PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

TESIS DOCTORAL:

LA FUNCION DE CALCIO EN LA VÍA DE SEÑALIZACIÓN MEDIADA POR NITRATO EN RAÍCES DE *ARABIDOPSIS THALIANA*.

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias Biológicas con mención en Genética Molecular y Microbiología.

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2015

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. Rodrigo A. Gutiérrez for his advice, guidance and encouragement throughout my work and also for all the opportunities he provided me.

I would like to thank to Dra. Ana Laxalt of the Universidad Nacional de Mar del Plata for her cooperation and guidance in my investigation with phosphatidic acid in response to nitrate.

I would like to specially acknowledge Jose Miguel, Carolina, Elena and Andrea for help me in this thesis and great ideas to improve this work. I would also like to thank all the members of the Plant Systems Biology Lab at Pontificia Universidad Católica de Chile or also nicknamed as “La Furia PSBL”.

I would like to thank to my family for give me all the opportunities to develop myself in all I wanted. I would like to thank especially to my wife Macarena for loving me and for help me during this special stage with her supporting in every important decision.

This work would not have been possible without the financial support of CONICYT, which awarded me a 4-year Doctoral scholarship and a Doctoral thesis support scholarship of the “VicerrectoríaAdjunta de Investigación y Doctorado” at the Faculty of Biological Sciences in Pontificia Universidad Católica de Chile.

This work was funded by International Early Career Scientist program from Howard Hughes Medical Institute, Fondo de Desarrollo de Areas Prioritarias (FONDAP) Center for Genome Regulation (15090007), Millennium Nucleus Center for Plant Functional Genomics (P10-062-F), Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1141097 and PhD fellowship from ComisiónNacional de InvestigaciónCientífica y Tecnológica (AT-24121649) to E.R.

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GLOSSARY

ABA: Abscisic acid

ANOVA: Analysis of variance

ARF: Auxin response factor

C: Carbon

CIPK: CBL- interacting protein kinase

CNGC: Cyclic nucleotide-gated channel

CaM: Calmodulin

CDPK: Ca^{2+}-dependent protein kinases

CBL: Calcineurin-like protein

cAMP: Cyclic AMP

cGMP: Cyclic GMP

cADPR: Cyclic ADP ribose

CDS: Coding sequence

Col-0: Columbia 0 ecotype.

Coelenterazine (CTZ): 3,2-Dihydro-2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazolo [1,2-a] pyrazin-3-one

Ca^{2+}: calcium,

$[Ca^{2+}]_{cyt}$: cytoplasmic Ca^{2+} concentration

DAG: Diacylglycerol

DGPP: Diacylglycerolpyrophosphate

DGK: Diacylglycerol kinase

EGTA: Ethylene glycol tetraacetic acid

GFP: Green fluorescent protein

HATS: High affinity transport systems

iGluR: Glutamate receptor

IP_3: inositol 1, 4, 5-trisphosphate

KNO_3: Potassium nitrate

KCl: Potassium chloride

LATS: Low affinity transport systems

$LaCl_3$: Lanthanum Chloride

LR: Lateral root

MS: Murashige and Skoog Medium

miRNA: micro RNA

N: Nitrogen

NLP: NIN-like protein

NR: Nitrate reductase

NRT1.1/AtNPF6.3: Nitrate transporter 1.1

NaCl: Sodium chloride

NIR: Nitrite reductase

NIA1: Nitrate reductase 1

NRT: Nitrate transporter

PRG: Primary nitrate response

PM: Plasma membrane

PLC: Phospholipase C

^{32}Pi: Inorganic ^{32}P-orthophosphate

PLD: Phospholipase D

PIP: phosphatidylinositol phosphate

PIP2: phosphatidylinositol bisphosphate

PA: Phosphatidic acid

PCR: Polymerase chain reaction

PRG: Primary response gene

qPCR: quantitative PCR

R: Receptor

ROCC: Receptor-operated calcium channel

RMA: robust multiarray analysis

TLC: Thin layer chromatography

U73122: 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione

U73343: 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione

VDCC: Voltage-dependent calcium channel

WT-AQ: 35S::Aequorin line

ABSTRACT

Understanding how plants sense and respond to changes in nitrogen (N) availability is the first step towards developing strategies for biotechnological applications such as to improve nitrogen-use efficiency. However, components involved in N signaling pathways remain poorly characterized. Calcium is a second messenger in signal transduction pathways in plants but its role in nitrate responses has not been addressed. Using aequorin reporter plants we show that nitrate treatments transiently increase cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). We found that nitrate also induces a rise in cytoplasmic concentration of inositol 1, 4, 5-trisphosphate (IP_3). Increases in IP_3 and $[Ca^{2+}]_{cyt}$ levels in response to nitrate treatments were blocked by U732122, a pharmacological inhibitor of phospholipase C (PLC), but not the non-functional analog U73343. In addition, increases in IP_3 and $[Ca^{2+}]_{cyt}$ levels in response to nitrate treatments were abolished in mutants of the nitrate transceptor NRT1.1/AtNPF6.3. Expression of nitrate-responsive genes was severely affected by pretreatments with Ca^{2+} channel blockers and PLC inhibitors. These results indicate that Ca^{2+} act as a second messenger in the nitrate-signaling pathway of *Arabidopsis thaliana*. Our results suggest a model where NRT1.1/AtNPF6.3 and a phospholipase C activity mediate the increase of Ca^{2+} in response to nitrate required for changes in expression of prototypical nitrate-responsive genes.

RESUMEN

Entender como las plantas censan y responden a cambios en la disponibilidad de nitrógeno (N) es el primer paso para desarrollar estrategias para aplicaciones biotecnológicas tales como mejorar la eficiencia del uso del nitrógeno. Sin embargo, los componentes involucrados en las vías de señalización de N son pobremente conocidos. Calcio es un segundo mensajero en vías de transducción de señales en plantas pero su rol en la respuesta a nitrato no ha sido evaluado. Usando una planta reportera que expresa la proteína Aequorina, hemos observado que tratamientos con nitrato incrementan la concentración de calcio citoplasmático ($[Ca^{2+}]_{cyt}$). Encontramos que nitrato también induce un aumento en los niveles de inositol trifosfato (IP_3). El incremento en los niveles de IP_3 y $[Ca^{2+}]_{cyt}$ en respuesta a nitrato fue bloqueado por U73122, un inhibidor de la actividad de la fosfolipasa C (PLC), pero no por el análogo no funcional U73343. Además, el incremento en los niveles de IP_3 y $[Ca^{2+}]_{cyt}$ en respuesta a nitrato fue inhibido en la mutante del receptor de nitrato NRT1.1/AtNPF6.3. La expresión de genes de respuesta nitrato fue severamente afectada por pre-tratamientos con bloqueadores de canales de calcio e inhibidores de la PLC. Estos resultados indican que el calcio actúa como un segundo mensajero en la vía de señalización de nitrato de *Arabidopsis thaliana*. Nuestros resultados sugieren un modelo en el que NRT1.1/AtNPF6.3 y la actividad de la PLC median el incremento de calcio en respuesta a nitrato, el cual a su vez produce el cambio en la expresión de genes de respuesta a nitrato.

1. INTRODUCTION

Plants are sessile organisms that evolved sophisticated responses to adapt to changing environmental conditions. Nutrient availability is an important factor for plant growth and productivity. Nitrogen (N) is the mineral nutrient required in the greatest amount by plants (Epstein and Bloom, 2005) and its availability, or lack thereof, limits plant growth and development (Alvarez et al., 2012), crop yield (Hirel et al., 2011) and primary production at a planetary scale (Gruber and Galloway, 2008). Available N forms, such as nitrate or ammonia, are in short supply in natural as well as agricultural systems. Plants have evolved sophisticated strategies to cope with heterogeneous N availability in the soil (Alvarez et al., 2012). Two sophisticated uptake systems have been found in plants: the low affinity transport systems (LATS) that acts in high nutrient concentrations (above 1 mM), and the high affinity transport systems (HATS) which acts at the micromolar range (Wang et al., 1993). N uptake is subjected to strict regulation according to plant demands (Crawford and Glass, 1998). Nitrate is the main source of N in agriculture and a potent signal that regulates the expression of hundreds of genes (Wang et al., 2004; Vidal and Gutiérrez, 2008; Ho and Tsay, 2010). Microarray analyses performed using nitrate, nitrite and other forms of N revealed thousands of genes regulated by N signals (Alvarez et al., 2012; Canales et al., 2014). These genes participate in many different plant processes including metabolism, growth and development (Vidal and Gutiérrez, 2008; Vidal et al., 2014b). Due to the importance of nitrate as primary N source for plants, the nitrate response has been the most thoroughly characterized. Roots are highly responsive to nitrate, with more than 1,000 genes identified that respond rapidly and at very low concentrations of external nitrate (250 μM) (Wang, 2003; Wang et al., 2004). Transcriptomics changes caused by nitrate treatments have been shown to correlate with

later changes in metabolite levels such as amino acids, indicating that changes in gene expression by nitrate are important for modulating plant metabolic processes (Wang et al., 2004). To discriminate between nitrate responsive genes and genes responding to N-signals downstream of nitrate reduction, the root and shoot response of nitrate reductase (NR)-null mutant plants was compared to the root and shoot response of wild-type plants (Wang et al., 2004). NR-null plants cannot reduce nitrate to nitrite, which is the first step in the N reduction and assimilation pathway. Therefore, regulation of gene expression by nitrate treatments in NR-null plants are the result of nitrate acting as a signal and not a result of another N form or signal produced after nitrate reduction or assimilation. In these experiments, 38% of the genes were regulated similarly by nitrate treatments in NR-null mutant and wild-type plants suggesting they were responding directly to nitrate (Wang et al., 2004). These genes were involved in specific processes such as energy, glycolysis and nitrogen and sulfur metabolism (Wang et al., 2004).

Cross-talk between nitrate and hormone signals.

Phytohormones are signal molecules produced by the plant to regulate cellular processes in targeted cells. Hormones have a role in the development of flowers, stems, leaves, roots, among other organs of plants. Moreover, it has also been shown that such cross-controls exist between nitrate and auxin, cytokinin, and ABA.

Auxin plays an important role in modulation of root architecture in response to nitrate. A previous report has found a regulatory module that includes miR167 and AUXIN RESPONSE FACTOR 8 (ARF8) involved in regulation of LR initiation and emergence in response to nitrate (Gifford et al., 2008). We have previously shown that regulatory module miR393/auxin receptor AFB3 has a role in coordinating primary and lateral root growth in response to nitrate availability (Vidal et al., 2010). It has been proved that NAC4

transcription factor is a key regulatory factor acting downstream of AFB3 in lateral root development (Vidal et al., 2013a), supporting the idea that nitrate and auxin can modulate LR architecture by miR393/AFB3/NAC4. Moreover, evidence showed that nitrate can regulate at the transcriptional level iso-pentenyl-transferase 3 (IPT3). This enzyme catalyzes the critical step of cytokinin biosynthesis and has been shown to be involved in the production of nitrate-induced cytokinins (Nero et al., 2009). Similarly, exogenous supply of cytokinin down-regulates the expression of NITRATE TRANSPORTER 2 (NRT2) genes, such as NRT2.1, NRT2.3, and NRT2.6 (Ruffel et al., 2014), but the impact of cytokinin and nitrate on modulation of root architecture is still unclear. On the other hand, abscisic acid (ABA) plays a central role in mediating the regulatory effects of high nitrate concentrations on root branching in Arabidopsis. Abscisic acid (ABA) seems to be required for this effect, since the NO_3^- inhibitory effect on LR is reduced by mutating either ABI4 or ABI5 genes (Nero et al., 2009). Indeed, recently the low affinity NO_3^- transporter NRT1.2, another member of the NRT family, has been identified as an ABA transporter in a heterologous cellular system (Kanno et al., 2012). NO_3^- does not seem to inhibit ABA transport in a yeast system (Kanno et al., 2013), but the impact of ABA on NO_3^- transport by NRT1.2 has still to be determined.

NITRATE TRANSPORTER 1.1 (NRT1.1) has been proposed also to facilitate uptake of the phytohormone auxin. Nitrate inhibits NRT1.1-dependent auxin uptake, thereby NRT1.1 represses lateral root growth at low nitrate availability by promoting basipetal auxin transport out of these roots (Krouk et al., 2010b). In addition, NRT1.1 also has roles in regulation of root system architecture independent from nitrate uptake. NRT1.1 is involved in root colonization of nitrate-rich patches by promoting LR elongation in response to a localized high nitrate supply (Remans et al., 2006b), and this effect is mediated by the MADS box transcription factor ANR1 (Remans et al., 2006b). Also,

NRT2.1 is involved in Lateral Root (LR) repression in media that contain a high C/N ratio (Little et al., 2005) and in LR initiation control in response to a low nitrate supply (Remans et al., 2006a). This signaling function is shown to be independent of nitrate transport (Little et al., 2005; Remans et al., 2006a).

Nitrate perception and signal transduction

A few primary nitrate response genes (PRGs) have been identified, characterized by rapid changes in gene expression independent of protein synthesis in response to nitrate treatments (Sakakibara et al., 1997). Krouk et al. 2010a monitored genome-wide responses at early time points after exposing plants to 1 mM nitrate. Results of these experiments showed that the initial nitrate response includes genes involved in translation, suggesting that nitrate initially triggers a reprogramming of the transcriptome to ensure production of the necessary proteins for nitrate acquisition (Krouk et al., 2010a).

The best characterized sensor in nitrate signaling pathway is NITRATE TRANSPORTER 1.1 (NRT1.1). NRT1.1 can switch from low to high affinity by phosphorylation of threonine residue 101 (T101) (Ho et al., 2009). Under low nitrate conditions, CBL-INTERACTING PROTEIN KINASE (CIPK) 23 can phosphorylate T101, making NRT1.1 a high affinity nitrate carrier and leading to weak induction of *NRT2.1* gene expression (Ho et al., 2009). NRT2.1 is a high affinity nitrate transporter and one of the several PRGs that show a rapid induction of gene expression in response to nitrate treatments, independently of protein synthesis. Conversely, under high nitrate conditions, T101 is not phosphorylated, NRT1.1 functions as a low affinity carrier and NRT2.1 gene expression is strongly induced. The NRT1.1 protein is involved in both nitrate perception and transport (Wang et al., 2009; Dechorgnat et al., 2011; Gojon et al., 2011). These two functions can be decoupled in the *chl1-9* mutant allele carrying a point

mutation that changes the proline residue 492 to leucine. This mutation reduces NRT1.1 nitrate uptake in all affinity ranges but does not affect the primary nitrate response of NRT2.1. Thus nitrate transport activity is not required for the signaling function of NRT1.1 (Ho et al., 2009). Our laboratory has demonstrated that gene expression in response to nitrate of AFB3, NAC4, TGA1 and TGA4 depends on the nitrate transport function of NRT1.1/AtNPF6.3 (Alvarez et al., 2014; Vidal et al., 2014a). Thereby, both transport and signaling function of NRT1.1/AtNPF6.3 are important to control gene expression in response to nitrate. It has been shown that NRT1.1 is required for normal expression in response to nitrate of more than 100 genes in Arabidopsis roots (Wang et al., 2009). CIPK8 is a kinase whose expression depends on NRT1.1. CIPK8 is an important regulator of early nitrate response genes (Hu et al., 2009). *cipk8* mutants show 40% reduction in the plant response to nitrate treatments, suggesting CIPK8 functions upstream of PRGs (Hu et al., 2009). CIPKs act in concert with CBL proteins, plant-specific calcium binding proteins that activate CIPKs to phosphorylate downstream targets (Shi et al., 1999; Albrecht et al., 2001; Pandey et al., 2008). The function of CIPK proteins in gene expression in response to nitrate prompts a possible role for calcium as a second messenger in nitrate signaling pathways. Early experiments using maize and barley detached leaves showed that nitrate response genes could be altered by pretreating the leaves with calcium chelator EGTA or calcium channel blocker $LaCl_3$ (Sakakibara et al., 1997; Sueyoshi et al., 1999). These results suggest that nitrate signaling partly depends on cellular calcium levels and suggest a mechanism that requires transient increase in calcium levels to activate CBL-CIPK proteins which in turn trigger changes in protein phosphorylation patterns activating gene expression responses in Arabidopsis. However, the role of calcium in the nitrate response has not been thoroughly addressed.

Another important regulatory factor of the nitrate signaling pathway is NLP7. NLP7 belongs to the NIN-like protein family (NLPs) (Schauser et al., 1999). The expression of genes involved in N metabolism depends on NLP7 (Castaings et al., 2009) and recently it was demonstrated that NLP7 binds to the promoter of 851 genes in response to nitrate (Marchive et al., 2013). We recently used an integrative bioinformatics approach to demonstrate that TGA1 and TGA4 transcription factors are regulators of the nitrate response (Alvarez et al., 2014). TGA1 and TGA4 are bZIP transcription factors and are regulated by nitrate in Arabidopsis root. In addition, TGA1 and TGA4 transcription factors act downstream of NRT1.1 transport activity to transcriptionally regulate NRT2.1 in a nitrate dependent manner. TGA1 and TGA4 also regulate lateral root development and primary root growth (Alvarez et al., 2014). On the other hand, members of the LATERAL ORGAN BOUNDARY DOMAIN (LBD37/38/39), were found to repress anthocyanin biosynthesis and many other nitrate responsive genes, including key genes required for nitrate uptake and assimilation (Rubin et al., 2009). SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9) was identified as a negative regulator of *NIR* and *NIA2* using a systems biology approach (Krouk et al., 2010a). In this work, new nitrate responsive-genes at early time points were identified, including the SPL9 transcription factor, using high-resolution transcriptomics profiling in response to nitrate treatment.

In this proposal, we would like to address the hypothesis that a canonical signaling pathway involving Ca^{2+}, protein kinases and transcription factors exist to transduce the nitrate signal and induce changes in gene expression in plants. (Figure 1).

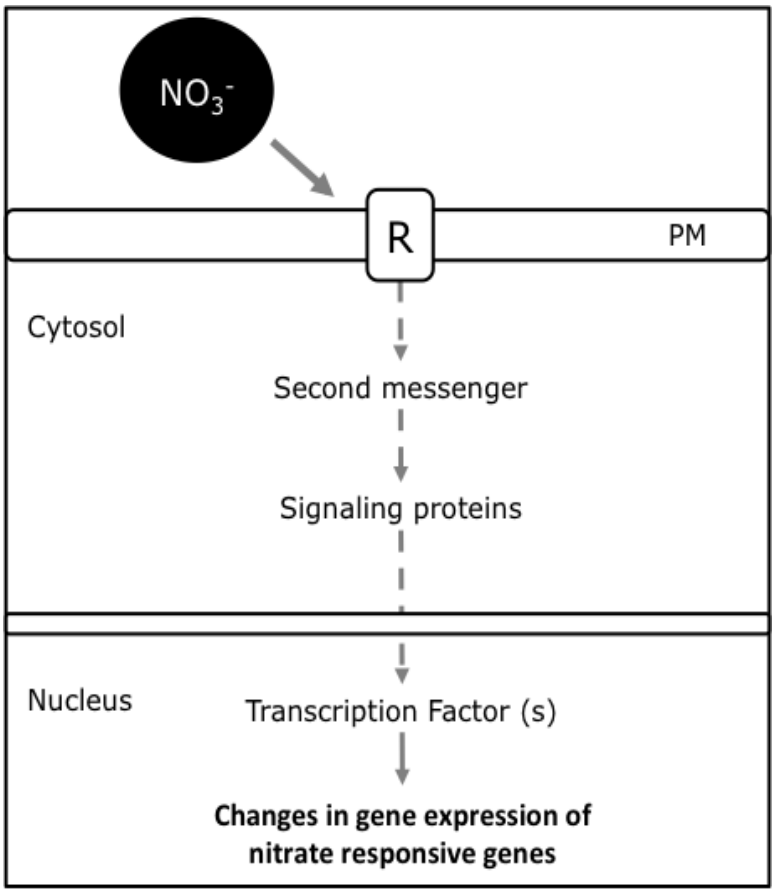

Figure 1.Simplified representation of a "canonical pathway" for nitrate signaling. An receptor (R) would perceive the nitrate signal and activate a signal transduction pathway that involves protein phosphorylation cascades that ultimately activate transcription factors that regulate expression of nitrate responsive genes. R: Receptor. PM: Plasma membrane.

Ca^{2+} in plant signaling pathways

Ca^{2+} is a key essential second messenger in signal transduction pathways in plants and other organisms (Harper et al., 2004; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Hepler, 2005). Multiple abiotic and biotic cues elicit specific and distinct spatiotemporal patterns of change in the cytosolic free Ca^{2+} concentration in plants (Sanders et al., 2002; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Hepler, 2005). Abiotic stimuli include light, UV/B irradiation, high temperature, touch, hyperosmotic stress, drought and oxidative stress (Bush, 1995; Plieth, 2001; Snedden and Fromm, 2001). Biotic stimuli include hormones such as abscisic acid (ABA), gibberellin, fungal elicitors, and nodulation factors (Bush, 1995; Plieth, 2001; Snedden and Fromm, 2001). Critical information regarding the nature and intensity of the stimuli can be relayed by changes in the characteristics of the "Ca^{2+} signature" consisting of differences in Ca^{2+} oscillation frequency, amplitude and localization. Ca^{2+} concentration in the cytoplasm is typically in the micromolar range and is finely controlled by regulating influx and efflux. Free Ca^{2+} in the cytosol can rapidly increase due to influx from outside the cell as well as release from internal stores in subcellular compartments such as vacuole or endoplasmic reticulum (Kiegle et al., 2000; Dodd et al., 2010). Influx is facilitated by Ca^{2+}-permeable channels in the plasma membrane and a strong electrochemical gradient due to concentrations in the millimolar range outside the cell and in internal stores such as vacuole (Kiegle et al., 2000; Dodd et al., 2010).

Ca^{2+} signatures are detected, decoded and transmitted to downstream responses by a set of Ca^{2+} binding proteins that function as Ca^{2+} sensors. Binding of Ca^{2+} to the protein sensors alter their structural and/or enzymatic properties, regulating their interaction with target proteins that affect solute transport and enzymatic activities, cytoskeletal orientation, protein phosphorylation cascades and changes in gene expression (White and Broadley,

2003; Dodd et al., 2010). In plants, some of the best studied calcium sensors are calmodulin (CaM), CaM-related proteins (Bouché et al., 2002), Ca^{2+}-dependent protein kinases (CDPK) (Harper et al., 2004) and calcineurin-like proteins (CBL) (Luan et al., 2002). Calmodulin is a small (17 kDa), highly conserved, acidic protein with two globular domains each containing two EF hands connected by a flexible α-helical spacer (Zielinski, 1998). Calmodulin binds to many different proteins implicated in diverse physiological processes including cation transporters (involved in cytosolic Ca^{2+} homeostasis) (Snedden and Fromm, 2001), cytoskeletal rearrangements and cell division (Bush, 1995), phytohormone (Reddy et al., 2000), disease resistance (Snedden and Fromm, 2001) and stress tolerance (Monshausen et al., 2009). Calmodulin can also regulate gene expression by binding to specific transcription factors such as EICBP (Ethylene-Induced CaM Binding Protein) that is involved in ethylene signal transduction (Reddy et al., 2000). CDPKs are ubiquitous in plants. They have four EF hands that bind Ca^{2+} to activate their serine/threonine kinases activity. CDPKs are capable of decoding Ca^{2+} signals by phosphorylating diverse target proteins including membrane solute transporters, ion and water channels, NADPH oxidases, metabolic enzymes, cytoskeletal proteins, proteases and DNA-binding proteins (Cheng et al., 2002; Sanders et al., 2002). The CBL Ca^{2+} sensor is a plant-specific family. Upon Ca^{2+} binding, they activate serine/threonine CIPKs which in turn activate downstream signal transduction cascades (Albrecht et al., 2001). It has been proposed that myristoylation of CBLs alter their cellular location and intracellular interaction (Luan et al., 2002). It is thought that particular CBLs transduce specific environmental or developmental signals (White and Broadley, 2003). CBL–CIPK complexes are known to regulate several stress responses, such as salt, cold, drought, abscisic acid signaling and K^+ shortage (Gong et al., 2004; Li et al., 2006; Pandey et al.,

2008) and are potential components of the nitrate signaling pathway (Ho et al., 2009; Hu et al., 2009).

Calcium transport Proteins

The Ca^{2+} concentrations on both sides of the cell membrane are finely controlled by regulating influx and efflux (channels, pumps, and transporter exchangers). Therefore, environmental changes causing the opening of the calcium channel gate creates a flow of Ca^{2+} ions and pumps and carriers are responsible for efflux to return Ca^{2+} concentration to basal levels in the cytosol (Knight et al., 1996; Polisensky and Braam, 1996; Alexandre et al., 1999; Meimoun et al., 2009; Chin et al., 2013; Choi et al., 2014a). The form of a Ca^{2+} signature that is potentially specific to relevant decoders is determined by the interplay between influx through channels and efflux through pumps and carriers. Moreover, the cellular and tissue location of ion channels play an important role in determining stimulus specificity (Wood et al., 2000; Marti et al., 2013). In animals, transport of Ca^{2+} into cells is controlled by three types of channels: voltage-dependent calcium channels (VDCCs), receptor-operated calcium channels (ROCCs), and mechanical-stimulation–gated channels. Electrophysiological, biochemical and sequence analyses have revealed the existence of VDCC and ROCC in plants (Sanders et al., 2002; Nagata et al., 2004). VDCC channels are controlled by the cell membrane voltage. Polarization or depolarization of the membrane voltage opens the gate subunits and permits a flood of calcium ions through the cell membrane. In plants, calcium–permeable channels that are activated by membrane depolarization and hyperpolarization have been revealed by diverse stimulus (Ward et al., 2009). On the other hand, the ROCCs channels family (cyclic nucleotide-gated channels [CNGCs] and Glutamate receptors [iGluR]) seem to have been conserved in plants and animals (Sanders et al., 2002; Nagata et al., 2004). Ionotropic glutamate receptors (iGluR)

families have 20 genes identified in Arabidopsis. Glutamate receptor homologs are required for glutamate-induced depolarization and intracellular Ca^{2+} elevation in roots (Ward et al., 2009). Physiological and genetic studies revealed that this channel is required for proper Ca^{2+}-fluxes underlying pollen tube growth and fertility, plant defense and wounding, among other processes (Steinhorst and Kudla, 2014).The Arabidopsis genome appears to encode 20 members of Cyclic nucleotide-gated channel (GNGC) (cyclic AMP [cAMP]) and cyclic GMP [cGMP])-gated channels (Nagata et al., 2004). Interestingly, GNGC have been implicated in diverse function in plants, including response to biotic and abiotic stress, as well as pollen tube growth (Guo et al., 2008; Tunc-Ozdemir et al., 2012; Chin et al., 2013). On the other hand, ryanodine and INOSITOL TRIPHOSPHATE (IP_3) receptors in plants have not homolog proteins compared with the animal channels (Nagata et al., 2004). In animals, these receptors opened the calcium channel gate when the second messengers Cyclic ADP Ribose (cADPR) and IP_3 join to the channels (Hymel et al., 1988; Finch and Augustine, 1998). Electrophysiological analysis has revealed the existence of ryanodine and IP_3 receptors and calcium oscillation but their molecular identification has not been possible (Blatt et al., 1990; Gilroy et al., 1990; Sanders et al., 2002). Recent evidence suggest that the IP_3 could be further phosphorylated into IP_6 (Laxalt and Munnik, 2002; Lemtiri-Chlieh et al., 2003; Meijer and Munnik, 2003; Munnik and Vermeer, 2010). The IP_6-induced increase in cytoplasmic calcium was shown to involve endomembrane for calcium release from internal stores and that the ability of IP_6 to release Ca^{2+} was ~100 times more potent than IP_3 (Lemtiri-Chlieh et al., 2003).

Several studies suggest that changes in $[Ca^{2+}]_{cyt}$ is mediated by IP_3 and the PI-PLC pathway in plants (Sanchez and Chua, 2001; Perera, 2006; Perera et al., 2008; Zheng et al., 2012). Therefore, many process such as biotic and abiotic stress have involved PLC signaling pathway as important role in the response to environmental changes. ABA and

heat shock treatments cause a rapid intracellular Ca^{2+} increase which is preceded by a transient increase in the levels of inositol 1, 4, 5-trisphosphate (IP_3) (Sanchez and Chua, 2001; Zheng et al., 2012). Understanding the role of PLC and Ca^{2+} signaling pathway in the nitrate signaling pathway is the first step to understand the mechanistic relationships between classical Ca^{2+} regulatory components and nitrate responsive genes in *Arabidopsis thaliana* (Figure 1).

In this thesis, our combined cell biology and molecular genetics approach allowed us to identify steps in the nitrate-signaling pathway that involves Ca^{2+} as second messenger in the regulation of prototypical nitrate responsive genes. We show that nitrate treatments cause a rapid increase of IP_3 and $[Ca^{2+}]_{cyt}$ levels and that blocking PLC activity inhibits both IP_3 and $[Ca^{2+}]_{cyt}$ increase after nitrate treatments. We provide evidence that NRT1.1/AtNPF6.3 is required for increasing both IP_3 and $[Ca^{2+}]_{cyt}$ in response to nitrate treatments. Altering $[Ca^{2+}]_{cyt}$ or blocking PLC activities hinders regulation of gene expression of nitrate responsive genes. Our results indicate Ca^{2+} is a second messenger in the nitrate-signaling pathway of Arabidopsis thaliana.

Hypothesis

Ca^{+2} acts as a second messenger in the nitrate signaling pathway in *Arabidopsis thaliana* roots.

General Aim

To demonstrate that calcium acts as a second messenger in the nitrate signaling pathway in *Arabidopsis thaliana* roots.

Specific Aims

1. To determine whether nitrate treatments trigger changes in cytoplasmic calcium concentration in Arabidopsis.

2. To evaluate the role of calcium as a second messenger in the nitrate response.

3. To identify factors that mediate changes in cytoplasmic calcium levels in response to nitrate treatments in Arabidopsis plants.

4. To identify genes regulated by calcium in response to nitrate treatments.

2. MATERIALS AND METHODS

Plant Materials.

Arabidopsis thaliana ecotype Col-0 was used for all experiments. The Arabidopsis line expressing cytoplasmic Aequorin (Gao et al., 2004) was obtained from Dr. Christoph Plieth, Christian-Albrechts-Universität zu Kiel, Germany. Aequorin is a haloprotein composed of two distinct units, apoaequorin and the prosthetic group coelenterazine. When Aequorin is bound to calcium, the coelenterazine is oxidized and generated blue light. The luminescence detected with a luminometer is used for determined the Ca^{2+} concentration. The *chl1-5* and *chl1-9* mutants were kindly donated by Dr. Yi-Fang Tsay, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan (Tsay et al., 1993). The *chl1-5* mutant has a deletion in the genome corresponding to an 18.31-kb DNA fragment, beginning in the last NRT1.1/AtNPF6.3 intron and ending after the At1g12090 gene. This mutant has been extensively used for functional characterization of the NRT1.1/AtNPF6.3 gene. The *chl1-9* plant is a point mutation of the NRT1.1/AtNPF6.3 transporter. *chl1-9* lines were generated by EMS mutagenesis and isolated by chlorate selection and was found to have normal levels of CHL1 mRNA and protein (Ho et al., 2009).The amino acid Pro 492 is replaced for Leu, generating a nitrate uptake defect in the *chl1-9* mutant.

Growth Conditions.

Plants were grown in hydroponic cultures under long-day (16/8-h light/dark) conditions at 22ºC (in Percival incubators) using MS basal salt media without N (M531, PhytoTechnology Laboratories LLC, Shawnee Mission, KS) supplemented with 0.5 mM ammonium succinate (Merck, catalog number 1.00682) and 0.1% sucrose for 15 day (Merck, catalog number 1.07687).

Plant Genotyping.

To investigate the role of *chl1-5* and *chl1-9* in the nitrate response, we generated a stable transgenic line that expressed aequorin in the *chl1-5* and *chl1-9* mutant background. Aequorin-expressing *chl1-5* lines (*chl1-5*-AQ) and *chl1-9* lines (*chl1*-9-AQ) were generated by crosses between both NRT1.1 lines and a 35S::aequorin line (WT-AQ). Transgenic lines were selected on Murashige and Skoog medium containing 50 µg mL^{-1} kanamycin. *chl1-5*-AQ lines are genotyped using polymerase chain reaction (PCR) to amplify specific regions from genomic DNA using the following sets of primers: Forward primer 5'- TAT CCT TCA CAC ACA TGC AC-3', Reverse 1 primer 5'- AAT GCA GTC ATG CAG TTT ATG CC-3' or Reverse 2 primer 5'- CTG CCA CAC ACT GAA CAA TTC C-3'. The primer combination Forward-Reverse 1 generates a 1100 base pairs (bp) amplification product only when using genomic DNA from the *chl1-5* mutant. The combination of Forward-Reverse 2 primers only generates a 1469 bp amplification product from genomic DNA extracted from WT plants. Because the *chl1-5* mutant has a deletion in the genome, the primer combination Forward-Reverse 1 would generate a PCR product of 19.4-kb with wild-type genomic DNA, but a 1.1-kb fragment was amplified when using *chl1-5* genomic DNA as template (Muños et al., 2004). We designed another primer, reverse 2, using the intergenic DNA sequence to identify the wild-type locus. The combination of Forward-Reverse 2 primers only generates an amplification product when using DNA extracted from wild-type plants. *chl1-9*-AQ lines are genotyped using polymerase chain reaction (PCR) to amplify specific regions from genomic DNA using the following sets of primers: Forward primer 5'-AGACATTGGACCGTTCCATC-3' and Reverse primer 5'-ATCGGCAATCCATGGATGAG-3'. The amplification product is 500pb using genomic DNA from *chl1*-9-AQ plants. The point mutation in *chl1-9* changes

the Proline 492 to Leucine. Hence, we sequenced the amplification products to identify the codon change from CCT (Pro) to CTT (Leu).

Chemical Treatment of Plants

U73122 (catalog number U6881), U73343 (catalog number U6756), EGTA (catalog number E3889) and $LaCl_3$ (catalog number 211605) were purchased from Sigma-Aldrich (St Louis, USA). Before harvesting plant material for analysis of gene expression, Col-0, *chl1-5* and *chl1-9* seedlings of 15 day were pre-treated in petri dishes for 1 h in the presence of 10µM U73122, 10µM U73343, 10 mM EGTA or 5 mM $LaCl_3$. We evaluated the effect of PLC inhibitor (U73122), non-functional PLC inhibitor analog (U73343), channel blockers ($LaCl_3$) and calcium chelating agent (EGTA) in WT-AQ lines. U73122 and U733343 were dissolved in 0.1% (v/v) DMSO (catalog number 317275) and $LaCl_3$ was dissolved in water (Merck, catalog number 1.15333). For $LaCl_3$ treatments, the medium was replaced with water to prevent unwanted precipitation with media components. Plants were then treated for 1 h with 5mM KNO_3 or 5mM KCl and then roots were harvested and immediately frozen in liquid N_2. For aequorin measurements and the inositol-1,4,5-triphosphate (IP_3) levels, the plants were pre-treated with all pharmacological agents for 1h and then treated for the indicated period of time with 5mM KNO_3 or 5mM KCl.

***In vivo* Reconstitution of Aequorin and Ca^{2+}-Dependent Luminescence Measurements**

Reconstitution of aequorin in vivo with coelenterazine (CTZ) was performed as described previously (Knight et al., 1996). Synthetic native CTZ was obtained from Sigma-Aldrich (cat N° C2230). Briefly, we incubated 14 days old seedlings overnight in the dark with 2.5 µM CTZ. To measure calcium increases, excised root organs and whole seedlings were

placed in a 5 mL glass cuvette to measure luminescence immediately after treatments. Luminescence measurements were performed using a Sirius single-tube luminometer (Berthold Detection Systems). Five washed root organs and whole seedlings were used for each experiment. Luminescence was recorded every 0.2 s during ten minutes. The first two minutes were used to stabilize the plants in the luminometer and then the plants were treated with 5mM KNO_3 or 5mM KCl as control for three minutes. Finally, we added 1 M $CaCl_2$ and 10% ethanol to discharge remaining aequorin in the plants, and measured luminescence for 5 additional minutes. To convert luminescence into Ca^{2+} concentrations, we calculated the Ca^{2+} concentrations according to the calibration equation of Knight H. et al (1996):

$$pCa = 0.332588(-\log k) + 5.5593$$

Where k is equal to luminescence counts per second of the plants divided by total remaining counts in plants treated with 1 M $CaCl_2$ and 10% ethanol.

Inositol-1,4,5-Triphosphate (IP_3) Assays

IP_3 was measured as described previously (Heilmann and Perera, 2013). Briefly, plants were treated for 10 seconds with 5mM KNO_3 or 5mM KCl and then roots were harvested and frozen immediately in liquid N_2. Frozen tissue (approximately 0.1 g) was grounded to powder under liquid N_2 and incubated with 200 µL of 10% perchloric acid on ice for 20 min. Perchloric acid was obtained from Sigma-aldrich (catalog number 244252). Samples were centrifuged to remove precipitates and supernatants were transferred to new tubes and the pH adjusted to 7.5 using 1.5 M KOH/60 mM HEPES. IP_3 was measured using the Inositol-1,4,5-triphosphate [^{3}H] radioreceptor assay kit (NEK 064 PerkinElmer) according to the instructions of the manufacturer. Briefly, the basic principle of radioreceptor assay kit is a competitive ligand binding, where a radioactive ligand competes with a non-

radioactive ligand for a membrane preparation containing the IP_3 receptor. The decrease of ^3H inositol trisphosphate ($[^3H]IP_3$) bound to the receptor is inversely proportional to the increase of the amount of unlabelled ligand bound to the receptor. Then, we can calculate the IP_3 concentration in the different samples by interpolation into a standard curve using IP_3 standards of known concentration.

RNA Isolation and RT-qPCR.

RNA was isolated from whole roots with the Trizol® reagent according to the instructions of the manufacturer (catalog number 15596-026, Invitrogen, Carlsbad, CA, USA). cDNA synthesis was carried out using the Improm-II reverse transcriptase (catalog number A3802) according to the instruction of the manufacturer (Promega, Madison, USA). RT-qPCR was carried out using the Brilliant® SYBR® Green QPCR Reagents on a Stratagene MX3000P qPCR system. The RNA levels were normalized relative to the *clathrin adaptor complexes medium subunit family protein* (At4g24550).

Gene Expression Analysis.

cDNA synthesis, array hybridization and normalization of the signal intensities were performed according to the instructions provided by Affymetrix. Data was normalized in the R software using robust multiarray analysis (RMA) (Irizarry et al., 2003). Normalized data was subjected to a two-way ANOVA analysis with a $p<0.05$ and a false discovery rate of 5%. For the ANOVA analysis, we used a model considering the expression of a given gene Y as $Yi = \beta0 + \beta1T + \beta2G + \beta3TG + \varepsilon$, $\beta0$ being the global mean, $\beta1$, $\beta2$ and $\beta3$ the effects of the treatment, the genotype and the interaction between these two factors respectively. The variable ε was defined as the unexplained variance. Next, we analyzed our data using the Sungear tool available in the VirtualPlant webpage (Poultney et al.,

2006; Katari et al., 2009). Sungear allows performing comparative studies of multiple data sets to determine genes that are unique or that are shared by different gene lists. In this case, sungear generated a triangle, representing all genes with a significant factor in the ANOVA model at the vertices (T, F o TF factor). Circles inside the triangle represent genes with the different ANOVA models found, significant factors indicated by the vertices pointed by the arrows around the vessels. Based on the sungear analysis, we can identify four main signaling groups: group 1, group 2, group 3 and group 4. Genes belonging to each group were exported to PlantGSEA toolkit (Yi et al., 2013). PlantGSEA allows a rigorous analysis of over-representation using Fisher's exact test for declaring a GO (Gene Ontology) category and the generation of a P-value. In order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool (Supek et al., 2011).

Construction of 35S::PLC4:GFP gene fusion.

To investigate the role of *PLC4* in the nitrate response, we generated a stable transgenic line that expressed 35S::PLC4 in Col-0 background. For the chimeric 35S::PLC4:GFP gene fusion, the CDS of *PLC4* (At5g58700) was amplified from cDNA from the *A. thaliana* ecotype Col-0. The following primers were used to amplify *PLC4* CDS and were designed to introduce NcoI and SpeI restriction sites: *PLC4* CDS forward, 5'- GCC CCA TGG AAG GAA AA and reverse, 5'-CAC TAG TGA CAA ACT CGA AG. The PCR product and plasmid were digested with NcoI and SpeI, and DNA fragments were ligated into pCAMBIA 1302 (CAMBIA, Canberra, Australia). The construct was verified by DNA sequencing and then introduced into *Agrobacterium tumefaciens* GV3101. *A. tumefaciens*-mediated transformation of *Arabidopsis* plants was accomplished using the floral dip protocol (Clough and Bent, 1998). Transformants were selected on Murashige and Skoog

medium containing 50 µg mL^{-1} hygromycin. T3 lines were screened by hygromycin resistance analysis.

Phosphatidic acid assay.

PA was measured as described previously (Munnik and Zarza, 2013). Briefly, 14 day-old seedlings were pre-labelled overnight with inorganic ^{32}P-orthophosphate (^{32}Pi) (NEK0534H002MC, Perkin Elmer). At the beginning day 15, seedling were treated with KNO_3 or KCl for 30, 60, 120, 300 and 600 seconds. As positive control we used treatment with 300 mM NaCl for 30 minutes. Plant lipids were extracted with organic solvent and were separated by alkaline thin layer chromatography (TLC). TLC were exposed to a Phosphoimager screen for 1 h. Labelled phospholipids were measured through densitometry of each lipid and were normalized for total lipids.

3. RESULTS

Nitrate treatments increase cytoplasmic calcium concentration in plant root and seedling.

Calcium is an essential second messenger in plant signaling processes (Bush, 1995; Trewavas and Malho, 1998). Increase in $[Ca^{2+}]_{cyt}$ has been recorded in cellular responses to several stimuli (Sanders et al., 1999). As a first step to determine whether calcium acts as a second messenger in the nitrate signaling pathway, we measured $[Ca^{2+}]_{cyt}$ in Arabidopsis root organs and whole seedlings treated with nitrate. Plants expressing cytoplasmic aequorin (WT-AQ) (Gao et al., 2004) were grown hydroponically for two weeks with ammonium as the only N source. We treated plants with 5 mM KNO_3 (or 5 mM KCl) at dawn on the 15^{th} day and luminescence was measured every 0.2 s thereafter. Nitrate treatment increases $[Ca^{2+}]_{cyt}$ in root organs (Fig. 2A) and whole seedlings (Fig2B). A lower peak was observed in KCl treatments (Fig. 2A-B). After reaching a maximum, $[Ca^{2+}]_{cyt}$ decreased to near basal levels (Fig. 2A-B). It is known that abiotic and biotic cues such as sugar, salt and drought stress can increase strong and transiently $[Ca^{2+}]_{cyt}$ in roots and leaves (Furuichi et al., 2001; Choi et al., 2014b; Johnson et al., 2014). This increase in $[Ca^{2+}]_{cyt}$ can be partially abolished by the use of Ca^{2+} channel blockers such as lanthanum. Pretreatment of WT-AQ plant roots and seedlings with 5 mM $LaCl_3$ for 1 hour inhibited the $[Ca^{2+}]_{cyt}$ increase observed in response to nitrate treatment (Fig.3A-B). A similar response was observed in pretreatments with EGTA (Fig.3C). These results indicate that nitrate treatments cause a specific increase in $[Ca^{2+}]_{cyt}$ in root cells and seedlings of Arabidopsis thaliana.

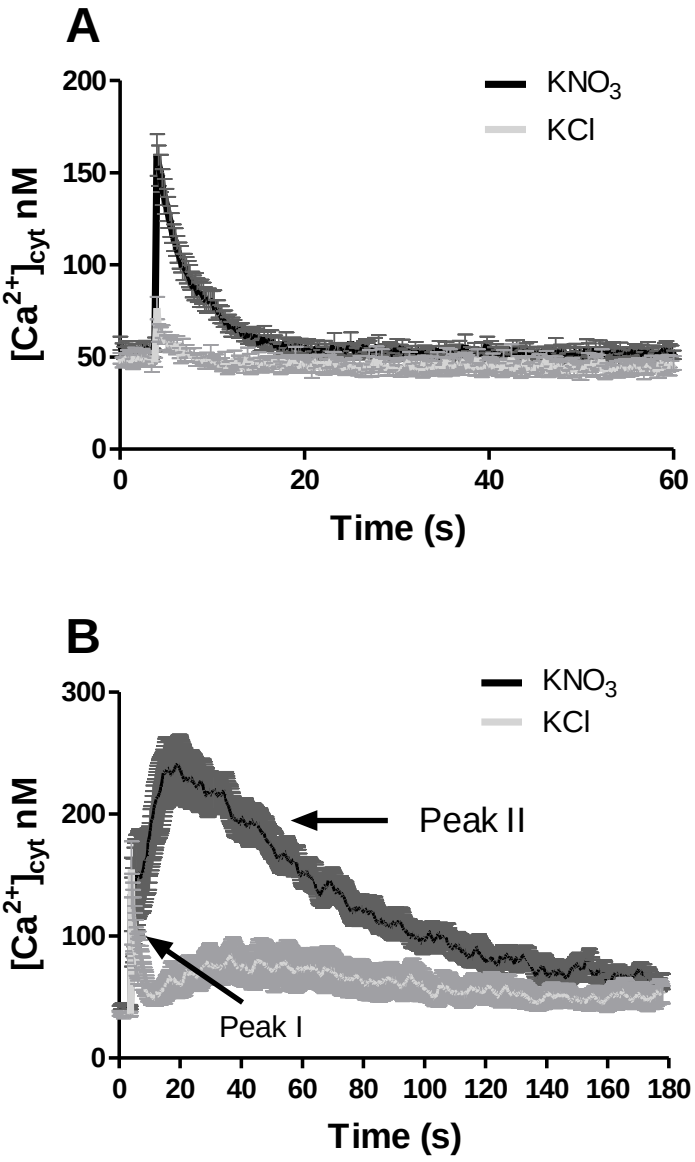

Figure 2. Nitrate treatments increase $[Ca^{2+}]_{cyt}$ levels. Wild-type plants expressing cytoplasmic aequorin were grown hydroponically for two weeks with 1 mM ammonium as the only N source. Aequorin was reconstituted by incubating plants in 2,5 µM coelenterazine overnight in dark. Cytosolic Ca^{2+} concentrations were monitored in A) root organs and B) whole seedlings over time in response to 5 mM KNO_3 or 5 mM KCl treatments. Plotted values correspond to the mean of at least three independent biological replicates ± standard deviation.

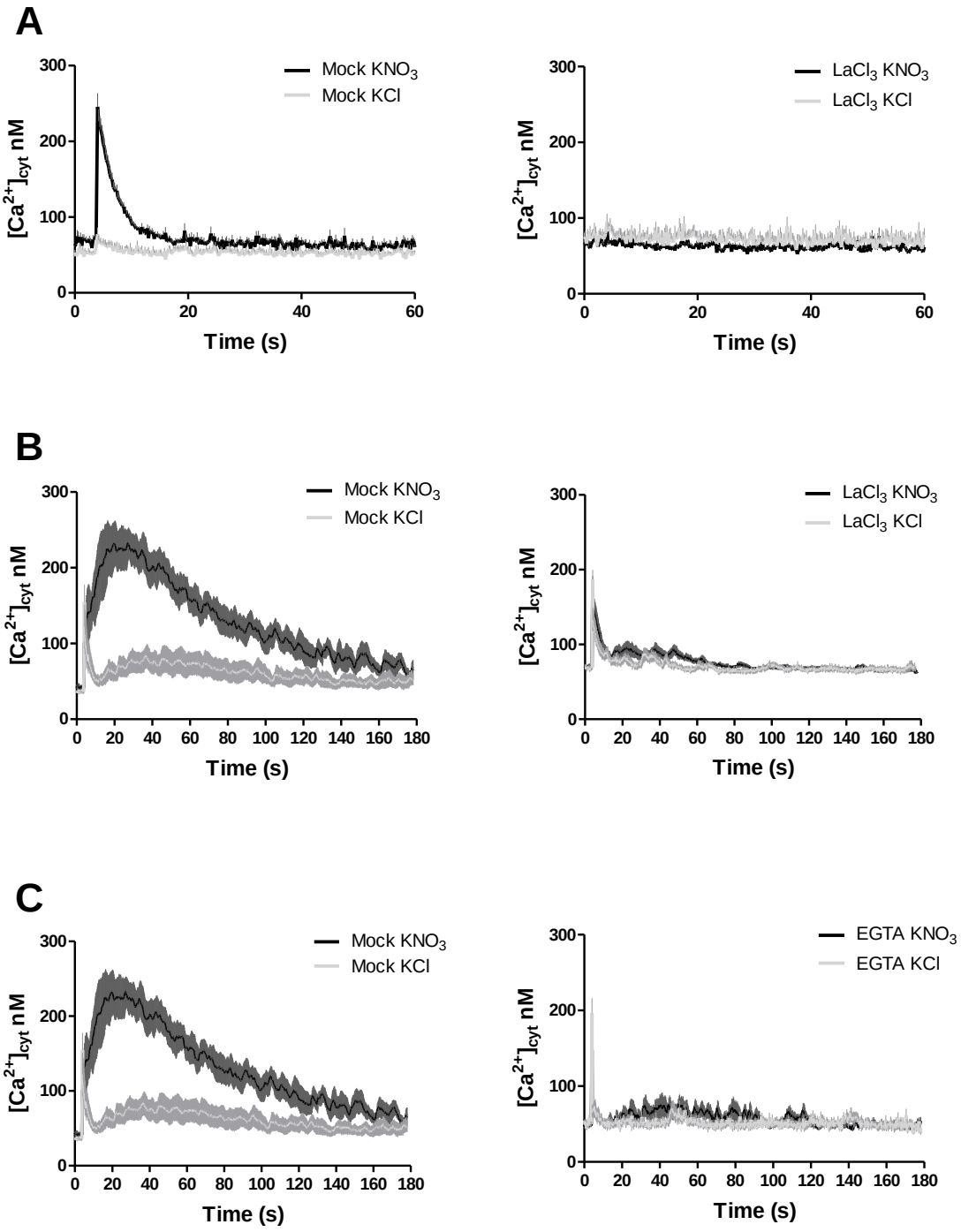
A
Mock KNO3
Mock KCl
LaCl3 KNO3
LaCl3 KCl
[Ca2+]cyt nM
Time (s)
B
Mock KNO3
Mock KCl
LaCl3 KNO3
LaCl3 KCl
[Ca2+]cyt nM
Time (s)
C
Mock KNO3
Mock KCl
EGTA KNO3
EGTA KCl
[Ca2+]cyt nM
Time (s)

Figure 3: $LaCl_3$ and EGTA treatments altered the increase $[Ca^{2+}]_{cyt}$ levels in response to nitrate. Wild-type plants expressing cytoplasmic aequorin were grown hydroponically for two weeks with 1 mM ammonium as the only N source. WT-AQ was reconstituted by incubating plant roots in 2,5 µM coelenterazine overnight in dark. WT-AQ were pretreated with Mock (H_20), $LaCl_3$ and EGTA for 1 hour and then treated for the indicated period of time with 5mM KNO_3 or 5mM KCl. Cytosolic Ca^{2+} concentrations were monitored over time in A) root organs and B) whole seedlings or C) with EGTA in whole seedling. Plotted values correspond to the mean of at least three independent biological replicates ± standard deviation.

PI-PLC activity is required for changes in cytoplasmic calcium levels in response to nitrate treatments in *Arabidopsis thaliana*.

In order to identify components of the signal transduction pathway mediating changes in cytoplasmic calcium levels in response to nitrate, we first determined whether a phospholipase C (PLC)-dependent pathway was implicated in this $[Ca^{2+}]_{cyt}$ increase. We evaluated the effect of a PLC inhibitor (U73122) and a non-functional PLC inhibitor analog (U73343) in WT-AQ lines in response to KNO_3 or KCl treatments. WT-AQ plants were pre-treated for 1 h with 10 µm U73122 or U73343 and luminescence of plant root and seedling were recorded after KNO_3 or KCl treatments using the same experimental conditions described above. The presence of PLC inhibitor (U73122) altered the $[Ca^{2+}]_{cyt}$ increase in response to nitrate treatments in plant roots and seedlings (Fig. 4A-B). However, treatments with non-functional analog (U73343) did not affected the $[Ca^{2+}]_{cyt}$ increase in Arabidopsis roots and seedlings (Fig. 4A-B). These results suggest that products of PLC enzyme activity or metabolites produced thereof trigger the $[Ca^{2+}]_{cyt}$ increase in response to nitrate treatments. As an independent confirmation that a PLC activity was induced under our experimental conditions, we measured IP_3 content after nitrate treatments in Arabidopsis roots. Wild-type plants were grown and treated with KNO_3 or KCl under the same experimental conditions described above and were quickly collected and frozen in liquid nitrogen. Treatment with 5 mM KNO_3 resulted in a 3-fold increase of IP_3 levels as compared to the KCl control 10 s after the treatment (Fig. 4C). Pretreatment of plants with U73122 (but not with U73343) completely blocked IP_3 increase in response to nitrate (Fig.4C). These results indicate that PLC activity is required for IP_3 accumulation as well as increase in $[Ca^{2+}]_{cyt}$ in response to nitrate treatments under our experimental conditions.

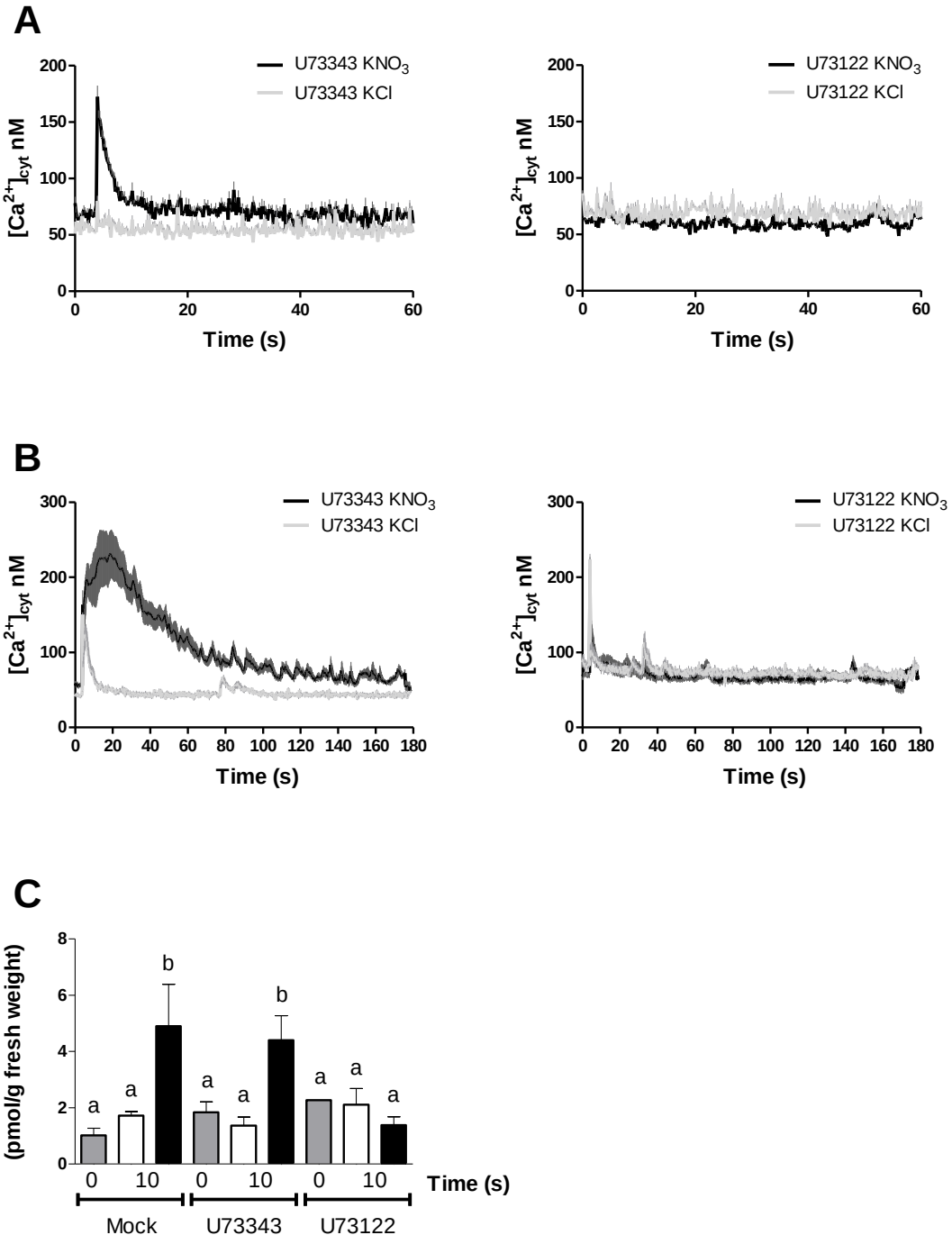
A
U73343 KNO3
U73343 KCl
U73122 KNO3
U73122 KCl
[Ca2+]cyt nM
Time (s)
B
U73343 KNO3
U73343 KCl
U73122 KNO3
U73122 KCl
[Ca2+]cyt nM
Time (s)
C
Inositol Triphosphate (pmol/g fresh weight)
Time (s)
Mock
U73343
U73122

Figure 4. A PLC inhibitor blocks increases in $[Ca^{2+}]_{cyt}$ and inositol-1,4,5-trisphosphate (IP_3) levels in response to nitrate treatments. Wild-type plants expressing cytoplasmic aequorin (WT-AQ) were grown hydroponically for two weeks with 1mM ammonium as the only nitrogen source and $[Ca^{2+}]_{cyt}$ and IP_3 levels were assayed as described in the main text. A) WT-AQ root organs and B) whole seedlings were pretreated with U73122 (PLC inhibitor) and U73343 (non functional analog) for 1 hour and then we were monitored the $[Ca^{2+}]_{cyt}$ levels over time after 5mM KNO_3 or 5mM KCl. (C) Plants were pretreated with Mock, U73122 (inhibitor of PLC) and U73343 (analogous no functional) and we evaluated the IP_3 content in response to 5mM KNO_3 or 5mM KCl treatment. Plotted values correspond to means of three independent biological replicates ± standard deviation. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. The letter indicates means that significantly differ between control and treatment conditions ($P < 0.05$)

NRT1.1 is a positive regulator of the $[Ca^{2+}]_{cyt}$ increase in response to nitrate treatments.

Several lines of evidence indicate that NRT1.1/AtNPF6.3 is a nitrate sensor in Arabidopsis plants (Ho et al., 2009; Wang et al., 2009; Gojon et al., 2011). To determine whether the increase in $[Ca^{2+}]_{cyt}$ in response to nitrate requires a functional NRT1.1/AtNPF6.3, we generated a stable transgenic line that constitutively expresses aequorin in a *nrt1.1*-null background. Aequorin-expressing *chl1-5* lines (*chl1-5*-AQ) were generated by crossing the *chl1-5* mutant (Tsay et al., 1993) and a transgenic line containing the 35S::Aequorin construct (WT-AQ) (Gao et al., 2004). We measured $[Ca^{2+}]_{cyt}$ in *chl1-5*-AQ plants in response to nitrate using the same experimental design described in the previous section. As we show in Figure 5B, the $[Ca^{2+}]_{cyt}$ increase by nitrate was significantly reduced in the *chl1-5*-AQ line, as compared to wild-type plants. As *chl1-5* is a mutant that does not express NRT1.1/AtNPF6.3 transcript (Tsay et al., 1993), these results did not distinguish whether the effect on $[Ca^{2+}]_{cyt}$ was caused by a defect in nitrate signaling or nitrate transport. We generated a *chl1-9*-AQ to evaluate $[Ca^{2+}]_{cyt}$ and distinguished between these two possibilities. A point mutation in *chl1-9* reduces NRT1.1/AtNPF6.3 nitrate uptake but does not affect the signaling function of NRT1.1 (Ho et al., 2009). As shown in Figure 5C, $[Ca^{2+}]_{cyt}$ are lower in *chl1-9*-AQ plant root as compared to wild-type in response to nitrate treatments, and are comparable with the results obtained for the *chl1-5*-AQ line. These results indicate that $[Ca^{2+}]_{cyt}$ increase by nitrate depends on NRT1.1/AtNPF6.3 nitrate transport.

In order to evaluate whether NRT1.1/AtNPF6.3 was part of the nitrate-PLC-Ca^{2+} pathway, we measured IP_3 content in *chl1-5* and *chl1-9* mutant root organs after nitrate treatments. *chl1-5* and *chl1-9* plants were grown for 15 days and were treated with 5 mM KNO_3 or KCl as control and IP_3 content was measured. In contrast to the increase in IP_3

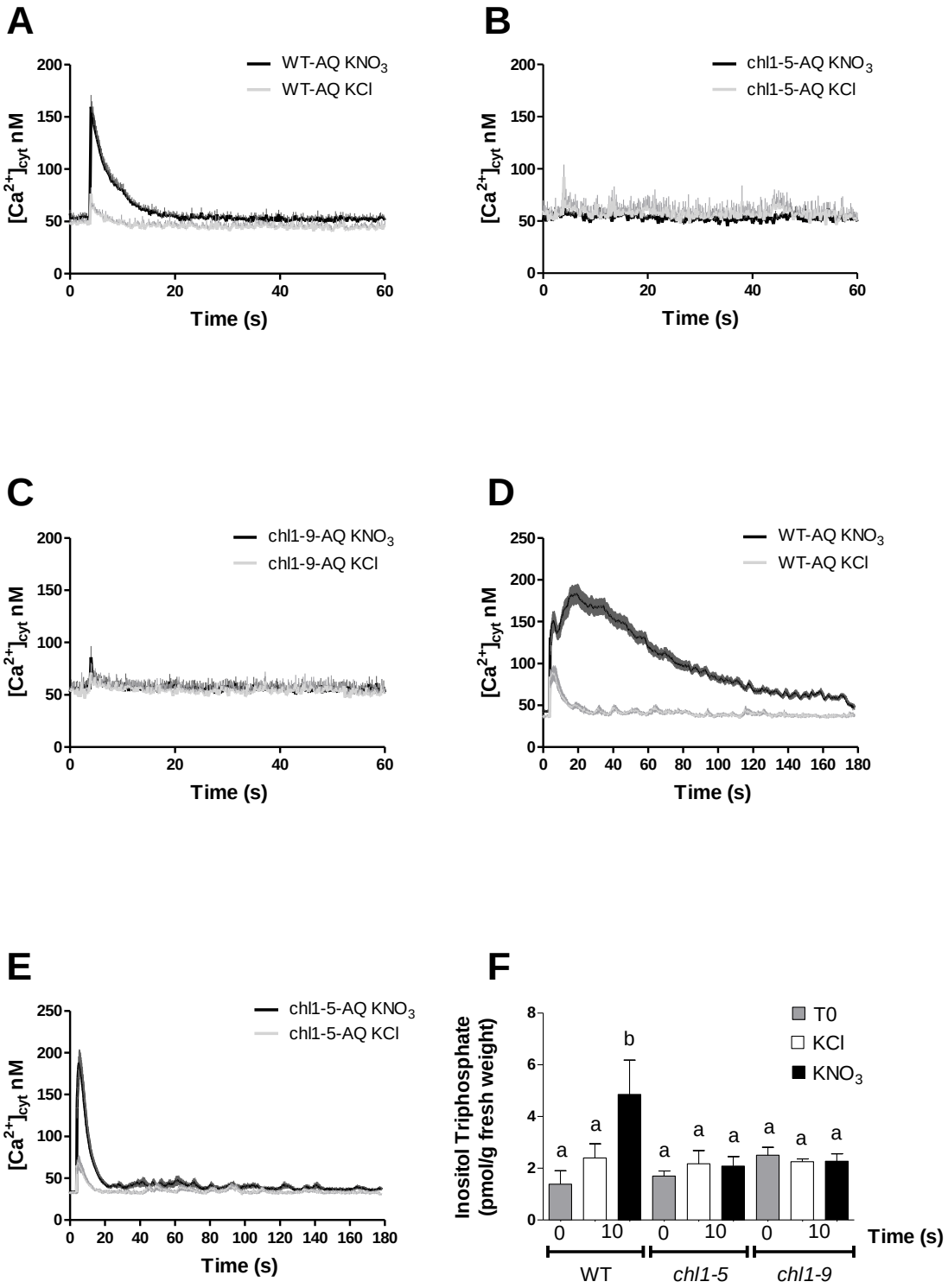
A
WT-AQ KNO3
WT-AQ KCl
[Ca2+]cyt nM
Time (s)
B
chl1-5-AQ KNO3
chl1-5-AQ KCl
[Ca2+]cyt nM
Time (s)
C
chl1-9-AQ KNO3
chl1-9-AQ KCl
[Ca2+]cyt nM
Time (s)
D
WT-AQ KNO3
WT-AQ KCl
[Ca2+]cyt nM
Time (s)
E
chl1-5-AQ KNO3
chl1-5-AQ KCl
[Ca2+]cyt nM
Time (s)
F
Inositol Triphosphate (pmol/g fresh weight)
T0
KCl
KNO3
Time (s)
WT
chl1-5
chl1-9

Figure 5. NRT1.1 is required for increases in $[Ca^{2+}]_{cyt}$ and inositol-1,4,5-trisphosphate (IP_3) levels in response to nitrate treatments. Wild-type, *chl1-5* and *chl1-9* plants were grown hydroponically for two weeks with ammonium as the only nitrogen source and $[Ca^{2+}]_{cyt}$ and IP_3 content were assayed as described in the main text. A) WT-AQ, B) *chl1-5*-AQ and C) *chl1-9*-AQ root organs and D) WT-AQ and E) *chl1-5*-AQ whole seedling were reconstituted by incubating plants in 2,5 µM coelenterazine overnight in darkness. At the beggining day were monitored the Cytosolic Ca^{2+} concentrations over time after 5mM KNO_3 or 5mM KCl treatment. F) Wild-type, *chl1-5* and *chl1-9* plants were treated with 5mM KNO_3 and 5mM KCl as control for 10s and then we evaluated de IP_3 content. Plotted values correspond to means of at least three independent biological replicates ± standard deviations. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. The letter indicates means that significantly differ between control and treatment conditions ($P < 0.05$)

levels in wild-type plant roots, there was no significant increases in IP_3 content in *chl1-5* and *chl1-9* mutant plant roots after KNO_3 treatments (Fig. 5F). This result indicates that accumulation of IP_3 in Arabidopsis roots in response to nitrate treatments also requires NRT1.1/AtNPF6.3 nitrate transport activity for activation of a PLC in response to nitrate treatments.

Nitrate-induced gene expression is mediated by NRT1.1, PLC and Ca^{2+}.

In order to evaluate the impact of this signaling pathway on nitrate regulation of gene expression, we analyzed the expression of characteristic nitrate-responsive genes in WT, *chl1-5* and *chl1-9* plant roots treated with the calcium channel blocker $LaCl_3$ or the PLC inhibitor U73122. Total RNA was isolated and mRNA levels were measured for selected genes using reverse transcription and quantitative real time polymerase chain reaction. In Fig. 6, *NRT2.1*, *TGA1*, *NIR*, *NRT3.1*, *AFB3* and *NIA1* gene expression is induced after KNO_3 treatments. As has been previously described (Ho et al., 2009; Wang et al., 2009; Alvarez et al., 2014; Vidal et al., 2014a), the induction of these genes by nitrate was significantly reduced in the *chl1-5* and *chl1-9* mutant as compared with wild-type plants. To determine whether the expression of these nitrate-responsive genes is mediated by PLC activity and Calcium, we pretreated WT, *chl1-5* and *chl1-9* plants with the PLC inhibitor U73122 or the calcium channel blocker $LaCl_3$, and then we treated with 5 mM KNO_3 or 5 mM KCl. Similarly, nitrate induction of *NRT2.1*, *TGA1*, *NRT3.1*, *NIR* and *NIA1* were significantly reduced in the presence of U73122 or $LaCl_3$ and are comparable with results obtained for the *chl1-5* and *chl1-9* mutants (Fig. 6 and 7). In contrast, the induction of *AFB3* by nitrate was not significantly affected by U73122 and $LaCl_3$. This indicates that NRT1.1/AtNPF6.3, a PLC activity and the increase in cytosolic calcium levels are required for changes in gene expression in response to nitrate treatments in Arabidopsis. These results suggest the existence of a Ca^{2+}-dependent and a Ca^{2+}-independent pathways downstream of NRT1.1/AtNPF6.3 to control gene expression of nitrate-responsive genes

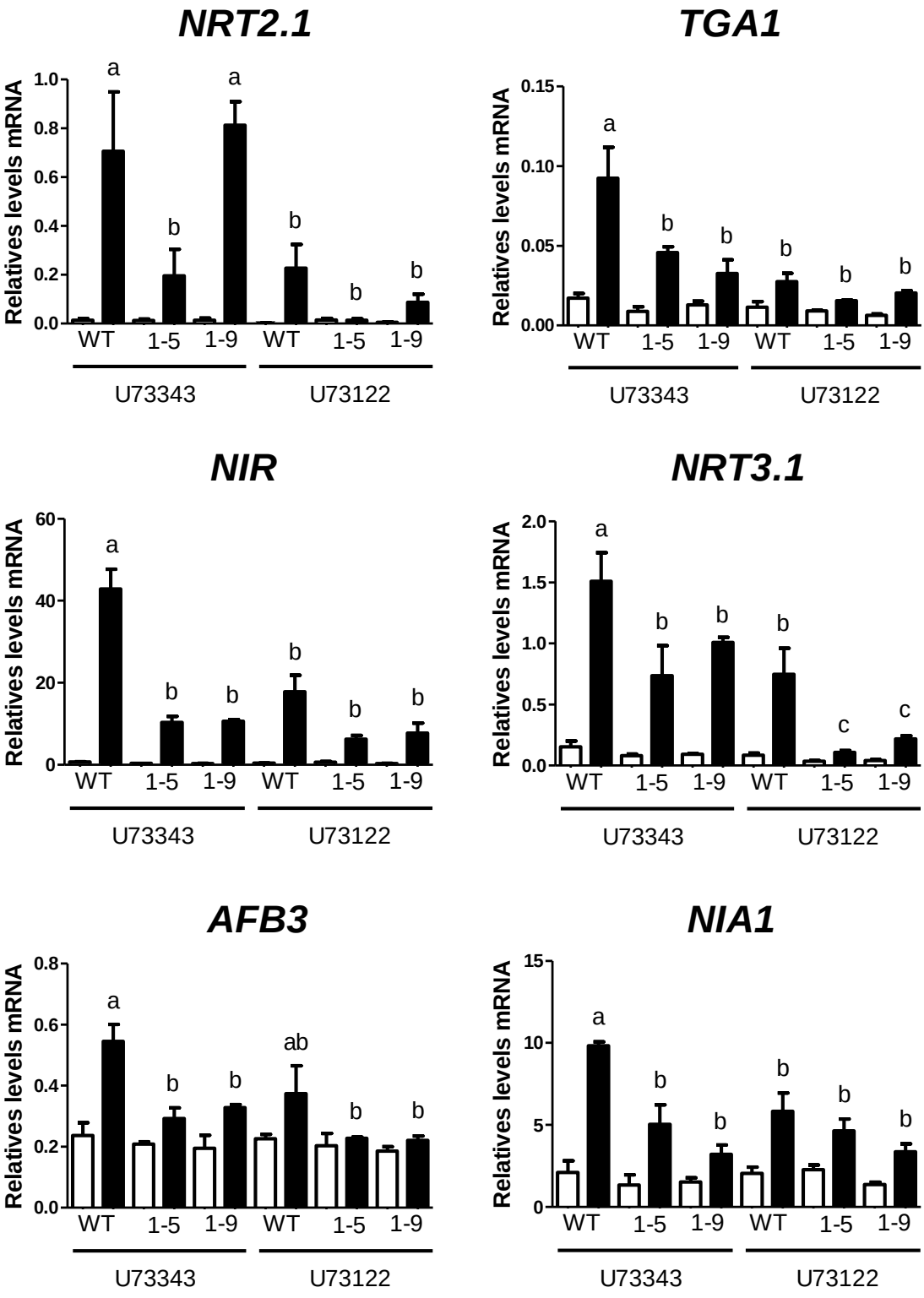
NRT2.1
TGA1
NIR
NRT3.1
AFB3
NIA1
Relatives levels mRNA
WT
1-5
1-9
U73343
U73122

Figure 6. Induction of gene expression in response to nitrate treatments is mediated by NRT1.1 and PLC activity in roots. Col-0, *chl1-5* and *chl1-9* plants were grown for 15 days. Plant roots were pre-treated for 1 h with 10 µM U73122 (PLC inhibitor) or 10 µM U73343 (non-functional PLC inhibitor analog) and then treated for 1h with 5 mM KNO_3 or 5 mM KCl. Plotted values correspond to means of three independent biological replicates ± standard deviations. White bars represent KCl treatment, and black bars represent KNO_3 treatment. We evaluated the gene expression of *NRT2.1*, nitrate transporter 2.1; *TGA1*, transcription factor; *NIR*, nitrite reductase, *NRT3.1*, nitrate transporter 3.1; *AFB3*, auxin receptor and *NIA1*, nitrate reductase. The letters indicates means that significantly differ between control and treatment conditions ($P < 0.05$).

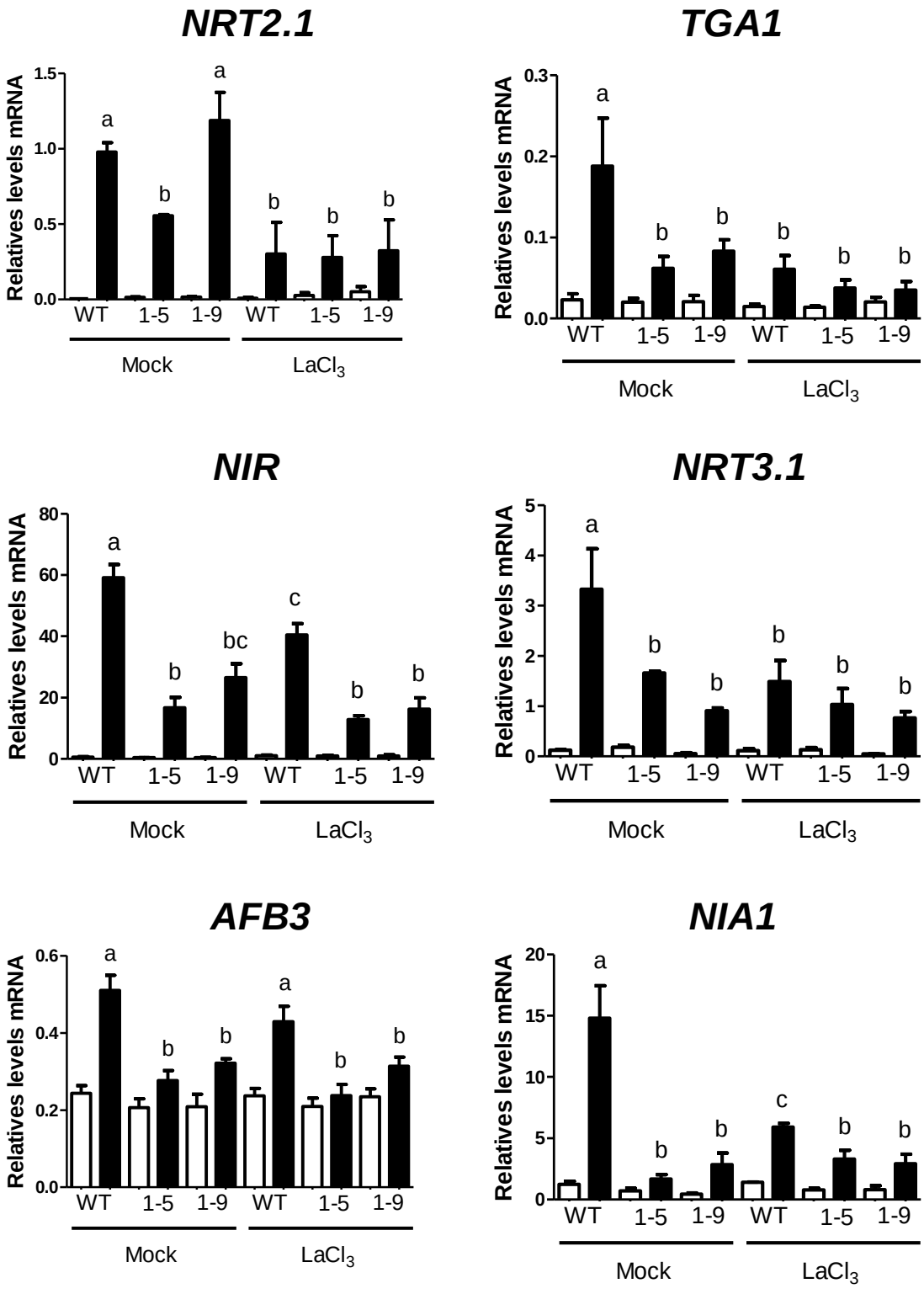
NRT2.1
TGA1
NIR
NRT3.1
AFB3
NIA1
Relatives levels mRNA
WT
1-5
1-9
Mock
LaCl3

Figure 7. Induction of gene expression in response to nitrate treatments is mediated by NRT1.1 and Ca^{2+} in roots. Col-0, *chl1-5* and *chl1-9* plants were grown for 15 days. Plants were pre-treated for 1 h with 5 mM $LaCl_3$ (channels blockers) and then treated for 1 h with 5 mM KNO_3 or 5 mM KCl as control. Plotted values correspond to means of three independent biological replicates ± standard deviations. White bars represent KCl treatment, and black bars represent KNO_3 treatment. We evaluated the gene expression of *NRT2.1*, nitrate transporter 2.1; *TGA1*, transcription factor; *NIR*, nitrite reductase, *NRT3.1*, nitrate transporter 3.1; *AFB3*, auxin receptor and *NIA1*, nitrate reductase. Letters indicate means that significantly differ between control and treatment conditions ($P < 0.05$).

Transcriptome changes associated to PLC activity are important for the nitrate response.

To assess the role of PLCs in the nitrate response we analyzed the global gene expression profile of plants treated with U73122 (PLC inhibitor) and U73343 (non-functional analog). Col-0 plants were grown in ammonium succinate as the only N source for 14 days and were pre-treated at the beginning of the light period on day 15 with 10 µM U73122 or U73343 for 1 hour. We then treated the plants with 5 mM KNO_3 or 5 mM KCl for 1h. Total RNA was isolated from roots and prepared for Affymetrix ARAGENE gene chip. ARAGENE gene chip 1.0 ST array have whole-transcriptome coverage of *Arabidopsis thaliana*. The whole-transcriptome analysis approach allows to detect multiple transcript isoforms, such as splice variants, non-polyadenylated transcripts and truncated transcripts. Gene expression data were normalized using RMA and differential gene expression was determined using a two-way analysis of variance (ANOVA). Since our primary goal was to identify genes whose nitrate regulation was altered in the presence of U73122, we used an ANOVA model considering the expression of a given gene Y as $Yi = \beta0 + \beta1T + \beta2F + \beta3TF + \varepsilon$, β0 being the global mean, β1, β2 and β3 being the effects of the treatment with KNO_3(T), Pharmacological treatment (F) and the interaction between those two factors (TF), respectively, and ε being the unexplained variance. We found 611 genes showing significant T factor, 1079 genes showing significant F factor and 228 genes exhibiting a significant TF interaction factor.

To simplify the analysis of our results and to have a first insight into how PLC activity regulates gene expression in response to nitrate, we analyzed our data using the Sungear tool available in the VirtualPlant webpage (Poultney et al., 2006; Katari et al., 2009). Sungear allows for the visualization and analysis of multiple data sets to determine genes that are unique or that are shared by different gene lists. In this case, sungear

generated a triangle, representing the genes with significant T, F or TF factors (Figure 9). Every vertices in the triangle represents a factor of the anova model, and the circles inside the triangle (vessels) represent the number of genes with anova models with significant factors, indicated by the arrow around the vessels. Using Sungear, we found that 228 genes have TF as a significant factor, indicating that PLC activity is necessary for the expression in response to nitrate of such genes (group 1). In addition, 233 genes were affected by nitrate and U73122 treatments as the significant factors, indicating that the nitrate response of these genes is altered by nitrate signal or PLC activity (group 2). Moreover, we observed that 242 genes are not affected by U73122, indicating that the nitrate response of these genes is not influenced by PLC activity (group 3). Finally, we visualized that 666 genes are affected only by the U73122 treatment, indicating that PLC activity is also important for the regulation of the expression of genes that not respond to nitrate (group 4). To uncover regulatory functions of genes whose response depends on PLC activity, we performed a Gene ontology (GO) analysis of genes present in the different groups. We identified GO terms using PlantGSEA tool (http://structuralbiology.cau.edu.cn/PlantGSEA) and in order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool (Canales et al., 2014). PlantGSEA is a method for interpreting biological meaning of a list of genes by computing over 20.290 defined genes sets derived from different resources of annotation systems. PlantGSEA allows a statistical analysis of over-representation generating a P-value and a FDR value. The lower the P-value is, the higher the significance of the overrepresentation.

According to our GO analysis, group 1 genes participate in inorganic anion transport, organic cation transport, nitrate transport and phosphorylation, among others (table 1). Moreover, group 2 genes participate in the iron response and ion transport,

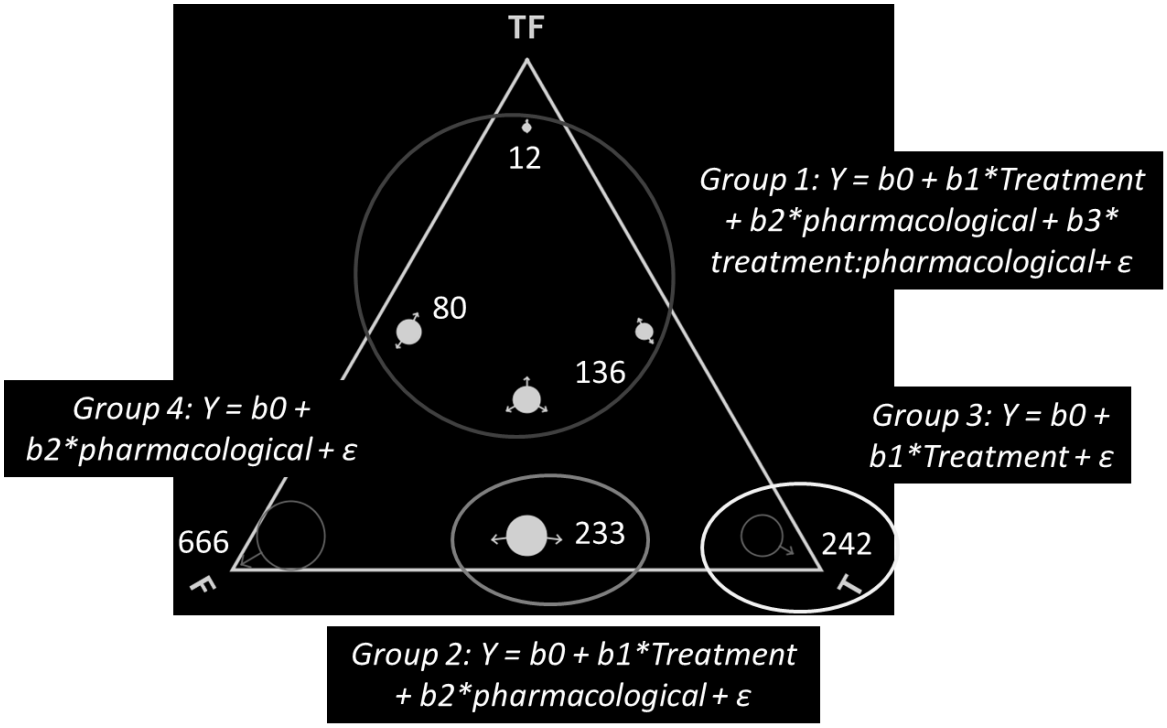

Figure 8. Sungear analysis of genes with significant Treatment (T), Pharmacological (F) and Treatment:pharmacological (TF) interaction factors reveal that PLC activity regulates the expression of nitrate-responsive genes and genes that do not respond to nitrate. The list of genes with significant factors obtained by two-way ANOVA of Affymetrix data is represented in a Venn diagram using the VirtualPlant tool Sungear (http://www.virtualplant.org). The Sungear triangle shows the factors (T, F, and TF) at the vertices (anchors). The circles inside the triangle (vessels) represent the genes controlled by the different factors, as indicated by the arrows around the vessels. The area of each vessel (size) is proportional to the number of genes associated with that vessel. The number of genes in the vessel is shown next to the corresponding vessel.

cellular response to lipid, cellular response to nitrate and flavonoid metabolic process (table 2). Group 3 is a set of genes that only respond to nitrate and GO analysis indicates that they participate in response to cadmium ion, monosaccharide metabolic process, isocitrate metabolic process, tricarboxylic acid metabolic process, amino acid homeostasis, oxidoreduction coenzyme metabolic process, primary root development, second-messenger-mediated signaling, among others. These categories are almost identical to those found for genes that respond directly to nitrate (Wang, 2003; Canales et al., 2014). The most consistent biological functions are those related to nitrate transport, carbon metabolism and root development that has been studied in the context of nitrate responses (table 3). Finally, group 4 genes participate in many other processes such as response to chitin, carbohydrate, light, hormone stimulus, defense response, heat response, hyperosmotic salinity response, among other perturbations (table 4). Altogether, these results indicate that PLC activity is important in a myriad of biological processes that has been previously studied in response to multiple abiotic and biotic cues (Stevenson et al., 2000; Munnik and Testerink, 2008; Chen et al., 2011). These results also suggest that PLC activity is important for gene expression in response to nitrate.

Table 1.

Term ID	Description	p-value
GO:0010200	response to chitin	1,79E-10
GO:0015698	inorganic anion transport	1,25E-05
GO:0015695	organic cation transport	7,70E-05
GO:0015706	nitrate transport	9,60E-05
GO:0010363	regulation of plant-type hypersensitive response	0,00011898
GO:0030001	metal ion transport	0,00013507
GO:0006612	protein targeting to membrane	0,00015994
GO:0071323	cellular response to chitin	0,0002299
GO:0009808	lignin metabolic process	0,00059509
GO:0016310	phosphorylation	0,00130998
GO:0009812	flavonoid metabolic process	0,00149146
GO:0009627	systemic acquired resistance	0,00198796
GO:0009813	flavonoid biosynthetic process	0,00319113
GO:0006694	steroid biosynthetic process	0,00361642
GO:0006468	protein phosphorylation	0,00485091
GO:0009873	ethylene-activated signaling pathway	0,00503336
GO:0000041	transition metal ion transport	0,00588938
GO:0071407	cellular response to organic cyclic compound	0,00818574
GO:0009750	response to fructose	0,00903585

Gene ontology analysis of group 1 genes. The gene table was obtained from Sungear analysis of the genes with significant TF factor. We identified GO terms using PlantGSEA toolkit with significance of $p<0.01$ and FDR 1%. In order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool.

Table 2.

Term ID	Description	p-value
GO:0006826	iron ion transport	0,00012283
GO:0071396	cellular response to lipid	0,00015238
GO:0071249	cellular response to nitrate	0,0002299
GO:0009812	flavonoid metabolic process	0,00149146
GO:0006821	chloride transport	0,00157228
GO:0072330	monocarboxylic acid biosynthetic process	0,00171082
GO:0010363	regulation of plant-type hypersensitive response	0,00174303
GO:0072523	purine-containing compound catabolic process	0,00176341
GO:0010583	response to cyclopentenone	0,00195713
GO:0009627	systemic acquired resistance	0,00198796
GO:0006612	protein targeting to membrane	0,00219763
GO:0009813	flavonoid biosynthetic process	0,00319113
GO:0046189	phenol-containing compound biosynthetic process	0,00344142
GO:0030001	metal ion transport	0,00360589
GO:0006094	gluconeogenesis	0,00390913
GO:0019761	glucosinolate biosynthetic process	0,0042584
GO:0010200	response to chitin	0,00429835
GO:0046482	para-aminobenzoic acid metabolic process	0,0045184
GO:0006576	cellular biogenic amine metabolic process	0,00489103
GO:0000041	transition metal ion transport	0,00588938
GO:0009699	phenylpropanoid biosynthetic process	0,00782203
GO:0009141	nucleoside triphosphate metabolic process	0,00955554
GO:0072511	divalent inorganic cation transport	0,00977351

Gene ontology analysis of group 2 genes. The gene table was obtained from Sungear analysis of the genes with both T and F significant factor. We identified GO terms using PlantGSEA toolkit with significance of $p<0.01$ and FDR 1%. In order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool.

Table 3

Term ID	Description	p-value
GO:0046686	response to cadmium ion	4,59E-08
GO:0005996	monosaccharide metabolic process	3,92E-07
GO:0006102	isocitrate metabolic process	0,00010818
GO:0072350	tricarboxylic acid metabolic process	0,0003553
GO:0080144	amino acid homeostasis	0,00046565
GO:0006733	oxidoreduction coenzyme metabolic process	0,00067183
GO:0080022	primary root development	0,00070458
GO:0010107	potassium ion import	0,00077153
GO:0055081	anion homeostasis	0,00093942
GO:0019722	calcium-mediated signaling	0,00144829
GO:0045333	cellular respiration	0,00148835
GO:0005991	trehalose metabolic process	0,00172693
GO:0009051	pentose-phosphate shunt, oxidative branch	0,00212259
GO:0015740	C4-dicarboxylate transport	0,00212259
GO:0019321	pentose metabolic process	0,00299649
GO:0019932	second-messenger-mediated signaling	0,00369548
GO:0009117	nucleotide metabolic process	0,00370288
GO:0019752	carboxylic acid metabolic process	0,00424834
GO:0006835	dicarboxylic acid transport	0,00574245
GO:0051348	negative regulation of transferase activity	0,00656604
GO:0044242	cellular lipid catabolic process	0,00733785
GO:0071616	acyl-CoA biosynthetic process	0,00868126
GO:0031400	negative regulation of protein modification process	0,00890922
GO:0009082	branched-chain amino acid biosynthetic process	0,00978161

Gene ontology analysis of group 3 genes. The gene table was obtained from Sungear analysis of the genes with significant T factor. We identified GO terms using PlantGSEA toolkit with significance of $p<0.01$ and FDR 1%. In order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool.

Table 4.

Term ID	Description	p-value
GO:0010200	response to chitin	4,56E-105
GO:0010363	regulation of plant-type hypersensitive response	1,71E-48
GO:0009642	response to light intensity	2,57E-44
GO:0030968	endoplasmic reticulum unfolded protein response	8,83E-44
GO:0042542	response to hydrogen peroxide	3,06E-35
GO:0046189	phenol-containing compound biosynthetic process	6,45E-32
GO:0006457	protein folding	2,09E-26
GO:0000165	MAPK cascade	1,35E-25
GO:0006605	protein targeting	3,87E-25
GO:0010310	regulation of hydrogen peroxide metabolic process	1,65E-19
GO:0009693	ethylene biosynthetic process	1,96E-19
GO:0071396	cellular response to lipid	1,06E-17
GO:0016310	Phosphorylation	1,50E-16
GO:0032787	monocarboxylic acid metabolic process	2,50E-15
GO:0042538	hyperosmotic salinity response	4,56E-13
GO:0046394	carboxylic acid biosynthetic process	5,18E-12
GO:0009873	ethylene-activated signaling pathway	4,29E-10
GO:0015698	inorganic anion transport	1,67E-07
GO:0010583	response to cyclopentenone	2,23E-07
GO:0009646	response to absence of light	2,79E-07
GO:0043090	amino acid import	4,03E-07
GO:0042343	indole glucosinolate metabolic process	6,71E-07
GO:0009816	defense response to bacterium, incompatible interaction	2,16E-06
GO:0010337	regulation of salicylic acid metabolic process	2,39E-06
GO:0070370	cellular heat acclimation	5,99E-06
GO:0009813	flavonoid biosynthetic process	1,24E-05
GO:0009812	flavonoid metabolic process	1,76E-05
GO:0015748	organophosphate ester transport	2,52E-05
GO:0015802	basic amino acid transport	7,66E-05
GO:0015706	nitrate transport	8,67E-05
GO:0015696	ammonium transport	9,37E-05
GO:0009695	jasmonic acid biosynthetic process	0,00019197
GO:0009694	jasmonic acid metabolic process	0,00023974
GO:0070588	calcium ion transmembrane transport	0,00024768
GO:0016311	dephosphorylation	0,00024913
GO:0015914	phospholipid transport	0,00033979
GO:0006470	protein dephosphorylation	0,00043212
GO:0000041	transition metal ion transport	0,00053405
GO:0043543	protein acylation	0,00054917
GO:0031365	N-terminal protein amino acid modification	0,00056039

GO:0052544	defense response by callose deposition in cell wall	0,00059326
GO:0042158	lipoprotein biosynthetic process	0,00078418
GO:0006464	cellular protein modification process	0,0008582
GO:0043562	cellular response to nitrogen levels	0,00088486
GO:0019375	galactolipid biosynthetic process	0,0009596
GO:0019374	galactolipid metabolic process	0,0010312
GO:0072503	cellular divalent inorganic cation homeostasis	0,00131452
GO:0006826	iron ion transport	0,00137318
GO:0010185	regulation of cellular defense response	0,0019085
GO:0042344	indole glucosinolate catabolic process	0,0019085
GO:0010120	camalexin biosynthetic process	0,00232579
GO:0010106	cellular response to iron ion starvation	0,00289282
GO:0042436	indole-containing compound catabolic process	0,00458373
GO:0010731	protein glutathionylation	0,0061489
GO:0015700	arsenite transport	0,0061489
GO:0052386	cell wall thickening	0,00730882
GO:0035304	regulation of protein dephosphorylation	0,00778299
GO:0010962	regulation of glucan biosynthetic process	0,00906829
GO:0043484	regulation of RNA splicing	0,00906829

Gene ontology analysis of group 4 genes. The gene table was obtained from Sungear analysis of the genes with significant F factor. We identified GO terms using PlantGSEA toolkit with significance of $p<0.01$ and FDR 1%. In order to focus on specific functions, we only considered GO terms at level 6, 7 and 8 and removed redundant terms using the REVIGO tool.

Nitrate regulates the expression of *PLC4* and *PLC5* genes.

In order to identify components of the signal transduction pathway mediating changes in response to nitrate, we first asked which phospholipase C (PLC) may be implicated in the nitrate signaling pathway. Arabidopsis has nine actively transcribed PI-PLC genes. *AtPLC2* is expressed constitutively, but expression of the remaining eight PI-PLC genes has been shown to be regulated by salt, cold and dehydration stress, ABA and other perturbations (Tasma et al., 2008). *AtPLC4* and *AtPLC5* gene expression is regulated by nitrate in Arabidopsis microarray data (Wang, 2003; Wang et al., 2004; Vidal et al., 2013a; Alvarez et al., 2014; Canales et al., 2014). As a first step to analyze the possible role of these phospholipases C in the nitrate response, we measured *PLC4* and *PLC5* mRNA levels in time-course experiments after nitrate treatments in our experimental conditions. Wild-type plants were grown hydroponically for two weeks with 1 mM ammonium as the only N source. At the beginning of the light period of the 15th day, plants were exposed to 5 mM KNO_3 or KCl. Root organs were harvested for RNA isolation 1 and 2 hours thereafter. We measured transcript levels of *PLC4* and *PLC5* genes using quantitative real time reverse transcription polymerase chain reaction (RT-qPCR). As shown in Figure 9, both *PLC4* and *PLC5* mRNAs accumulated after KNO_3 but not after KCl treatments, indicating that expression of these genes is regulated by nitrate treatments in roots. These results indicate that nitrate treatments affect *PLC4* and *PLC5* gene expression in roots under our experimental conditions, and support the idea that one or more PLCs are implicated in Arabidopsis root nitrate signaling.

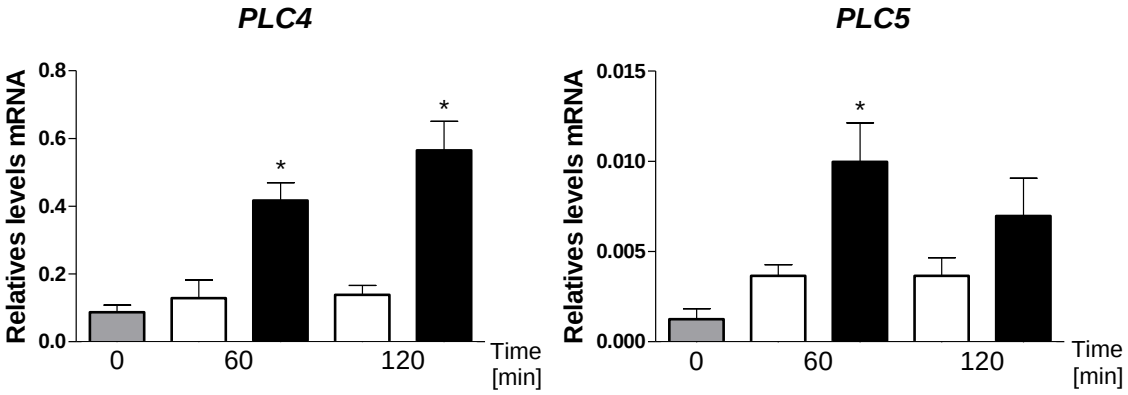

Figure 9: *PLC4* and *PLC5* respond to nitrate in Arabidopsis roots. Plants were grown hydroponically for two weeks with 1 mM ammonium as the only nitrogen source. At the beginning of the light period of the 15th day, plants were harvested (time 0) or exposed to 5mM KNO_3 or 5 mM KCl for the indicated times. Plotted values correspond to means of three independent biological replicates ± standard deviations. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. Letters indicate means that significantly differ between control and treatment conditions ($P < 0.05$)

PLC4 modulates the expression of nitrate responsive genes.

Our network predicts relevant relationships that are affected by PLC activity in response to nitrate. To address this prediction, we generated plants over-expressing the *PLC4* gene and evaluated the expression of *NRT2.1*, *NIR* and *NRT3.1* genes in response to 5mM KNO_3 or 5mM KCl treatments using RT-qPCR. *NRT2.1* and *NRT3.1* were induced by the nitrate treatments in wild-type plants. However, nitrate induction of *NRT2.1* and *NRT3.1* was significantly higher in the *PLC4* overexpressor plants 2 h after KNO_3 treatments (Figure 10). In contrast, *NIA1* and *NIR* genes did not show differences in expression between wild-type and *PLC4* overexpressor plants in response to nitrate treatments (Figure 11). These results indicate that *PLC4* is important for normal changes in gene expression of *NRT2.1* and *NRT3.1* in response to nitrate treatments in Arabidopsis. In addition, PLC4 activity, NRT1.1/AtNPF6.3 and the increase in cytosolic calcium may be in the same signaling pathway because they regulate the same genes. A plausible scenario is that nitrate transported by NRT1.1 activates PLC activity that increases in the cytosolic calcium levels, and finally to modulation of gene expression. In the case of PLC4 overexpressors, plants expressed ectopically the PLC4 gene and could have increased levels of cytoplasmic calcium when nitrate is sensed.

Not all targets were affected as expected. *NIR* gene expression was induced by nitrate treatments similarly in wild-type and PLC4 overexpressor lines. This gene was suggested by our gene network analysis as a putative target of PLC activity. It is possible that other PLC can regulate the NIR gene expression.

In summary, our results suggest a scenario in which nitrate can modulate changes in gene expression, with NRT1.1, PLC, IP_3 and Ca^{2+} acting as signaling components according to two different models (i) Activation of NRT1.1 and PLC activity dependent of

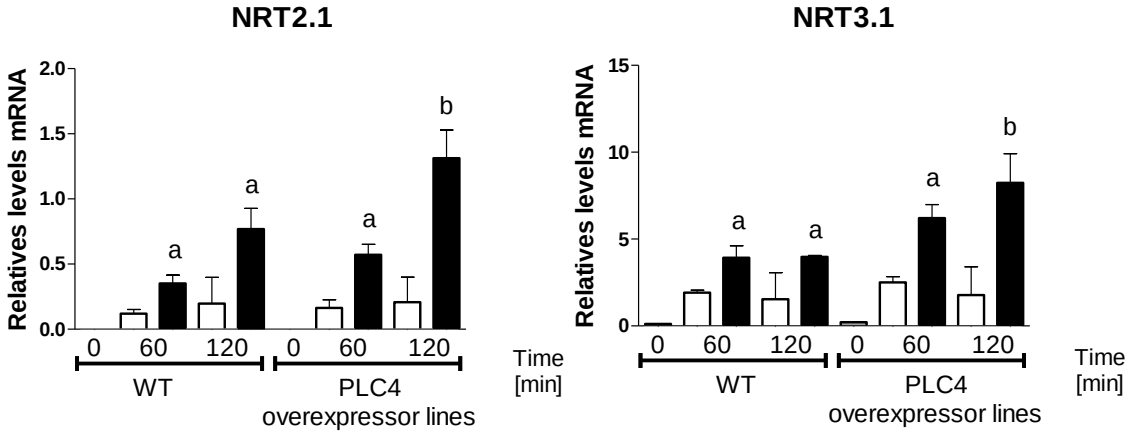

Figure 10. PLC4 overexpression enhances nitrate response of *NRT2.1* and *NRT3.1* genes. Wild-type Col-0 or *PLC4* overexpressor plants were grown hydroponically for two weeks with 1 mM ammonium as the only nitrogen source. At dawn of the 15th day, plants were treated with either 5mM KNO_3 or 5 mM KCl. Root organs were harvested for total RNA isolation 2h afterwards as described in materials and methods. mRNA levels were measured by RT-qPCR. Plotted values correspond to means of three independent biological replicates ± standard deviations. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. We evaluated the gene expression of *NRT2.1*, nitrate transporter 2.1 and *NRT3.1*, nitrate transporter 3.1. Letters indicate means that significantly differ between control and treatment conditions ($P < 0.05$).

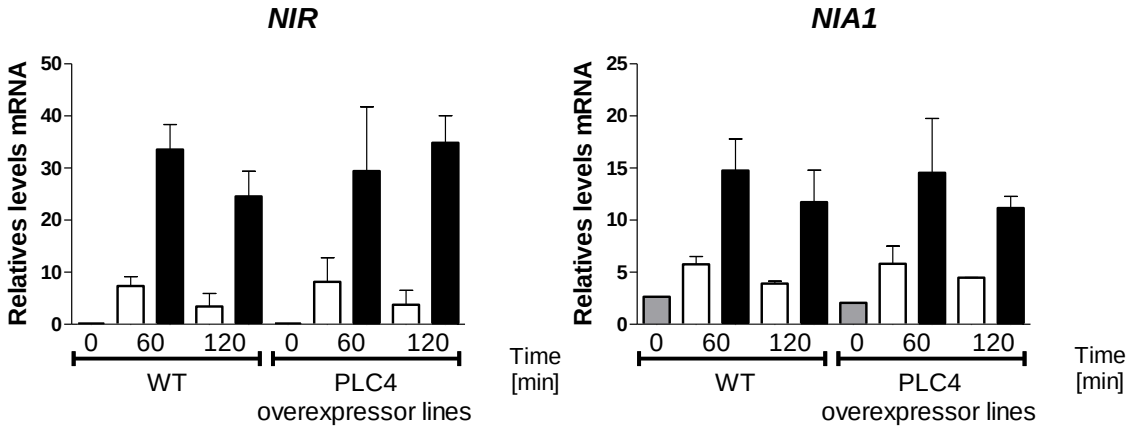

Figure 11. PLC4 overexpression do not enhance nitrate induction of *NIR* or *NIA1* gene expression. Wild-type Col-0 or *PLC4* overexpressor plants were grown hydroponically for two weeks with 1 mM ammonium as the only nitrogen source. At dawn of the 15th day, plants were treated with either 5mM KNO_3 or 5 mM KCl. Root organs were harvested for total RNA isolation 2h afterwards, as described in materials and methods. mRNA levels were measured by RT-qPCR. Plotted values correspond to means of three independent biological replicates ± standard deviations. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. We evaluated the gene expression of *NIR*, nitrite reductase and *NIA1*, nitrate reductase. No significant difference ($P < 0.05$) were found between control and treatment conditions.

nitrate signal that can increase IP_3 levels which in turn result in elevations of $[Ca^{2+}]_{cyt}$ and (ii) Ca^{2+}-independent pathways downstream of NRT1.1/AtNPF6.3 to control gene expression of nitrate-responsive genes (Fig. 12).

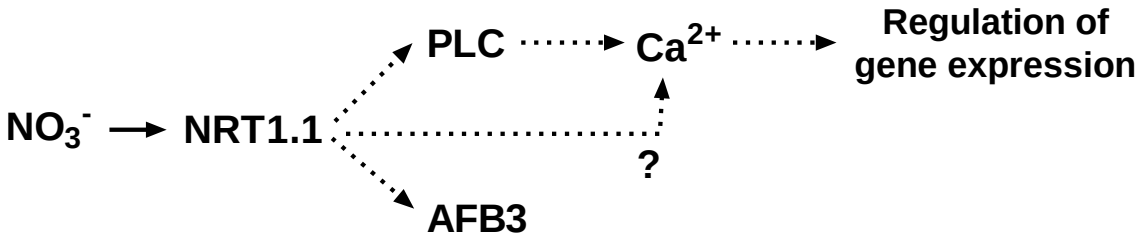

Figure 12. A simplified model of the nitrate signaling pathway. Nitrate is sensed by NRT1.1 and (i) activates a PLC activity that increase $[Ca^{2+}]_{cyt}$ levels or (ii) Ca^{2+}-independent pathways. Increases in $[Ca^{2+}]_{cyt}$ activate gene expression of nitrate responsive genes.

Phosphatidic acid is not involved in the nitrate signaling pathway.

It is known that PLC activity generates as products IP_3 and diacylglycerol (DAG) (Alexandre et al., 1999; Hirose et al., 1999). DAG can generate diacylglycerolpyrophosphate (DGPP) through diacylglycerol kinase (DGK), and DGPP generates phosphatidic acid (PA) through PLD activity (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001). PA is a second messenger involved in many processes in plants (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001). To evaluate the putative role of DAG and PA with signaling pathway in response to nitrate, we measured PA content in Arabidopsis seedlings. To determine the PA content, we grew Arabidopsis plants in the same conditions mentioned above and at 14 days seedlings were pre-labelled overnight with inorganic ^{32}P-orthophosphate (^{32}Pi). Next day, plant lipids were extracted and separated by alkaline thin layer chromatography (TLC). Labelled phospholipids were measured in a phosphoimager. In figure 13, the signaling lipids phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP2) and PA do not show significant differences between KNO_3 and KCl treatments. In contrast, the NaCl treatment produces an increase of PIP2 and PA, comparable with results obtained by others (Munnik T. et al., 2000). These results suggest that PA is not involved in the nitrate signaling pathway and that DAG is not phosphorylated by DAGK in response to nitrate.

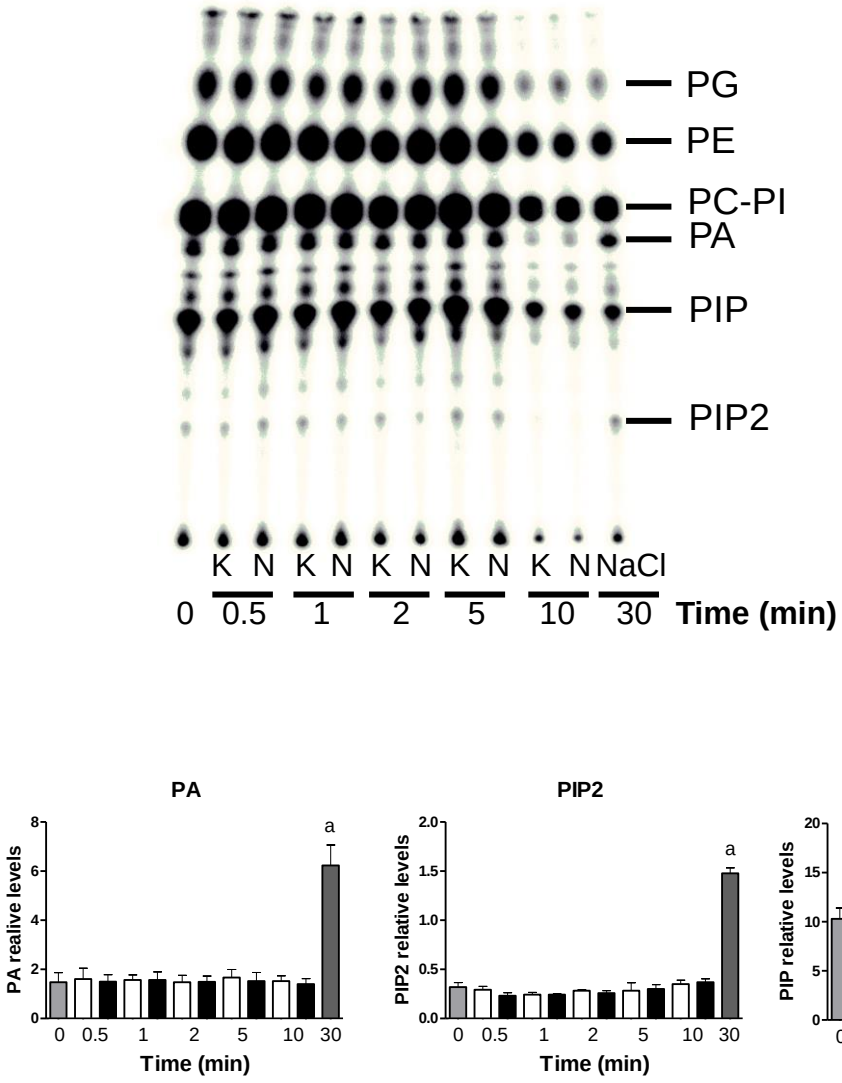

Figure 13. Phosphatidic acid is not induced by nitrate treatment. Arabidopsis seedlings were metabolically pre-labelled with ^{32}P-orthophosphate overnight, and subsequently at the beginning of the next day we treated seedling with KNO_3 (black bars) and KCl (white bars) for the indicated times, or with 300 mM NaCl (black grey bar) for 30 minutes. Lipids were then extracted and separated on alkaline TLC, to visualize phospholipids. A) Alkaline TLC in response to KNO_3, KCl and NaCl. B) Quantification of PA, PIP_2 and PIP in response to nitrate treatment. Plotted values correspond to means of three independent biological replicates ± standard deviations. Letters indicates means that significantly differ from the control ($P < 0.05$).

3. DISCUSSION

Calcium is a second messenger implicated in various signaling pathways in plants (Sanders et al., 2002; Harper et al., 2004; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Hepler, 2005; Dodd et al., 2010). Changes in $[Ca^{2+}]_{cyt}$ can be induced by diverse environmental stimuli, including salt and oxidative stress, cold, light, hormones and bacterial and fungal pathogens (Polisensky and Braam, 1996; Stoelzle et al., 2003; Chen and Kao, 2012; Choi et al., 2014a; Gilroy et al., 2014). Despite progress in identifying components of the nitrate-signaling pathway (Little et al., 2005; Castaings et al., 2009; Ho et al., 2009; Hu et al., 2009; Rubin et al., 2009; Wang et al., 2009; Vidal et al., 2010; Vidal et al., 2013b; Vidal et al., 2013a; Alvarez et al., 2014; Vidal et al., 2014a), relatively little is known about how plants sense and transduce the nitrate signal to regulate gene expression. In this work, we found that nitrate treatments trigger changes in $[Ca^{2+}]_{cyt}$ dynamics. Furthermore, nitrate treatments increase IP_3 levels, which correlates with an increase in $[Ca^{2+}]_{cyt}$. This effect was abolished in *chl1-5* and *chl1-9* mutant plants indicating NRT1.1/AtNPF6.3 transporter activity is required for increased Ca^{2+} and IP_3 in response to nitrate treatments. We found that gene expression in response to nitrate is affected by a PLC inhibitor and a Ca^{2+} channel blocker, suggesting the existence of a signaling pathway for nitrate sensing and signal transduction involving a perception event at or downstream of NRT1.1/AtNPF6.3, activation of a PLC activity, and calcium increases as a second messenger to regulate gene expression.

In animals, extracellular signals are perceived by membrane receptors, leading to PI-PLC activation and an increase in IP_3. IP_3 is a primary effector of calcium release, interacting with receptors on intracellular calcium storage sites to release calcium into the cytoplasm (Alexandre et al., 1999; Hirose et al., 1999). Several studies suggest that

changes in $[Ca^{2+}]_{cyt}$ is mediated by IP_3 and the PI-PLC pathway in plants (Sanchez and Chua, 2001; Perera, 2006; Perera et al., 2008; Zheng et al., 2012). We found that the nitrate-inducted increase in $[Ca^{2+}]_{cyt}$ is abolished by U73122, specific PLC inhibitor. We observed similar effects of NRT1.1 mutations and U73122 treatment on $[Ca^{2+}]_{cyt}$ increase after nitrate treatment, suggesting that NRT1.1, PLC and IP_3 are involved in the same pathway mediating the nitrate response. Evidences show that plant PLCs contain Ca^{2+} binding motifs, and are activated by Ca^{2+} (Hunt et al., 2004; Tasma et al., 2008), it is also possible that PLCs are activated by stimulus-triggered $[Ca^{2+}]_{cyt}$ increases rather than by cell-surface receptors such as G protein-coupled receptors or receptor tyrosine kinases (Urano and Jones, 2014). Other mode of regulation can be PLC phosphorylation. Proteomic analysis of plasma membrane phosphorylated proteins in response to flagellin (flg22) identified PLC2 (Nuhse et al., 2003). On the other hand, protein-protein interaction between NRT1.1 and PLC2 has been observed in membrane-based Interactome Database (MIND) (https://associomics.dpb.carnegiescience.edu/Associomics/Home.html). In addition, unpublished results obtained in own laboratory have shown that PLC2 is phosphorylated after 5 minutes of nitrate treatment (Vega et al, 2015. Unpublished data). These evidence might suggest that there is a relationship between NRT1.1 and PLCs, although experimental data are necessary to test this hypothesis.

Arabidopsis has nine actively transcribed PI-PLC genes. *AtPLC2* is expressed constitutively, but expression of the remaining eight PI-PLC genes has been shown to be regulated by salt, cold and dehydration stress, ABA and other perturbations (Tasma et al., 2008). In particular, the expression of *AtPLC4* and *AtPLC5* is regulated by nitrate in Arabidopsis root organs (Wang, 2003; Wang et al., 2004; Vidal et al., 2013a; Alvarez et al., 2014; Canales et al., 2014). Our results show that inhibition of PLC activity blocks the increase in cytosolic IP_3 and Ca^{2+} levels in response to nitrate treatments. These results

support the idea that one or more PLCs are implicated in Arabidopsis root nitrate signaling. The mechanism by which PLC catalyzes the generation of DAG and IP_3 in animals is well understood (Alexandre et al., 1999; Hirose et al., 1999). In *Saccharomyces cerevisiae*, PLC activity is an important component in a nitrogen-sensing signaling pathway, in thermotolerance and in carbon pathways (Schomerus and Kuntzel, 1992; Flick and Thorner, 1998; Ansari et al., 1999). This is consistent with work showing that IP_3 and DAG levels are increased when starved yeast cells are placed in a nitrogen-containing medium (Schomerus and Kuntzel, 1992). However, although an accumulation of IP_3 can be detected in plants in response to various stimuli and this increase in IP_3 levels correlates with increases in cytoplasmic Ca^{2+} levels (Sanchez and Chua, 2001; Zheng et al., 2012), no homologs of animal IP_3 receptors have been described in Arabidopsis (Nagata et al., 2004). Recent evidence suggests that IP_3 can be further phosphorylated into IP_6 (Laxalt and Munnik, 2002; Lemtiri-Chlieh et al., 2003; Meijer and Munnik, 2003; Munnik and Vermeer, 2010). Thus, IP_3 levels may function directly or via its phosphorylated product IP_6 in nitrate-mediated Ca^{2+} release. Similarly, DAG accumulation can lead to an increase in phosphatidic acid (PA), probably by action of a phospholipase D (PLD) activity (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001). PA has been shown to act as second messenger in plant signal pathways (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001) and previous work have demonstrated that PLDε and PA participate in N signaling during nitrogen deprivation in *Arabidopsis thaliana* (Hong et al., 2009). However, under our conditions PA does not increase in response to nitrate in Arabidopsis.

In Arabidopsis, the nitrate transporter NRT1.1/AtNPF6.3 acts as a nitrate sensor, determining the expression of nitrate primary response genes according to external nitrate availability (Ho et al., 2009; Wang et al., 2009). Mutations in NRT1.1/AtNPF6.3 and U73122 and $LaCl_3$ treatments have similar inhibitory effects on $[Ca^{2+}]_{cyt}$ increase by

nitrate, suggesting that NRT1.1/AtNPF6.3 and PLC belong to the same signal transduction pathway to control cytoplasmic calcium levels in response to nitrate. In addition, we have not observed an additional effect of U73122 and $LaCl_3$ on nitrate regulation of gene expression in chl1-5 and chl1-9 backgrounds. NRT1.1/AtNPF6.3 has been shown to be required for the nitrate response of more than 100 nitrate-responsive genes (Wang et al., 2009). We found that normal responses to nitrate of *NIR*, *NRT2.1*, *TGA1*, *NRT3.1* and *NIA1* depends on NRT1.1/AtNPF6.3, PLC activity and Ca^{2+}. These results suggest the existence of a PLC and Ca^{2+} dependent signaling pathway downstream of NRT1.1/AtNPF6.3.

Treatment of detached maize and barley leaves with protein kinase inhibitors has been shown to alter the nitrate regulation of nitrate-responsive genes (Sakakibara et al., 1997; Sueyoshi et al., 1999). Furthermore, nitrate treatments induce changes in phosphorylation levels of proteins (Engelsberger and Schulze, 2012; Wang et al., 2012). Transcriptomics analysis of the nitrate response has shown that several protein kinases and phosphatases are regulated by nitrate availability (Canales et al., 2014), and the Ca^{2+}-dependent protein kinase CIPK8 controls the nitrate response of primary nitrate-responsive genes downstream of NRT1.1 (Hu et al., 2009). Interestingly, the gene network modules reveal that a PLC activity modulates in response to nitrate the expression of genes participating in phosphorylation events. This suggests protein kinases and phosphatases might act downstream the nitrate-NRT1.1-PLC-Ca^{2+} pathway to control gene expression.

We have previously shown that the regulatory factors AFB3 and TGA1 are located downstream of NRT1.1/AtNPF6.3 function in the Arabidopsis root nitrate response (Alvarez et al., 2014; Vidal et al., 2014a). As our results indicate, TGA1 and its target NRT2.1 would operate downstream of NRT1.1/AtNPF6.3 and a calcium-dependent signaling pathway, while AFB3 would operate downstream of NRT1.1/AtNPF6.3 via a

calcium-independent signaling pathway. This observation is consistent with previous results that indicate AFB3-mediated and TGA1-mediated responses act independently to control root system architecture in response to nitrate (Alvarez et al., 2014; Vidal et al., 2014a). These results suggest bifurcation into at least two different signaling pathways downstream of NRT1.1/AtNPF6.3 activation by a nitrate signal.

In sum, our combined cellular biology and molecular genetics approaches allowed us to identify a mechanism that involves Ca^{2+} as second messenger in the regulation of nitrate responsive genes. Although the relationship between IP_3 and calcium has been addressed in plants, our study reveals a role for calcium and phosphoinositides in the response to nitrate. Our work also provides evidence that nitrate sensed by NRT1.1/AtNPF6.3 activates a PLC activity that increase $[Ca^{2+}]_{cyt}$ levels. Increased $[Ca^{2+}]_{cyt}$ activates gene expression of nitrate responsive genes. Mapping components in the nitrate-signaling pathway contributes to our understanding of how plants sense and respond to changes in nitrogen (N) availability and provide new targets for improving N-use efficiency in crops.

5. CONCLUSION

In this thesis, we found that Ca^{2+} act as a second messenger in the nitrate signaling pathway in *Arabidopsis thaliana*. We found that NRT1.1 and PLC are signaling components of the nitrate signaling pathway that act upstream of calcium function. Our work provides evidence that nitrate is sensed by NRT1.1 and activates a PLC activity that increases $[Ca^{2+}]_{cyt}$ levels. Increased $[Ca^{2+}]_{cyt}$ activates gene expression of nitrate responsive genes. We showed that PLC activity is important for gene expression of hundreds of genes that are involved in different processes. We also determine that PLC4 is involved in the regulation of sentinel-responsive genes in response to nitrate.

These findings increase our understanding of how plants sense and respond to changes in nitrogen (N) availability.

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