sensor protein kinases; IOS1: Impaired oomycete susceptibility 1; SNRK2.4: Sucrose nonfermenting1-Related Kinase 2.4; CPL3: RNA polymerase II C-terminal domain phosphatase-like 3; MAPK: mitogen-activated PK; MAPKK: MAPK kinase; MAPKKK: MAPKK kinase; CDKC: cyclin-dependent kinase C; RNA II: RNA

polymerase II; CTD: carboxyl-terminal domain; PNR: primary nitrate responses; PHS1: Propyzamide hypersensitive 1; NLP: NIN-Like Protein.

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**Table 1** | Genome-wide meta-analysis of nitrate-responsive genes encoding PKs and PPs<sup>a</sup>

| Gene ID <sup>c</sup> | Description  | Total <sup>b</sup> | Induced | Repressed |
|----------------------|--|--------------------|---------|-----------|
| AT2G2698             | CBL-interacting protein kinase 3 ( <i>CIPK3</i> )                            | 20                 | 20      | 0         |
| AT3G1656             | Protein phosphatase 2C family protein  | 16                 | 16      | 0         |
| AT4G3295             | Protein phosphatase 2C family protein  | 15                 | 15      | 0         |
| AT4G3847             | ACT-like protein tyrosine kinase family protein ( <i>STY46</i> )             | 14                 | 14      | 0         |
| AT2G3004             | mitogen-activated protein kinase kinase kinase 14 ( <i>MAPKKK14</i> )        | 14                 | 14      | 0         |
| AT1G4916             | Protein kinase superfamily protein ( <i>WNK7</i> )                           | 14                 | 14      | 0         |
| AT3G1751             | CBL-interacting protein kinase 1 ( <i>CIPK1</i> )                            | 12                 | 12      | 0         |
| AT5G2601             | Protein phosphatase 2C family protein  | 11                 | 2       | 9         |
| AT5G5413             | Calcium-binding endonuclease/exonuclease/phosphatase family                  | 10                 | 10      | 0         |
| AT2G1782             | histidine kinase 1 ( <i>HK1</i> )  | 9                  | 9       | 0         |
| AT1G0715             | mitogen-activated protein kinase kinase kinase 13 ( <i>MAPKKK13</i> )        | 8                  | 8       | 0         |
| AT2G1770             | ACT-like protein tyrosine kinase family protein ( <i>STY8</i> )              | 7                  | 0       | 7         |
| AT1G3027             | CBL-interacting protein kinase 23 ( <i>CIPK23</i> )                          | 6                  | 6       | 0         |
| AT2G2303             | SNF1-related protein kinase 2.9 ( <i>SNRK2.9</i> )                           | 6                  | 6       | 0         |
| AT1G7230             | Leucine-rich receptor-like protein kinase family protein ( <i>PSY1R</i> )    | 5                  | 5       | 0         |
| AT3G1141             | protein phosphatase 2CA ( <i>PP2CA</i> )                                     | 5                  | 5       | 0         |
| AT1G7350             | MAP kinase kinase 9 ( <i>MKK9</i> )  | 5                  | 0       | 5         |
| AT4G2999             | Leucine-rich repeat transmembrane protein kinase protein                     | 4                  | 4       | 0         |
| AT1G5351             | mitogen-activated protein kinase 18 ( <i>MPK18</i> )                         | 4                  | 4       | 0         |
| AT5G6708             | mitogen-activated protein kinase kinase kinase 19 ( <i>MAPKKK19</i> )        | 4                  | 4       | 0         |
| AT3G2275             | Protein kinase superfamily protein   | 4                  | 4       | 0         |
| AT2G3002             | Protein phosphatase 2C family protein ( <i>AP2C1</i> )                       | 4                  | 4       | 0         |
| AT5G3824             | Protein kinase family protein  | 4                  | 2       | 2         |
| AT1G3326             | Protein kinase superfamily protein   | 4                  | 0       | 4         |
| AT2G3418             | CBL-interacting protein kinase 13 ( <i>CIPK13</i> )                          | 3                  | 3       | 0         |
| AT2G2509             | CBL-interacting protein kinase 16 ( <i>CIPK16</i> )                          | 3                  | 3       | 0         |
| AT4G2141             | cysteine-rich RLK (RECEPTOR-like protein kinase) 29 ( <i>CRK29</i> )         | 3                  | 3       | 0         |
| AT4G1146             | cysteine-rich RLK (RECEPTOR-like protein kinase) 30 ( <i>CRK30</i> )         | 3                  | 3       | 0         |
| AT3G5876             | Integrin-linked protein kinase family ( <i>ILK4</i> )                        | 3                  | 3       | 0         |
| AT5G0182             | CBL-interacting protein kinase 16 ( <i>CIPK14</i> )                          | 3                  | 0       | 3         |
| AT1G7604             | calcium-dependent protein kinase 29 ( <i>CPK29</i> )                         | 2                  | 2       | 0         |
| AT2G3849             | CBL-interacting protein kinase 22 ( <i>CIPK22</i> )                          | 2                  | 2       | 0         |
| AT1G4910             | Leucine-rich repeat protein kinase family protein                            | 2                  | 2       | 0         |
| AT1G5181             | Leucine-rich repeat protein kinase family protein                            | 2                  | 2       | 0         |
| AT5G6271             | Leucine-rich repeat protein kinase family protein                            | 2                  | 2       | 0         |
| AT2G2825             | Protein kinase superfamily protein ( <i>NCRK</i> )                           | 2                  | 2       | 0         |
| AT3G0707             | Protein kinase superfamily protein   | 2                  | 2       | 0         |
| AT3G2869             | Protein kinase superfamily protein   | 2                  | 2       | 0         |
| AT1G6358             | Receptor-like protein kinase-related family protein                          | 2                  | 2       | 0         |
| AT1G0500             | Phosphotyrosine protein phosphatases superfamily protein ( <i>PFA-DSP1</i> ) | 2                  | 2       | 0         |
| AT1G1622             | Protein phosphatase 2C family protein  | 2                  | 2       | 0         |
| AT3G1680             | Protein phosphatase 2C family protein ( <i>EGR3</i> )                        | 2                  | 2       | 0         |
| AT5G2511             | CBL-interacting protein kinase 25 ( <i>CIPK25</i> )                          | 2                  | 1       | 1         |
| AT5G5965             | Leucine-rich repeat protein kinase family protein                            | 2                  | 1       | 1         |
| AT1G0788             | Protein kinase superfamily protein ( <i>ATMPK13</i> )                        | 2                  | 1       | 1         |
| AT3G5935             | Protein kinase superfamily protein   | 2                  | 1       | 1         |
| AT3G1437             | Protein kinase superfamily protein ( <i>WAG2</i> )                           | 2                  | 1       | 1         |
| AT2G4018             | phosphatase 2C5 ( <i>PP2C5</i> )   | 2                  | 1       | 1         |

| Gene ID <sup>a</sup> | Description   | Total <sup>b</sup> | Induced | Repressed |
|----------------------|---|--------------------|---------|-----------|
| AT1G5180             | Leucine-rich repeat protein kinase family protein ( <i>SIF3</i> )               | 2                  | 0       | 2         |
| AT2G2330             | Leucine-rich repeat protein kinase family protein                               | 2                  | 0       | 2         |
| AT4G3395             | Protein kinase superfamily protein ( <i>OST1</i> )                              | 2                  | 0       | 2         |
| AT1G6693             | Protein kinase superfamily protein ( <i>LRK10L2</i> )                           | 2                  | 0       | 2         |
| AT5G6621             | calcium-dependent protein kinase 28 ( <i>CPK28</i> )                            | 1                  | 1       | 0         |
| AT5G5763             | CBL-interacting protein kinase 21 ( <i>CIPK21</i> )                             | 1                  | 1       | 0         |
| AT4G2440             | CBL-interacting protein kinase 8 ( <i>CIPK8</i> )                               | 1                  | 1       | 0         |
| AT2G2922             | Concanavalin A-like lectin protein kinase family protein ( <i>LECRK-III.1</i> ) | 1                  | 1       | 0         |
| AT4G2323             | cysteine-rich RLK (RECEPTOR-like protein kinase) 15 ( <i>CKR15</i> )            | 1                  | 1       | 0         |
| AT4G2140             | cysteine-rich RLK (RECEPTOR-like protein kinase) 28 ( <i>CRK28</i> )            | 1                  | 1       | 0         |
| AT4G0449             | cysteine-rich RLK (RECEPTOR-like protein kinase) 36 ( <i>CRK36</i> )            | 1                  | 1       | 0         |
| AT4G0454             | cysteine-rich RLK (RECEPTOR-like protein kinase) 39 ( <i>CRK39</i> )            | 1                  | 1       | 0         |
| AT4G2314             | cysteine-rich RLK (RECEPTOR-like protein kinase) 6 ( <i>CRK6</i> )              | 1                  | 1       | 0         |
| AT2G1919             | FLG22-induced receptor-like kinase 1 ( <i>SIRK</i> )                            | 1                  | 1       | 0         |
| AT1G7582             | Leucine-rich receptor-like protein kinase family protein ( <i>CLV1</i> )        | 1                  | 1       | 0         |
| AT2G2900             | Leucine-rich repeat protein kinase family protein                               | 1                  | 1       | 0         |
| AT3G4637             | Leucine-rich repeat protein kinase family protein                               | 1                  | 1       | 0         |
| AT5G4978             | Leucine-rich repeat protein kinase family protein                               | 1                  | 1       | 0         |
| AT2G1921             | Leucine-rich repeat transmembrane protein kinase protein                        | 1                  | 1       | 0         |
| AT2G0145             | MAP kinase 17 ( <i>MPK17</i> )  | 1                  | 1       | 0         |
| AT5G5438             | protein kinase family protein ( <i>THE1</i> )                                   | 1                  | 1       | 0         |
| AT3G6326             | Protein kinase superfamily protein ( <i>ATMRK1</i> )                            | 1                  | 1       | 0         |
| AT1G6869             | Protein kinase superfamily protein ( <i>PERK9</i> )                             | 1                  | 1       | 0         |
| AT1G6646             | Protein kinase superfamily protein  | 1                  | 1       | 0         |
| AT5G4168             | Protein kinase superfamily protein  | 1                  | 1       | 0         |
| AT4G1189             | Protein kinase superfamily protein ( <i>ARCK1</i> )                             | 1                  | 1       | 0         |
| AT1G6355             | Receptor-like protein kinase-related family protein                             | 1                  | 1       | 0         |
| AT4G4001             | SNF1-related protein kinase 2.7 ( <i>SNRK2.7</i> )                              | 1                  | 1       | 0         |
| AT5G2793             | Protein phosphatase 2C family protein ( <i>EGR2</i> )                           | 1                  | 1       | 0         |
| AT1G0716             | Protein phosphatase 2C family protein   | 1                  | 1       | 0         |
| AT2G3370             | Protein phosphatase 2C family protein   | 1                  | 1       | 0         |
| AT1G4826             | CBL-interacting protein kinase 17 ( <i>CIPK17</i> )                             | 1                  | 0       | 1         |
| AT5G4582             | CBL-interacting protein kinase 20 ( <i>CIPK20</i> )                             | 1                  | 0       | 1         |
| AT3G4533             | Concanavalin A-like lectin protein kinase family protein ( <i>LECRK-I.1</i> )   | 1                  | 0       | 1         |
| AT5G0155             | lectin receptor kinase a4.1 ( <i>LECRK-VI.3</i> )                               | 1                  | 0       | 1         |
| AT5G0156             | lectin receptor kinase a4.3 ( <i>LECRKA4.3</i> )                                | 1                  | 0       | 1         |
| AT1G5189             | Leucine-rich repeat protein kinase family protein                               | 1                  | 0       | 1         |
| AT5G4977             | Leucine-rich repeat protein kinase family protein                               | 1                  | 0       | 1         |
| AT1G0570             | Leucine-rich repeat transmembrane protein kinase protein                        | 1                  | 0       | 1         |
| AT1G2615             | proline-rich extensin-like receptor kinase 10 ( <i>PERK10</i> )                 | 1                  | 0       | 1         |
| AT1G6159             | Protein kinase superfamily protein  | 1                  | 0       | 1         |
| AT3G0901             | Protein kinase superfamily protein  | 1                  | 0       | 1         |
| AT4G0976             | Protein kinase superfamily protein ( <i>CEK3</i> )                              | 1                  | 0       | 1         |
| AT3G4592             | Protein kinase superfamily protein  | 1                  | 0       | 1         |
| AT3G4627             | receptor protein kinase-related   | 1                  | 0       | 1         |
| AT5G1052             | ROP binding protein kinases 1 ( <i>RBK1</i> )                                   | 1                  | 0       | 1         |
| AT3G2315             | Signal transduction histidine kinase, hybrid-type, ethylene sensor              | 1                  | 0       | 1         |
| AT1G6136             | S-locus lectin protein kinase family protein                                    | 1                  | 0       | 1         |
| AT1G6150             | S-locus lectin protein kinase family protein                                    | 1                  | 0       | 1         |

| Gene ID <sup>c</sup> | Description  | Total <sup>b</sup> | Induced | Repressed |
|----------------------|--|--------------------|---------|-----------|
| AT4G2730             | S-locus lectin protein kinase family protein                                 | 1                  | 0       | 1         |
| AT1G2123             | wall associated kinase 5 ( <i>WAK5</i> )                                     | 1                  | 0       | 1         |
| AT2G3296             | Phosphotyrosine protein phosphatases superfamily protein ( <i>PFA-DSP2</i> ) | 1                  | 0       | 1         |

<sup>a</sup>Differential gene expression (Canales *et al.*, 2014; Li *et al.*, 2017; Liu *et al.*, 2017). In our analysis, we applied  $\geq 2$ -fold cut off.

<sup>b</sup>Total number of experiments in which expression of a gene is either induced or repressed by nitrate.

<sup>c</sup>Genes that are induced or repressed by nitrate in more than three experiments are in bold.

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