The Calcium Ion Is a Second Messenger in the Nitrate Signaling Pathway of Arabidopsis[1]

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Understanding how plants sense and respond to changes in nitrogen availability is the first step toward developing strategies for biotechnological applications, such as improvement of nitrogen use efficiency. However, components involved in nitrogen signaling pathways remain poorly characterized. Calcium is a second messenger in signal transduction pathways in plants, and it has been indirectly implicated in nitrate responses. Using aequorin reporter plants, we show that nitrate treatments transiently increase cytoplasmic Ca^{2+} concentration. We found that nitrate also induces cytoplasmic concentration of inositol 1,4,5-trisphosphate. Increases in inositol 1,4,5-trisphosphate and cytoplasmic Ca^{2+} levels in response to nitrate treatments were blocked by U73122, a pharmacological inhibitor of phospholipase C, but not by the nonfunctional phospholipase C inhibitor analog U73343. In addition, increase in cytoplasmic Ca^{2+} levels in response to nitrate treatments was abolished in mutants of the nitrate transceptor NITRATE TRANSPORTER1.1/Arabidopsis (*Arabidopsis thaliana*) NITRATE TRANSPORTER1 PEPTIDE TRANSPORTER FAMILY6.3. Gene expression of nitrate-responsive genes was severely affected by pretreatments with Ca^{2+} channel blockers or phospholipase C inhibitors. These results indicate that Ca^{2+} acts as a second messenger in the nitrate signaling pathway of Arabidopsis. 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.

Plants are sessile organisms that evolved sophisticated sensing and response mechanisms to adapt to changing environmental conditions. Calcium, a ubiquitous second messenger in all eukaryotes, has been implicated in plant signaling pathways (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 concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) in plants (Sanders et al., 2002; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Hepler, 2005). Abscisic acid and heat shock treatments cause a rapid intracellular Ca^{2+} increase that is preceded by a transient increase in the level of inositol 1,4,5-trisphosphate (IP_3; Sanchez and Chua, 2001; Zheng et al., 2012). Ca^{2+} signatures are detected, decoded, and transmitted to downstream responses by a set of Ca^{2+} binding proteins that functions as Ca^{2+} sensors (White and Broadley, 2003; Dodd et al., 2010).

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). Despite progress in identifying genome-wide responses, only a handful of molecular components involved in nitrate signaling has been identified. Several pieces of evidence indicate that NITRATE TRANSPORTER1.1 (NRT1.1)/Arabidopsis (*Arabidopsis thaliana*) NITRATE TRANSPORTER1 PEPTIDE TRANSPORTER FAMILY6.3 (AtNPF6.3) is a nitrate sensor in Arabidopsis (Ho et al., 2009; Gojon et al., 2011; Bouguyon et al., 2015). NRT1.1/AtNPF6.3 is required for normal expression of more than 100 genes in response to nitrate in Arabidopsis roots (Wang et al., 2009). Downstream of NRT1.1/AtNPF6.3, CALCINEURIN B-LIKE INTERACTING SER/THR-PROTEINE KINASE8 (CIPK8) is required for normal nitrate-induced expression

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of primary nitrate response genes, and the CIPK23 kinase is able to control the switch from low to high affinity of NRT1.1/AtNPF6.3 (Ho et al., 2009; Hu et al., 2009; Ho and Tsay, 2010; Castaings et al., 2011). CIPKs act in concert with CALCINEURIN B-LIKE proteins, plant-specific calcium binding proteins that activate CIPKs to phosphorylate downstream targets (Albrecht et al., 2001). Early experiments using maize (*Zea mays*) and barley (*Hordeum vulgare*) detached leaves showed that nitrate induction of two nitrate primary response genes was altered by pretreating leaves with the calcium chelator EGTA or the calcium channel blocker $LaCl_3$ (Sakakibara et al., 1997; Sueyoshi et al., 1999), suggesting an interplay between nitrate response and calcium-related signaling pathways. However, the role of calcium as a second messenger in the nitrate signaling pathway has not been directly addressed.

We show that nitrate treatments cause a rapid increase of IP_3 and $[Ca^{2+}]_{cyt}$ levels and that blocking phospholipase C (PLC) activity inhibits both IP_3 and $[Ca^{2+}]_{cyt}$ increases 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 activity hinders regulation of gene expression of nitrate-responsive genes. Our results indicate that Ca^{2+} is a second messenger in the nitrate signaling pathway of Arabidopsis.

RESULTS

Nitrate Treatments Increase $[Ca^{2+}]_{cyt}$ Rapidly and Transiently in Arabidopsis Roots

Calcium is an essential second messenger in plant signaling processes (Bush, 1995; Trewavas and Malhó, 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 roots of Arabidopsis, where the transcriptomic and phenotypic responses to nitrate have been well documented (Wang et al., 2004; Gifford et al., 2008; Gutiérrez et al., 2008; Vidal et al., 2010, 2013a, 2013b, 2014; Alvarez et al., 2014). Plants expressing cytoplasmic aequorin (WT-AQ; Gao et al., 2004) were grown hydroponically for 2 weeks with ammonium as the only N source. Plant roots were excised, and luminescence was recorded every 0.2 s after treating the roots with 5 mM KNO_3 or KCl as control. As shown in Figure 1A, nitrate treatment elicited a rapid and transient increase in $[Ca^{2+}]_{cyt}$ in roots. KCl treatment also generated a rapid and transient peak; however, this calcium peak was considerably lower than the one obtained after nitrate treatments (Fig. 1A). After reaching a maximum, $[Ca^{2+}]_{cyt}$ decreased to near basal levels (Fig. 1A).

It is known that abiotic and biotic cues, such as sugar, salt, and drought stress, cause transient $[Ca^{2+}]_{cyt}$ in roots and leaves (Furuichi et al., 2001; Choi et al., 2014; Michal 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 chloride (Knight et al., 1996; Choi et al., 2014). Pretreatment of WT-AQ root and seedlings with 5 mM $LaCl_3$ for 1 h inhibited the $[Ca^{2+}]_{cyt}$ increase observed in response to nitrate treatment (Fig. 1B). These results indicate that nitrate treatments cause a specific increase in $[Ca^{2+}]_{cyt}$ in Arabidopsis.

Phosphatidylinositol-PLC Activity Is Required for Changes in $[Ca^{2+}]_{cyt}$ Levels in Response to Nitrate Treatments in Arabidopsis Roots

To identify components of the signal transduction pathway mediating changes in cytoplasmic calcium levels in response to nitrate, we first determined whether a PLC-dependent pathway was implicated in this $[Ca^{2+}]_{cyt}$ increase. We evaluated the effect of a PLC inhibitor (U73122) and a nonfunctional PLC inhibitor analog (U73343) in WT-AQ lines in response to KNO_3 or KCl treatments. WT-AQ plants were pretreated for 1 h with 10 μM U73122 or U73343, and luminescence of excised plant roots was recorded every 0.2 s after 5 mM KNO_3 or KCl treatment. The presence of the PLC inhibitor (U73122) altered the $[Ca^{2+}]_{cyt}$ increase in response to nitrate treatments (Fig. 2A). However, treatments with the nonfunctional analog

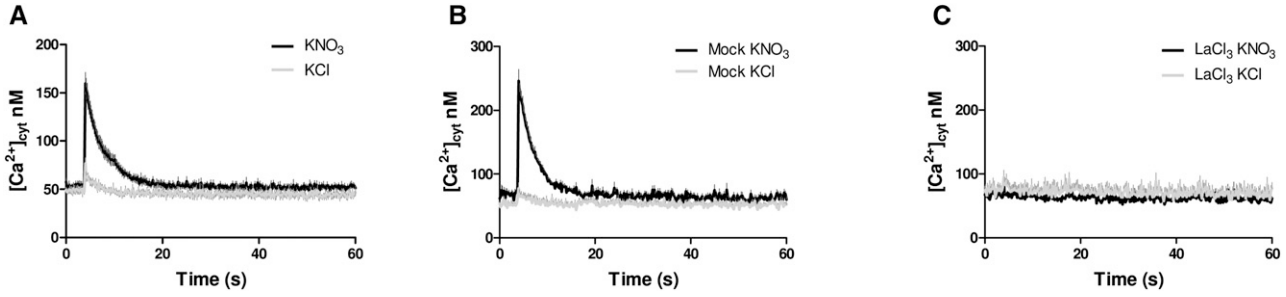

Figure 1. Nitrate treatments increase $[Ca^{2+}]_{cyt}$ in Arabidopsis roots. Wild-type plants expressing WT-AQ were grown hydroponically for 2 weeks with 1 mM ammonium as the only N source. Aequorin was reconstituted by incubating plant roots in 2.5 μM coelenterazine (CTZ) overnight in the dark. $[Ca^{2+}]_{cyt}$ levels were monitored in excised roots in response to 5 mM KNO_3 or KCl treatment (A) without pretreatment (B) or with pretreatment with the Ca^{2+} channel blocker lanthanum chloride ($LaCl_3$; C). Values plotted correspond to the means of at least three independent biological replicates of five plants per treatment ± SD.

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(U73343) did not affect the $[Ca^{2+}]_{cyt}$ increase in Arabidopsis roots (Fig. 2A). 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 of PLC activity implicated in nitrate signaling, 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 roots were quickly collected and frozen in liquid nitrogen. Treatment with 5 mM KNO_3 resulted in a 3-fold increase in IP_3 levels compared with the KCl control 10 s after the treatment (Fig. 2B). Pretreatment of plants with U73122 (but not with U73343) completely blocked IP_3 increase in response to nitrate (Fig. 2B). In addition, $LaCl_3$ reduced IP_3 levels in all tested conditions, suggesting that a calcium-dependent PLC activity is implicated (Supplemental Fig. S1).

These results indicate that a PLC activity is required for IP_3 accumulation as well as increasing $[Ca^{2+}]_{cyt}$ in response to nitrate treatments under our experimental conditions.

NRT1.1 Is a Positive Regulator of the $[Ca^{2+}]_{cyt}$ Increase in Response to Nitrate Treatments in Arabidopsis Roots

Several lines of evidence indicate that the nitrate transporter NRT1.1/AtNPF6.3 acts as 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 an *nrt1.1* mutant background. Aequorin-expressing *chlorate-resistant1-5* (*chl1-5*) line (*chl1-5-AQ*) was generated by crossing *chl1-5* (Gao et al., 2004) with a transgenic line containing the 35S::aequorin construct (WT-AQ). We measured $[Ca^{2+}]_{cyt}$ in *chl1-5-AQ* plant roots in response to nitrate using the same experimental strategy described above. As shown in Figure 3A, the increase in $[Ca^{2+}]_{cyt}$ elicited by nitrate was significantly reduced in the *chl1-5-AQ* line compared with in the wild-type plants.

We also evaluated $[Ca^{2+}]_{cyt}$ in response to nitrate treatments in aequorin reporter lines in the *chl1-9* mutant background. *chl1-9* has a P492L point mutation that has been shown to reduce NRT1.1/AtNPF6.3 nitrate uptake without affecting the signaling function of NRT1.1 over the NRT2.1 nitrate transporter (Ho et al., 2009). It was recently shown that this point mutation causes abnormal NRT1.1/AtNPF6.3 localization (Bouguyon et al., 2015). As shown in Figure 3B, $[Ca^{2+}]_{cyt}$ is lower in *chl1-9-AQ* roots compared with the wild type in response to nitrate treatments and comparable with the results obtained for the *chl1-5-AQ* line. These results indicate that the increase in $[Ca^{2+}]_{cyt}$ by nitrate depends on NRT1.1/AtNPF6.3.

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 plant roots after nitrate treatments. *chl1-5* and *chl1-9* plants were grown for 15 d and treated with 5 mM KNO_3 or KCl as control, and IP_3 content was measured. In contrast to the increase in IP_3 levels in wild-type roots, there was no significant increase in IP_3 content in *chl1-5* and *chl1-9* mutant roots after nitrate treatments (Fig. 3C). This result indicates that accumulation of IP_3 in Arabidopsis root in response to nitrate treatments also requires NRT1.1/AtNPF6.3 for activation of a PLC activity.

Nitrate-Induced Gene Expression Is Mediated by NRT1.1/NPF6.3, PLC, and Ca^{2+}

To determine the impact of this signaling pathway on nitrate regulation of gene expression, we analyzed the expressions of nitrate-responsive genes, which have been shown to play important roles in nitrate-dependent root growth (Ho et al., 2009; Alvarez et al., 2014; Vidal et al., 2014) in wild-type, *chl1-5*, and *chl1-9*

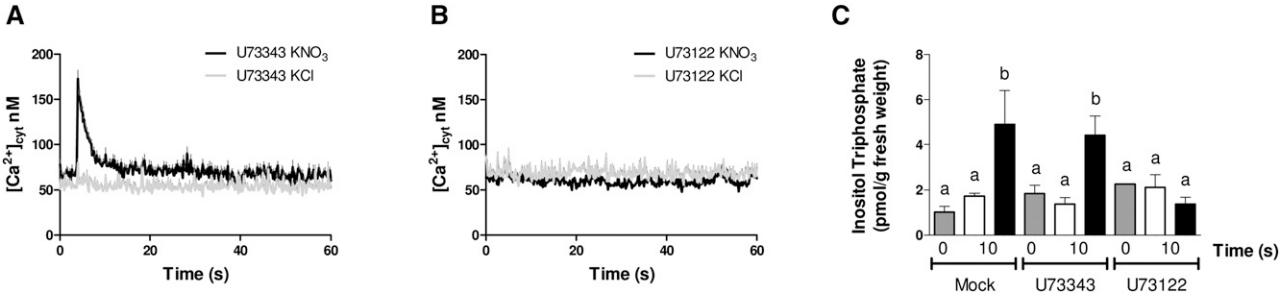

Figure 2. A PLC inhibitor blocks the increase in $[Ca^{2+}]_{cyt}$ and IP_3 levels in response to nitrate treatments in Arabidopsis roots. Wild-type plants expressing WT-AQ were grown hydroponically for 2 weeks with 1 mM ammonium as the only nitrogen source, and $[Ca^{2+}]_{cyt}$ and IP_3 levels were assayed as described in the text. $[Ca^{2+}]_{cyt}$ levels were monitored in excised roots pretreated with U73343 (nonfunctional analog; A) or U73122 (PLC inhibitor; B) after we treated with KNO_3 and KCl. C, Plants were pretreated with mock, U73122 (inhibitor of PLC), or U73343 (analogous no function), and we evaluated the IP_3 content in Arabidopsis roots in response to 5 mM KNO_3 or KCl. Values plotted correspond to the means of three independent biological replicates ± SD. Gray bars represent time zero (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. The letters indicate means that significantly differ between control and treatment conditions ($P < 0.05$).

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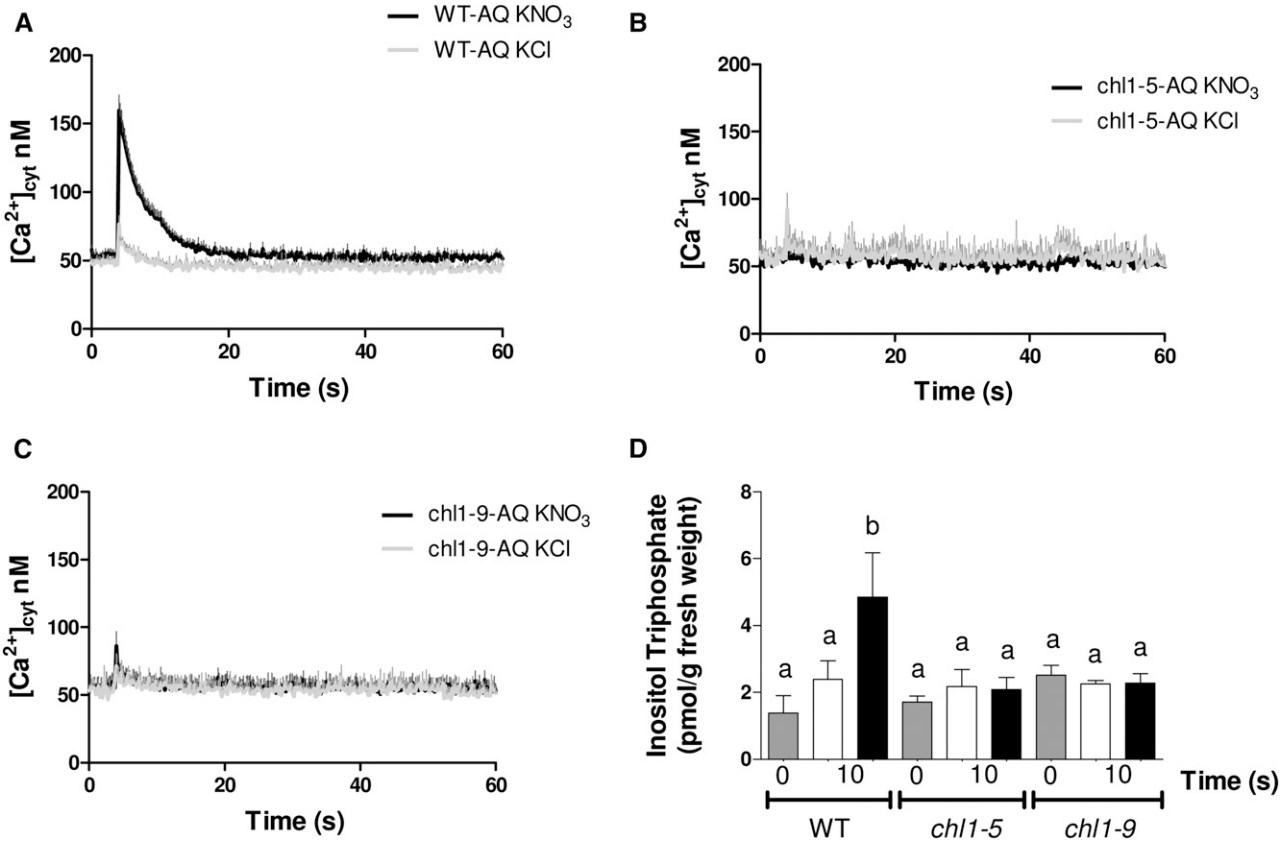

Figure 3. NRT1.1/AtNPF6.3 is required for increases in $[Ca^{2+}]_{cyt}$ and IP_3 levels in response to nitrate treatments in Arabidopsis roots. Wild-type, *chl1-5*, and *chl1-9* plants were grown hydroponically for 2 weeks with ammonium as the only nitrogen source, and $[Ca^{2+}]_{cyt}$ and IP_3 contents were assayed as described in the text. WT-AQ (A), *chl1-5-AQ* (B), and *chl1-9-AQ* (C) plants were reconstituted by incubating plant roots in 2.5 μM CTZ overnight in the dark. $[Ca^{2+}]_{cyt}$ levels were monitored over time. D, Wild-type (WT), *chl1-5*, and *chl1-9* plants were treated with 5 mM KNO_3 and 5 mM KCl as control for 10 s, and then, we evaluated the IP_3 content. Values plotted correspond to the means of at least three independent biological replicates ± SD. Gray bars represent time zero (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. The letters indicate means that significantly differ between control and treatment conditions ($P < 0.05$).

plants treated with the calcium channel blocker $LaCl_3$ or the PLC inhibitor U73122. Total RNA was isolated from roots, and mRNA levels were measured for selected genes using quantitative real-time reverse transcription (qRT)-PCR. As shown in Figure 4, *NRT2.1*, *TGACG SEQUENCE-SPECIFIC BINDING PROTEIN1* (*TGA1*), and *AUXIN SIGNALING F-BOX3* (*AFB3*) gene expressions are induced after KNO_3 treatments. Consistent with previous reports (Ho et al., 2009; Alvarez et al., 2014; Vidal et al., 2014), nitrate regulation of gene expression of these genes was significantly altered in the *chl1-5* and *chl1-9* mutants under our experimental conditions. Similarly, nitrate inductions of *NRT2.1* and *TGA1* were significantly reduced in the presence of $LaCl_3$ or U73122 but not mock or U73343 treatment (Fig. 4). Interestingly, induction of *AFB3* by nitrate was not significantly affected in the presence of U73122 or $LaCl_3$ (Fig. 4). In addition, *NITRITE REDUCTASE* (*NIR*) and *NRT3.1* gene expressions behaved similarly to *TGA1*, with altered response to nitrate treatments in *chl1-5* or *chl1-9* mutant plants and in the presence of U73122 or $LaCl_3$ (Supplemental Fig. S2). This indicates that NRT1.1/AtNPF6.3, a PLC activity, and an increase in $[Ca^{2+}]_{cyt}$ levels are required for changes in gene expression in response to nitrate treatments in Arabidopsis. Moreover, these results suggest the existence of Ca^{2+}-dependent and -independent pathways downstream of NRT1.1/AtNPF6.3 to control gene expression of nitrate-responsive genes (Fig. 5).

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), and changes in $[Ca^{2+}]_{cyt}$ are an important component of these calcium signaling networks. These changes can be induced by diverse environmental stimuli, including salt and oxidative stress, cold, light, hormones, and bacterial and fungal pathogens (Polisensky and Braam, 1996;

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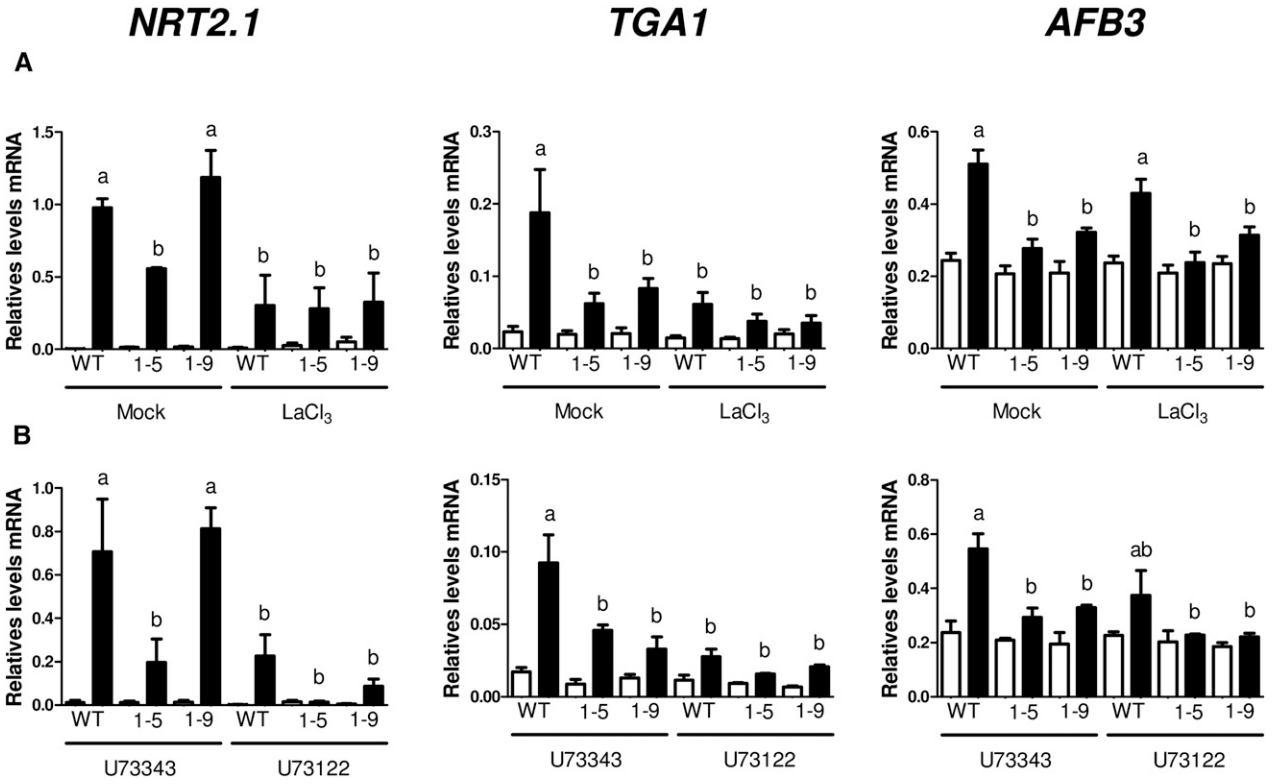

Figure 4. Regulation of gene expression in response to nitrate treatments is mediated by NRT1.1/AtNPF6.3, a PLC activity, and Ca^{2+} in Arabidopsis roots. Col-0, *chl1-5,* and *chl1-9* plants were grown for 15 d. Plants were pretreated with 5 mM $LaCl_3$ (A) or 10 μM U73122 or U73343 (B) and then, treated with 5 mM KNO_3 or KCl as control. Values plotted correspond to the means of three independent biological replicates ± SD. White bars represent KCl treatment, and black bars represent KNO_3 treatment. The *ADAPTOR PROTEIN4 μ-ADAPTIN* gene (At4g24550) was used as a normalization reference (Aceituno et al., 2008). The letters indicate means that significantly differ between control and pharmacological treatment ($P < 0.05$). WT, Wild type.

Stoelzle et al., 2003; Chen and Kao, 2012; Choi et al., 2014; Gilroy et al., 2014). We found that nitrate is also able to trigger changes in $[Ca^{2+}]_{cyt}$. Moreover, we found that nitrate treatments increase IP_3 levels, which correlate with an increase in $[Ca^{2+}]_{cyt}$. In animals, IP_3 is generated by the cleavage of phosphatidylinositol (PI) 4,5-bisphosphate by PI-PLC enzymes (Alexandre et al., 1999; Hirose et al., 1999). This effect was abolished in *chl1-5* and *chl1-9* mutant plants, indicating that NRT1.1/AtNPF6.3 function 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 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 as a second messenger to regulate gene expression.

Arabidopsis has nine actively transcribed PI-PLC genes. AtPLC2 is expressed constitutively, but expressions of the remaining eight PI-PLC genes have been shown to be regulated by salt, cold and dehydration stress, abscisic acid, and other perturbations (Tasma et al., 2008). Interestingly, the expression of AtPLC4 and AtPLC5 genes is regulated by nitrate in Arabidopsis roots (Wang et al., 2003, 2004; Vidal et al., 2013a, 2013b; Alvarez et al., 2014; Canales et al., 2014). Our results show that inhibition of PLC activity in plant roots blocks the increase in cytosolic IP_3 and Ca^{2+} levels in response to nitrate treatments. In addition, $LaCl_3$ also blocked the increase in IP_3 and $[Ca^{2+}]_{cyt}$ levels by nitrate treatments, suggesting a calcium-dependent PLC activity (Hunt et al., 2004). 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 diacylglycerol and IP_3 in animals is well understood (Alexandre et al., 1999; Hirose et al., 1999). However, although accumulation of IP_3 can be detected

Figure 5. A simplified model of the NRT1.1/AtNPF6.3 calcium-dependent and -independent nitrate signaling pathway. Nitrate is sensed by NRT1.1/AtNPF6.3 and activates a PLC activity that increases $[Ca^{2+}]_{cyt}$. Increase in $[Ca^{2+}]_{cyt}$ activates gene expression of nitrate-responsive genes.

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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). IP_3 can be further phosphorylated into inositol hexaphosphate (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 through 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 activity (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001). PA has been shown to act as second messenger in plant signaling pathways (Katagiri et al., 2001; Munnik, 2001; Sang et al., 2001), and previous work showed that phospholipase Dε and PA participate in N signaling during nitrogen deprivation in Arabidopsis (Hong et al., 2009). However, it is unclear whether PA has an effect over cytoplasmic calcium levels in Arabidopsis.

In Arabidopsis roots, the nitrate transporter NRT1.1/AtNPF6.3 is thought to be a nitrate sensor essential for regulation of gene expression in response to changes in external nitrate (Ho et al., 2009). Mutation of NRT1.1/AtNPF6.3 and U73122 treatments have a similar inhibitory effect over $[Ca^{2+}]_{cyt}$, which suggests that NRT1.1/AtNPF6.3 and PLC belong to the same signal transduction pathway to control cytoplasmic calcium levels in response to nitrate. We found that normal response to nitrate of *NIR*, *NRT2.1*, *TGA1*, and *NRT3.1* depends on NRT1.1/AtNPF6.3, PLC activity, and Ca^{2+}. However, we did not observe an additional effect of U73122 or $LaCl_3$ on nitrate regulation of gene expression in *chl1-5* or *chl1-9* mutant backgrounds. Our results indicate existence of a PLC-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). These studies are consistent with our results and suggest kinase targets of the nitrate-NRT1.1-Ca^{2+} pathway described here to control gene expression.

We have previously shown that regulatory factors AFB3 and TGA1 are downstream of NRT1.1/AtNPF6.3 function in the Arabidopsis root nitrate response (Alvarez et al., 2014; Vidal et al., 2014). As our results indicate, TGA1 and its target *NRT2.1* would operate downstream of NRT1.1/AtNPF6.3 through a calcium-dependent signaling pathway, whereas AFB3 would operate downstream of NRT1.1/AtNPF6.3 through a calcium-independent signaling pathway. This observation is consistent with previous results that indicate that AFB3- and TGA1-mediated responses act independently to control root system architecture in response to nitrate (Alvarez et al., 2014; Vidal et al., 2014). More recently, using transcriptomics and phenotypic analysis of NRT1.1/NPF6.3 mutants, Bouguyon et al. (2015) showed that multiple signaling pathways act downstream of NRT1.1/NPF6.3. Our results are also consistent with these observations and show that at least one signaling pathway downstream of NRT1.1/NPF6.3 depends on PLC, IP_3, and Ca^{2+}.

Our combined cell biology and molecular genetics approach allowed us to identify steps in the nitrate signaling pathway that involve Ca^{2+} as second messenger in the regulation of prototypical nitrate-responsive genes. Mapping components in the nitrate signaling pathway contributes to our understanding of how plants sense and respond to changes in N availability and provides unique targets for improving N use efficiency in crops.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) was used for all experiments. The Arabidopsis line expressing WT-AQ (Gao et al., 2004) was obtained from Christoph Plieth. The *chl1-5* and *chl1-9* mutants were donated by Yi-Fang Tsay. Plants were grown in hydroponic culture under long-day (16-h light/8-h-dark cycle) conditions at 22°C (in Percival incubators) using Murashige and Skoog salt medium without N (M531; Phytotechnology Laboratories) supplemented with 0.5 mM ammonium succinate and 0.1% (w/v) Suc. Plants were treated for the indicated periods of time at the beginning of the light cycle on day 15 with 5 mM KNO_3 or KCl as a control.

Chemical Treatment of Plants

U73122, U73343, and $LaCl_3$ were purchased from Sigma-Aldrich. Before harvesting plant material for analysis of gene expression, Col-0 seedlings were pretreated in petri dishes for 1 h in the presence of 10 μM U73122, 10 μM U73343, or 5 mM $LaCl_3$. Plants were then treated for the indicated periods of time with 5 mM KNO_3 or KCl. For aequorin measurements, plant pretreatment with all pharmacological agents was done 1 h before the addition of KNO_3 or KCl to excised roots. U73122 and U733343 were dissolved in 0.1% (v/v) dimethyl sulfoxide, and $LaCl_3$ was dissolved in water.

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

Reconstitution of aequorin in vivo with CTZ was performed as described previously (Knight et al., 1996). Synthetic native CTZ was obtained from Sigma-Aldrich. Briefly, for each experiment, we incubated 14-d-old seedlings overnight in the dark with 2.5 μM CTZ. Plant were washed with water, and roots were excised and placed in a cuvette to measure luminescence immediately after treatments. Luminescence was recorded for the duration of the experiment every 0.2 s. To convert luminescence into Ca^{2+} concentrations, 1 M $CaCl_2$ and 10% (v/v) ethanol were added to discharge the remaining aequorin. Calculations of Ca^{2+} concentrations were performed as previously mentioned (Knight et al., 1996). Luminescence measurements were performed using a Sirius Single-Tube Luminometer (Berthold Detection Systems).

IP_3 Assays

IP_3 was measured as described previously (Heilmann and Perera, 2013). Briefly, plants were treated with 5 mM KNO_3 or KCl for 10 s, and roots were harvested and frozen immediately in liquid N_2. Frozen tissue (approximately 0.1 g) was grounded to powder and incubated with 200 μL of 10% (v/v) perchloric

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acid on ice for 20 min. Samples were centrifuged to remove the precipitate, the supernatant was transferred to a new tube, and the pH was adjusted to 7.5 using 1.5 M KOH and 60 mM HEPES. IP_3 was measured using the Inositol 1,4,5-Triphosphate (3H) Radioreceptor Assay Kit (Perkin Elmer) according to the instructions of the manufacturer.

RNA Isolation and qRT-PCR

RNA was isolated from whole roots with the PureLink RNA Mini Kit (12183020; Life Technologies) according to the instructions of the manufacturer. Complementary DNA synthesis was carried out using the Improm-II Reverse Transcriptase according to the instructions of the manufacturer (Promega). qRT-PCR was carried out using the Brilliant SYBR Green QPCR Reagents on a Stratagene MX3000P qPCR System. The RNA levels were normalized relative to *ADAPTOR PROTEIN4 μ-ADAPTIN* (At4g24550; Aceituno et al., 2008).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. $LaCl_3$ reduced the IP_3 levels in roots.

Supplemental Figure S2. NRT1.1/AtNPF6.3, PLC activity, and Ca^{2+} are required for the nitrate-dependent up-regulation of NRT3.1 and NIR.

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