** O Botanical Studies**

ORIGINAL ARTICLE

Open Access

CrossMark

Tobacco plants expressing the maize nitrate transporter ZmNrt2.1 exhibit altered responses of growth and gene expression to nitrate and calcium

Abubakar Ibrahim[1,2], Xiao-Lu Jin[1], Yu-Bin Zhang[1,3], Jessica Cruz[1], Paveena Vichyavichien[1], Nwadiuto Esiobu[1] and Xing-Hai Zhang[1]*

Abstract

Background: Nitrate uptake is a highly regulated process. Understanding the intricate interactions between nitrate availability and genetically-controlled nitrate acquisition and metabolism is essential for improving nitrogen use efficiency and increasing nitrate uptake capacity for plants grown in both nitrate-poor and nitrate-enriched environments. In this report, we introduced into tobacco (*Nicotiana tabacum*) the constitutively expressed maize high-affinity transporter ZmNrt2.1 gene that would bypass the tight control for the endogenous nitrate-responsive genes. By using calcium inhibitors and varying levels of NO_3^-, Ca^{2+} and K^+, we probed how the host plants were affected in their nitrate response.

Results: We found that the ZmNrt2.1-expressing plants had better root growth than the wild type plants when Ca^{2+} was deficient regardless of the nitrate levels. The growth restriction associated with Ca^{2+}-deficiency can be alleviated with a high level of K^+. Furthermore, the transgenic plants exhibited altered expression patterns of several endogenous, nitrate-responsive genes, including the high- and low-affinity nitrate transporters, the Bric-a-Brac/Tramtrack/Broad protein BT2 and the transcription factor TGA-binding protein TGA1, in responding to treatments of NO_3^-, K^+ or inhibitors for the calcium channel and the cytosolic Ca^{2+}-regulating phospholipase C, as compared to the wild type plants under the same treatments. Their expression was not only responsive to nitrate, but also affected by Ca^{2+}. There were also different patterns of gene expression between roots and shoots.

Conclusion: Our results demonstrate the ectopic effect of the maize nitrate transporter on the host plant's overall gene expression of nitrate sensing system, and further highlight the involvement of calcium in nitrate sensing in tobacco plants.

Keywords: Calcium signaling, Maize high-affinity nitrate transporter ZmNrt2.1, Nitrate sensing, Nitrate responsive genes, Potassium, Tobacco

Background

Nitrogen is an essential element for all forms of life. Nitrate (NO_3^-) is a major form of nitrogen in the environment taken up by plants (Guerrero et al. 1981). Because

of high demand for nitrate by crop plants to achieve high yield, over 100 million tons of nitrogen-fertilizers are being applied annually to farm land worldwide in recent years (FAO 2015). Yet due to low affinity between nitrate and most types of soils, as much as 50–70% of the applied nitrogen may be leached from the soil (Peoples et al. 1995), causing pollution and eutrophication of natural water systems, especially near agricultural and urban areas (Unkles et al. 2004; Howarth and Marino 2006).

*Correspondence: xhzhang@fau.edu
[1] Department of Biological Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA
Full list of author information is available at the end of the article

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 2 of 12

Better understanding of how plants respond to nitrate availability in soil and regulate nitrate uptake is important in finding suitable solutions to over-fertilization as well as remedy for nitrogen deficiency.

Plants take up NO_3^- from soil by transporting it across the plasma membrane of the root epidermal and cortical cells. This process is carried out by various nitrate transporters through a proton-coupled gradient $2H^+/1NO_3^-$ symport mechanism (Meharg and Blatt 1995; Crawford and Glass 1998). Once inside the cell, most nitrate is first reduced to nitrite by nitrate reductases, and then to ammonium by nitrite reductases. Ammonium is metabolized into glutamine, a universal precursor for many metabolic processes (Guerrero et al. 1981; Good et al. 2004; Wang et al. 2012). Plants have developed multiple and independently operating systems to adjust for changing nitrate availability in the environment. There are at least three known nitrate uptake systems: a constitutive high-affinity transport system (cHATS), a NO_3^- inducible high-affinity transport system (iHATS), and a low-affinity constitutively expressed transport system (LATS) (Crawford and Glass 1998; Forde 2000). Previous studies have shown that the LATS operates when NO_3^- levels are high (> 1 mM), whereas the cHATS and iHATS operate at lower NO_3^- levels (< 1 mM) (Crawford and Glass 1998; Quaggiotti et al. 2003; Katayama et al. 2009). Although the cHATS has a higher affinity for NO_3^-, the iHATS is more active with a V_{max} 30 times that of cHATS and is responsible for a greater NO_3^- uptake (Kronzucker et al. 1996; Crawford and Glass 1998). Upon exposed to nitrate, nitrogen starved plants increase nitrate uptake several folds largely due to the iHATS (Tsay et al. 2007).

In Arabidopsis, at least four families of nitrate-transporting proteins have been studied: low-affinity nitrate transporter 1/peptide transporter (NRT1 or NPF), high-affinity nitrate transporter 2 (NRT2), chloride channel and slow anion channel associated homologues (Forde 2000; Quaggiotti et al. 2003; Krapp et al. 2014). Members of NRT1 family have been shown to play a variety of important physiological roles such as sustaining NO_3^- nitrate uptake and influx, transporting plant hormones, regulating NRT2 gene expression and HATS activity, and nitrate and calcium sensing (Muños et al. 2004; Remans et al. 2006; Chiba et al. 2015; Undurraga et al. 2017). Interestingly, unlike NRT2, NRT1.1 gene is not induced by nitrate deficiency nor suppressed by reduced nitrogen metabolism (Lejay et al. 1999). But Loqué et al. (2003) reported repression of NTR1.1 gene expression by the reduced NO_3^- product NO_2^- in Arabidopsis roots.

The NRT2 family is a part of the iHATS responsible for the influx of nitrate into the plant cell when nitrate becomes available in the environment. For example, the Arabidopsis NRT2.1 is crucial for NO_3^- influx especially when nitrate is deficient (Wirth et al. 2007). Structurally, NRT2 nitrate transporters belong to the nitrate-nitrite transporter family, a part of the Major Facilitator Superfamily of membrane transporters (Pao et al. 1998). Thus, the NRT2 membrane topology is made of two sets of six transmembrane domains connected by a cytosolic loop (Pao et al. 1998; Forde 2000). For several plants investigated, the transport activity of NRT2 protein is shown to require the direct interaction with another protein, NAR2 (Quesada et al. 1994; Zhou et al. 2000; Orsel et al. 2006). For example, in Arabidopsis both the NRT2.1 and NAR2.1 proteins were shown to localize and interact at the plasma membrane in the roots, where the active nitrate transporter is presumably formed as a NRT2.1/NAR2.1 heterooligomer (Wirth et al. 2007; Yong et al. 2010). In a study carried out in barley, nitrate and nitrite transport activity was only observed when HvNRT2.1 and HvNAR2.3 genes were both injected into the *Xenopus* oocytes expression system, and no transport activity was detected when HvNRT2.1 gene was injected alone or coupled with HvNAR2.1 or HvNAR2.2 genes (Tong et al. 2005). In maize roots, the accessory protein ZmNAR2.1 (ZmNRT3.1A) was important for iHATS activity and appeared to form an oligomer with ZmNRT2.1 (Pii et al. 2016). Although the two-component system in several species tested seems to be specific for NRT and NAR proteins, the rice OsNAR2.1 had a broader range of interactions with OsNRT2.1, OsNRT2.2 and OsNRT2.3a (Yan et al. 2011). Furthermore, the NRT2 genes in grass species are distinct from the Arabidopsis NRT2 by the absence of gene family clusters in the genomes and the lack of an intron, suggesting that the divergence of the NRT2 family occurred after the evolutionary split between dicots and monocots (Plett et al. 2010).

The iHATS including NRT2 is part of nitrate sensing system tightly controlled to maintain nitrogen homeostasis, where its activity dramatically increases upon first provision of NO_3^- and is quickly repressed after NO_3^- exposure (Crawford and Glass 1998; Quaggiotti et al. 2003; Medici and Krouk 2014). Down-regulation occurs through mRNA stability and with influx of other nitrogen metabolites such as ammonium, glutamate, glutamine, asparagine, and arginine (Imsande and Touraine 1994; Forde and Clarkson 1999). Growth and development is another signal for NRT2 regulation. For example, in Arabidopsis NRT2.1 protein levels remain stable in older plants and are not affected by environmental cues such as nutrient availability or darkness, while in younger (8-day old) seedlings the amount of NRT2.1 protein is decreased after 24 h of darkness (Laugier et al. 2012). Another study showed that light, sucrose or nitrogen treatments strongly affect both NRT2.1 transcription and HATS activity in Arabidopsis, but NRT2.1 protein

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 3 of 12

level remains largely steady in response to these treatments (Wirth et al. 2007). Yet a different study reported that cellular glucose elevates NRT2.1 protein levels and transport activity in Arabidopsis, independent of NRT2.1 transcription (de Jong et al. 2014). Furthermore, posttranscriptional control was reported to be important for NRT2.1/NAR2.1 transport system in Arabidopsis roots (Laugier et al. 2012) and NRT2.1-nitrate influx in *Nicotiana plumbaginifolia* (Fraisier et al. 2000). Regardless of the various points of view and some seemingly discrepancies in literature, it is clear that NRT2.1 is actively regulated at various levels of transcription and translation, and there is an intricate crosstalk between plant metabolism and nitrate gene expression throughout growth and development.

In plants, hundreds of genes, including the aforementioned NO_3^- uptake systems and nitrate transporters (NRT), respond to nitrate as a regulatory signal (Wang et al. 2004; Krapp et al. 2014; Medici and Krouk 2014). However, increasing evidence has shown that calcium is another essential player in the nitrate signaling network. For example, Ca^{2+} and calcium-binding proteins such as CIPKs are important in modulating NRT gene expression in response to cellular and environmental nitrate levels (Albrecht et al. 2001; Hu et al. 2009). The universal calcium-mobilizing second messenger, inositol 1,4,5-trisphosphate, is produced by phosphoinositide-specific phospholipase C (PLC) enzymes from hydrolyzing the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate (Streb et al. 1983; Hunt et al. 2004). Changes in cellular Ca^{2+} levels through the actions of PLC and membrane-bound calcium-permeable channels can significantly affect the expression of nitrate-responding genes (Sakakibara et al. 1997; Riveras et al. 2015). Thus, inhibitors such as U73122 for PLC (Franklin-Tong et al. 1996) and La^{3+} for several calcium channels (White 2000) have become a useful tool for analyzing Ca^{2+}- or nitrate-responsive genes.

It is obvious that nitrate uptake and metabolism in plants is tightly regulated by various signals at different levels (Wang et al. 2012; Krapp et al. 2014; Medici and Krouk 2014). Studies of the high-affinity nitrate transporter NRT2, a major nitrate uptake avenue for plants, and other nitrate responsive and regulatory genes will help better understand the intricate interactions between nitrate availability in the environment and genetically-controlled nitrate acquisition and metabolism. This knowledge is needed for achieving high nitrogen use efficiency and high capacity of nitrate uptake for plants in both nitrate-poor and anthropologically nitrate-enriched environments, in order to aim for an optimal balance between fertilizer usage, plant productivity and environmental protection (Good et al. 2004). As part of the effort

to investigate plant nitrate response and regulation, we introduced in tobacco plants a maize high-affinity transporter ZmNrt2.1 gene driven by the constitutive CaMV 35S promoter. Therefore, this ZmNrt2.1 transgene would bypass the tight transcriptional regulation exerted on host plant's endogenous nitrate-responsive genes. Thus, we hoped to use ZmNrt2.1, along with calcium inhibitors and varying availability of NO_3^-, Ca^{2+} and K^+, to probe changes of nitrate responses in the host plant. Our studies reveal the ectopic effect of ZmNrt2.1 on growth and nitrate sensing system involving several endogenous nitrate-responsive genes.

Materials and methods

Plant material

As described previously (Hill et al. 2016), plants of tobacco (*Nicotiana tabacum* L., cv. Havana petit) were grown in MS medium solidified with 2.7 g/L phytagel in a growth chamber at 24°C under 8 h of darkness and 16 h of cool white light of 150 μmol photons $m^{-2}\ s^{-1}$. The seedlings were used for various treatments as described below.

Construction of transformation vector expressing maize nitrate transporter

The coding sequence of the high-affinity nitrate transporter gene ZmNrt2.1 from maize (*Zea mays*, Genbank accession: NM_001111725; Quaggiotti et al. 2003), fused with the FLAG tag (DYKDDDDK) coding sequence, was synthesized by GenScript (USA). The 1.6-kb *Sma*I/*Sac*I-cut fragment was ligated into the *Sma*I/*Sac*I digested binary plasmid E1492 (Zhang et al. 2004) that is derived from pBI101 (Clontech, USA). The resultant plasmid p35S-ZmNrt (Additional file 1: Figure S1) was introduced to *Agrobacterium tumefaciens* strain EHA105.

Agrobacterium-mediated plant transformation

Plant transformation was carried out as previously described (Zhang et al. 2004; Tsai et al. 2010; Hill et al. 2016) using kanamycin (100 mg/L) as selection reagent. Eight independent, homozygous lines of transgenic plants were identified by screening T1 and T2 seeds germinated on kanamycin-containing medium. Based on the segregation ratios, plants with a single copy ZmNrt2.1 transgene were identified. Three independent lines of these plants were used for this study.

DNA, RNA and protein analysis

Genomic DNA was extracted from leaves of the wild type and transgenic plants using Plant DNeasy kit (Qiagen, USA). Polymerase chain reaction (PCR) was carried out to confirm the presence of ZmNrt2.1 in the transgenic plants.

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 4 of 12

Total RNA was extracted from shoots or roots of the wild type and transgenic plants in similar physiological conditions or under different treatments using Qiagen Plant RNeasy kit, including a DNase treatment to remove residual DNA. The first-strand cDNA was synthesized using M-MLV Reverse Transcriptase RNase H$^-$ kit (Solis BioDyne, Estonia) with random primers and oligo dT$_{18}$. Quantitative real-time PCR (qPCR) using SYBR Green (Life Technologies, USA) was carried out to examine the transcription of the transgene ZmNrt2.1 and several endogenous genes with gene-specific primers. Tobacco elongation factor 1α and ribosomal large subunit protein L25 (rpL25) cDNAs were used as reference for qPCR, according to Schmidt and Delaney (2010). The information for all the primers used in this study is listed in Additional file 1: Table S1.

To detect the presence of the ZmNrt protein, proteins were extracted from same leaf area of the wild type and transgenic plants according to Zhang et al. (2011) and Hill et al. (2016). Western blot analysis was done as described previously (Zhang et al. 2011, 2014), using monoclonal FLAG tag antibody (Cell Signaling, USA).

qPCR analysis of tobacco endogenous nitrate-responsive genes

To examine changes in expression of the endogenous nitrate-responsive genes in the wild type and transgenic tobacco plants, the homologous genes in tobacco were identified by analyzing the *Arabidopsis* and *Nicotiana* sequence databases. Gene specific primers (Additional file 1: Table S1) were designed for qPCR analysis of tobacco Bric-a-Brac/Tramtrack/Broad gene family member BT2 (GenBank Accessions: XM_016603770 and XM_016606879), leucine-zipper transcription factor TGA-binding protein TGA1 (KJ808740 and M62855), NAR2.1 (XM_009799779 and XM_009612602), NRT1.1 (AB102805 to AB102808) and NRT2.1 (AJ557583 and AJ557584).

Nitrate and protein determination

NO_3^- concentrations were determined from an aqueous extraction of 0.2 g frozen leaves and roots using the method described in Liu et al. (2014), spectrophotometrically measured at 410 nm using $NaNO_3$ as standard. Protein extraction and measurement was done according to Hill et al. (2016).

Nutrient treatments

Seven-day-old seedlings grown on MS medium were transferred to the medium with various NO_3^-, Ca^{2+} and K^+ concentrations, and grown for 14 days in a growth chamber at 24 °C under 8 h of darkness and 16 h of light at 150 μmol photons m^{-2} s^{-1}. Root length and biomass were measured. Samples of roots and shoots were also collected for gene expression assays.

Plant nutrient solutions were purchased from LaMotte (USA). The medium nutrient compositions are listed in Additional file 1: Table S2.

Inhibitor treatments

Twenty-one-day-old seedlings of the wild type and ZmNrt2.1-expressing plants grown on MS medium were removed from the medium, gently rinsed with water and incubated with gentle shaking for 1 h in water, 5 mM LaCl$_3$, 10 μM U73122 or 10 μM U73343, followed by incubation for 1 h in either 5 mM KNO$_3$ or 5 mM KCl, according to Riveras et al. (2015). Roots and shoots were collected, used immediately for RNA extraction or flash frozen in liquid N$_2$ and stored at − 80 °C. LaCl$_3$, U73122 and U73343 were purchased from Sigma-Aldrich (USA). LaCl$_3$ was dissolved in water. U73122 and U73343 were dissolved in a minimal amount of dimethyl sulfoxide and diluted in water to the final concentration. All the solutions were adjusted to pH 6.5.

Statistical analysis

At least three replicates for each assay were performed. The differences were detected using one way ANOVA coupled with Games-Howell (equal variance not assumed) and the Least Significant Difference (LSD) methods (equal variance assumed) with the corresponding patterns of experimental data. Statistical analysis was performed using SPSS software (ver. 16.0). The significant level was set at $P < 0.05$.

Results

Generation of transgenic plants expressing the maize high-affinity nitrate transporter ZmNrt2.1

The maize high-affinity nitrate transporter ZmNrt2.1 is composed of 524 amino acids, and shares 69% residue identity and 82% similarity with the tobacco 530-amino acid NtNrt2.1 (Additional file 1: Figure S2). Kanamycin-resistant transgenic plants generated via *Agrobacterium*-mediated transformation were initially screened by PCR, an example of which is shown in Additional file 1: Figure S3. All the transgenic plants grew and reproduced normally, with no obvious phenotypic differences as compared to the untransformed wild type plants. Homozygous lines of transgenic plants were used in this study.
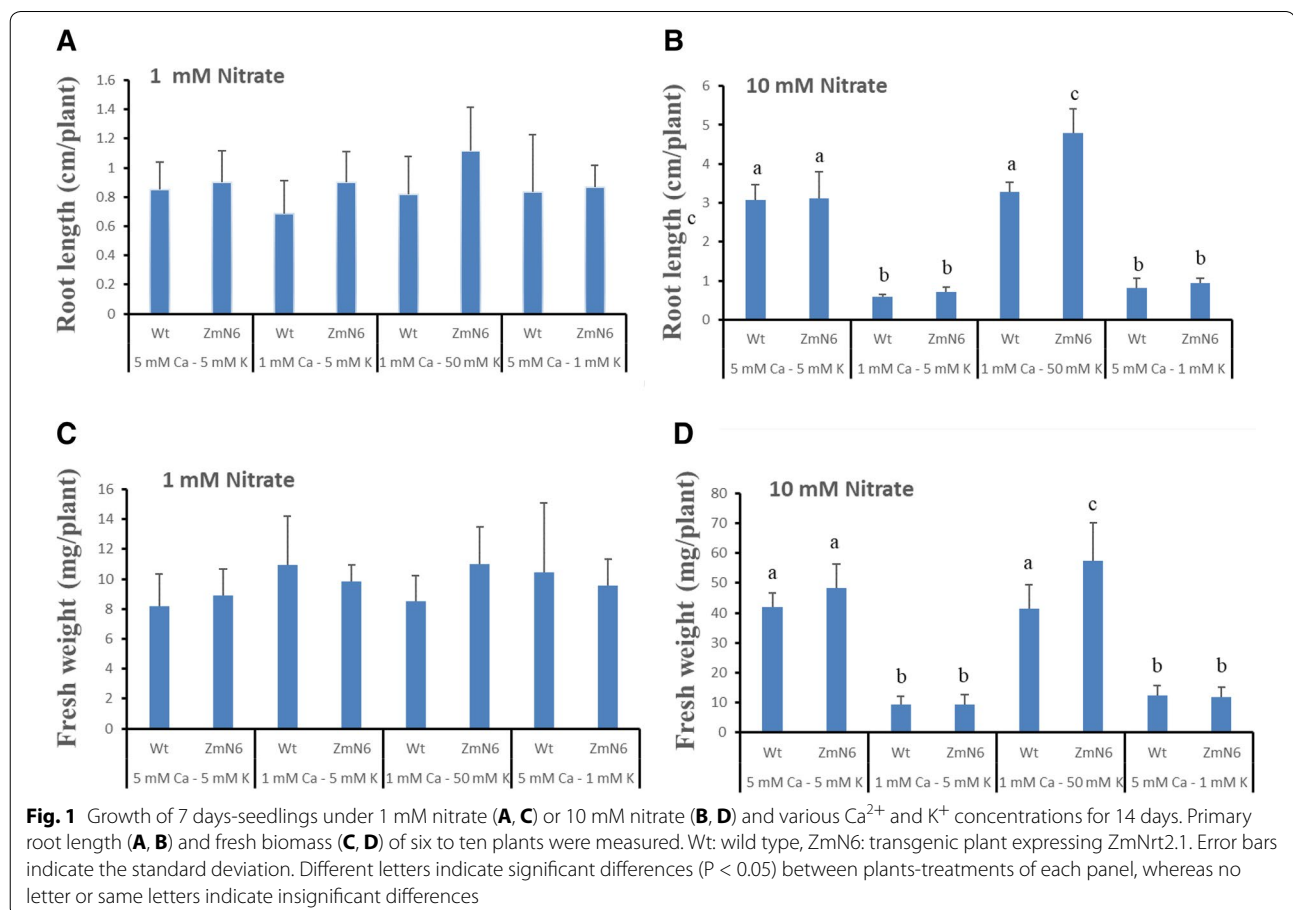
While RT-PCR analyses indicate an absence of ZmNrt2.1 transcription in the wild type plants, various levels of ZmNrt2.1 mRNA were detected in leaves and roots of the transgenic ZmN plants grown in the nutrient-rich MS medium (Additional file 1: Figures S4 and S5). As expected, the expression of the tobacco endogenous

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 5 of 12

nitrate transporter genes NtNrt2.1 (high-affinity) and NtNrt1.1 (low-affinity) was also detected in the leaf and root of both the wild type plants and transgenic plants (Additional file 1: Figures S4 and S5). Likewise, Western blot analyses detected the presence of FLAG-fused ZmNrt2.1 protein in the transgenic plants but not in the wild type plants (Additional file 1: Figure S5). These results demonstrate that as expected, both the wild type and transgenic plants expressed the endogenous genes such as NtNrt2.1 and NtNrt1.1, and the transgenic ZmN plants also expressed the maize ZmNrt2.1. Three independent ZmN lines with a single transgene, along with the untransformed wild type plants, were studied in parallel. Similar patterns of changes and responses were observed in all these ZmNrt2.1-expressing transgenic plant lines examined. Here, we present the data on the plant line ZmN6.

Growth response to various nitrate treatments

For the growth analysis, our control ("normal") medium contained NO_3^-, Ca^{2+} and K^+ at 10, 5 and 5 mM, respectively (see medium compositions in Additional file 1: Table S2). The nitrate contents of leaves or roots were largely similar between the wild type plants and the transgenic plants (Additional file 1: Figure S6). This is consistent with previous observations of similar NO_3^- contents between the wild type and CaMV 35S-NpNrt2.1 transgenic *Nicotiana plumbaginifolia* plants regardless of NO_3^- levels supplied (Fraisier et al. 2000). When grown under relatively low nitrate (1 mM) but normal Ca^{2+} and K^+ conditions, both the wild type and ZmNrt2.1-expressing plants had similar primary root growth. However, under low levels (both 1 mM) of NO_3^- and Ca^{2+}, the transgenic ZmN6 plants grew longer primary roots than the wild type (Fig. 1A, B), which is consistent with previous reports that constitutively expressing the NRT2.1 gene is associated with increased growth (Fraisier et al. 2000; Laugier et al. 2012). On the other hand, both the wild type and ZmN6 plants grew longer primary roots under 10 mM NO_3^- with normal levels of Ca^{2+} (5 mM) and K^+ (5 mM) than when either Ca^{2+} or K^+ was low. However, under 10 mM nitrate, Ca^{2+}-restricted root growth can be compensated with a high level (50 mM) of K^+ for both plants, and ZmN6 plants grew significantly longer primary roots than the wild type (Fig. 1A, B and Additional file 1: Figure S7).

Fig. 1 Growth of 7 days-seedlings under 1 mM nitrate (**A**, **C**) or 10 mM nitrate (**B**, **D**) and various Ca^{2+} and K^+ concentrations for 14 days. Primary root length (**A**, **B**) and fresh biomass (**C**, **D**) of six to ten plants were measured. Wt: wild type, ZmN6: transgenic plant expressing ZmNrt2.1. Error bars indicate the standard deviation. Different letters indicate significant differences (P < 0.05) between plants-treatments of each panel, whereas no letter or same letters indicate insignificant differences

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 6 of 12

Likewise, under 1 mM nitrate, regardless of Ca^{2+} or K^+ levels, both the wild type and ZmN6 plants had similar biomass (Fig. 1C, D). As expected, under 10 mM nitrate both plants had higher biomass when Ca^{+2} and K^+ were sufficient (Fig. 1D). Again, high (50 mM) K^+ level was able to overcome Ca^{2+}-deficient (but not low NO_3^-) caused growth restriction for both the ZmN and the wild type plants, when compared to the growth under 5 mM K^+, and the ZmN plants grew better than the wild type (Fig. 1C, D and Additional file 1: Figure S7). Overall, these observations point to a role of Ca^{2+} and K^+ in nitrate response and also hint at altered nitrate sensing in the ZmN plants involving both Ca^{2+} and K^+, indicating a consequence of ectopic expression of the maize nitrate transporter gene. This prompted us to further examine expression in roots and shoots of several endogenous genes in response to NO_3^-, Ca^{2+} and K^+.

Altered responses in expression of the endogenous, nitrate-responsive genes in roots

Under 1-h KNO_3 treatment, the calcium channel blocker $LaCl_3$ had largely no effect on the transcription of the endogenous nitrate transporter genes NtNrt2.1 and NtNrt1.1 in the roots of the ZmN transgenic plants, but $LaCl_3$ inhibited these genes in the wild type plants (Fig. 2A, C). By contrast, no inhibitory effect of $LaCl_3$ was observed for both plants when nitrate was absent (Fig. 2B, D). On the other hand, the phospholipase C (PLC) inhibitor U73122 that blocks increases in the cytosolic Ca^{2+} levels had no effect on NtNrt2.1 gene expression regardless of whether nitrate was present, similar as its nonfunctional analog U73343 (Fig. 2A, B). For NtNrt1.1, U73122 and U73343 treatments exhibited largely similar expression levels (Fig. 2C, D), suggesting a lack of inhibition. In stark contrast to the NtNrt2.1 gene, the transcription of NtNAR, a protein functionally associated with NtNrt2.1, was inhibited by $LaCl_3$ in the transgenic ZmN roots, but not the wild type roots, in the presence of either KNO_3 or KCl. U73122, on the other hand, variously inhibited NtNAR genes for both plants (Additional file 1: Figure S7). These results suggest that (1) calcium, nitrate and potassium all influenced the expression of these genes, and (2) the ZmNrt2.1-expressing plants showed altered responses of the native genes NtNrt2.1 and NtNAR to changes in the calcium channels and cytosolic Ca^{2+} levels, as compared to the wild type plants.
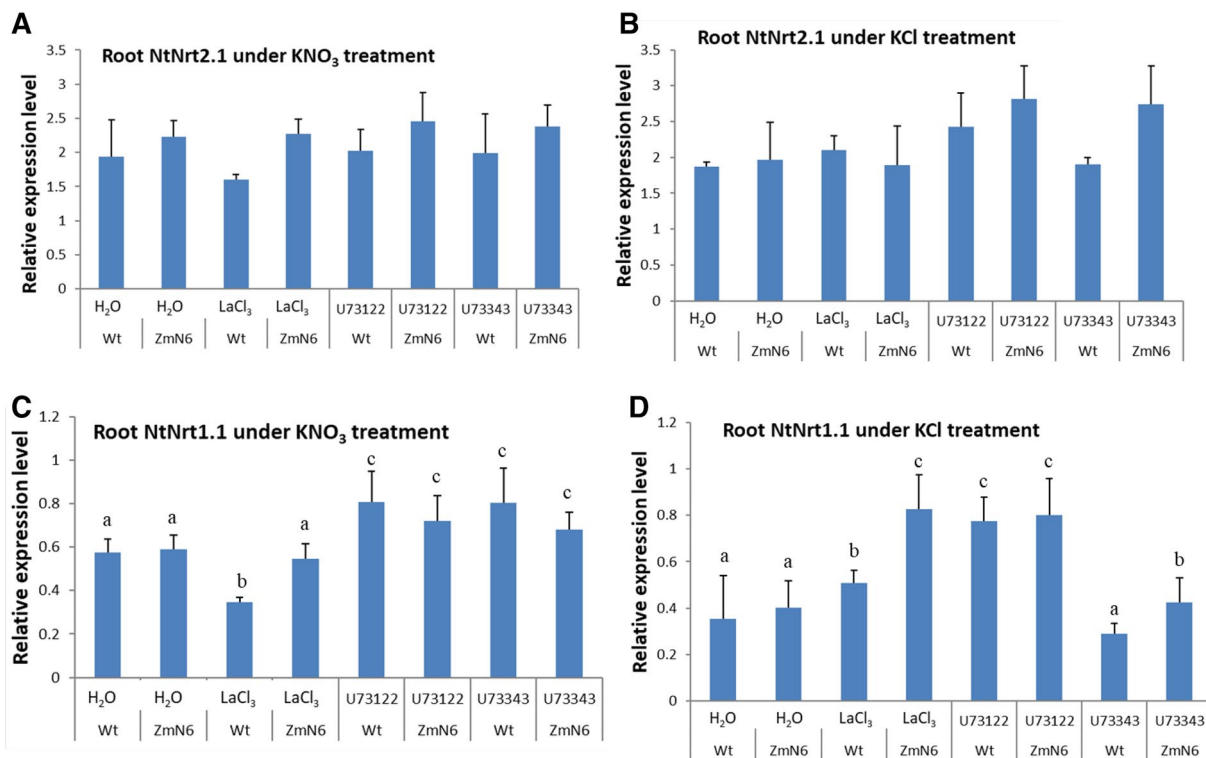

Fig. 2 Expression of root high-affinity (NtNrt2.1; **A**, **B**) and low-affinity (NtNrt1.1; **C**, **D**) nitrate transporter genes in response to calcium inhibitor and nitrate treatments. Three-week-old plants were incubated for 1 h in H_2O, 5 mM $LaCl_3$, 10 μM U73122 or 10 μM U73343, followed by 1 h in 5 mM KNO_3 or 5 mM KCl. The significant differences (P < 0.05) for multiple comparisons are shown with different letters separately for each panel, whereas no letter or same letters indicate insignificant variation

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 7 of 12

In Arabidopsis, a member of Bric-a-Brac/Tramtrack/Broad gene family, BT2, has been shown to be regulated by multiple signals including nitrogen nutrients and to suppress nitrate uptake (Mandadi et al. 2009; Araus et al. 2016). We examined the responses of the tobacco BT2 gene to calcium and nitrate. When treated with $LaCl_3$ or U73122 followed by KNO_3, the BT2 gene expression was elevated for both the wild type and ZmN plants (Fig. 3A), implicating a down-regulatory role by Ca channels and cytosolic $[Ca^{2+}]$. Under KCl without nitrate, there was also a significant increase in BT2 expression with $LaCl_3$ and U73122 treatments as compared to H_2O only treatment, but the wild type exhibited a stronger response than the ZmN plants (Fig. 3B). This indicates a potassium effect on BT2 genes when nitrate was absent and calcium signaling pathway was impaired, reflecting a complex crosstalk among NO_3^-, Ca^{2+} and K^+ in regulating genes for nitrate nutrition. Again, the ZmN transgenic plants exhibited altered responses different from the wild type plants.

Furthermore, the transcription factor TGA-binding protein, TGA1, is reported to be responsive to nitrate and may play a regulatory role in nitrate sensing (Ho and Tsay 2010; Alvarez et al. 2014). We found that the endogenous TGA1 expression in the ZmN roots was different from that in the wild type roots. Under KNO_3 treatment, TGA1 transcription in the ZmN plants was increased after incubation with $LaCl_3$ or U73122, whereas any enhancement of TGA1 expression was abolished by these inhibitors in the wild type plants (Fig. 3C). On the other hand, without NO_3^-, $LaCl_3$ and U73122 did not show inhibitory effect on TGA1 transcription in both plants (Fig. 3D). This suggests that while K^+ may not be directly involved in TGA1 gene expression, Ca^{2+} is important in mediating the nitrate-sensing TGA1 gene expression. What is revealing is that the ZmNrt2.1-expression plants under nitrate induction exhibited an altered pattern of TGA1 expression after treatment with Ca inhibitors $LaCl_3$ and U73122 (Fig. 3C), again highlighting the effect of the heterologous ZmNrt2.1 gene on the overall nitrate-related gene expression in the host plant.

Altered responses in expression of the endogenous, nitrate-responsive genes in shoots

Similarly, the shoots of the wild type and ZmN plants also exhibited some differences in gene expression as well
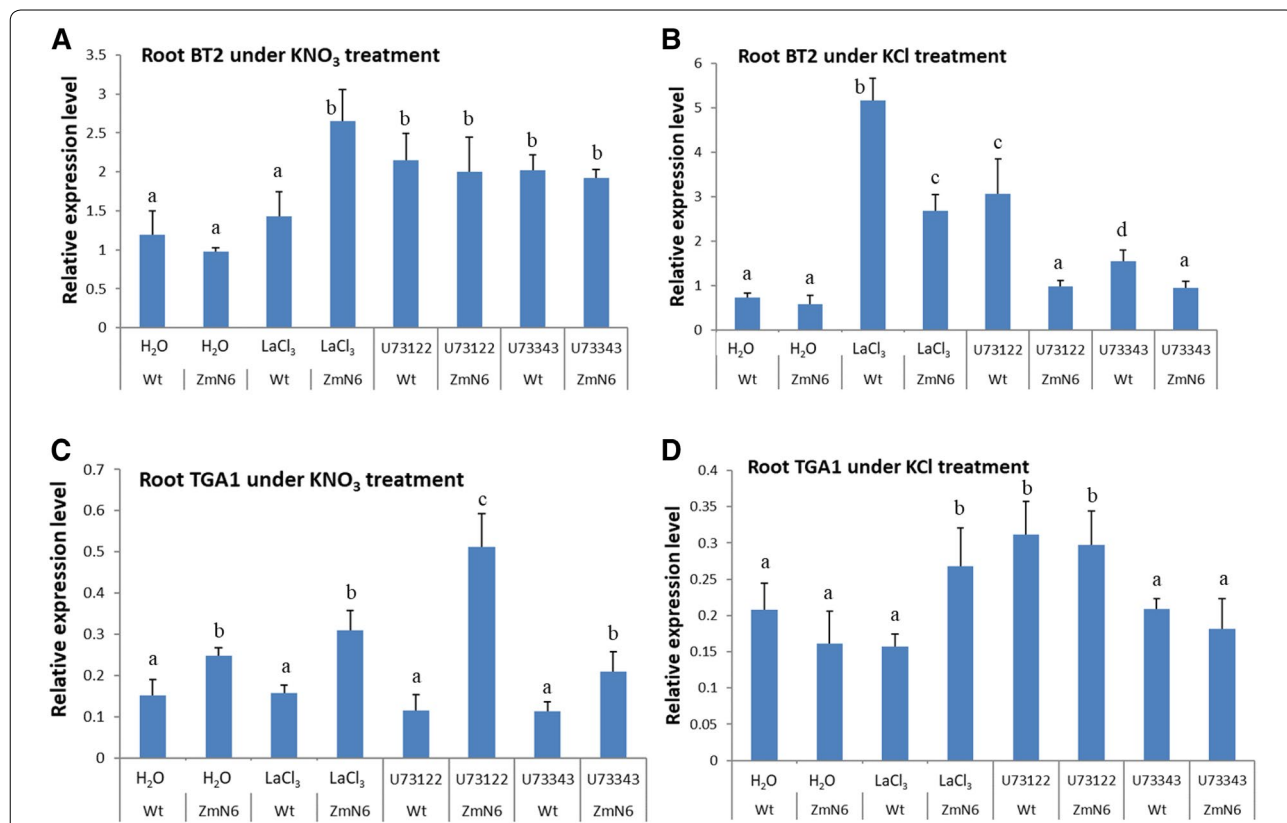

Fig. 3 Expression of root Bric-a-Brac/Tramtrack/Broad gene BT2 (**A**, **B**) and transcription factor TGA-binding protein gene TGA1 (**C**, **D**) genes in response to calcium inhibitor and nitrate treatments, as described in Fig. 2

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 8 of 12

as from the roots. In the presence of KNO_3, $LaCl_3$ did not inhibit NtNrt2.1 for both the wild type and ZmN plants (Fig. 4A), similar as in roots (Fig. 2A). U73122, however, almost completely abolished the NtNrt2.1transcription in the ZmN plants, whereas only partial inhibition was measured for the wild type (Fig. 4A). Without NO_3^-, $LaCl_3$ and U73122 almost completely inhibited NtNrt2.1 expression for both plants as compared to H_2O only treatment (Fig. 4B), which is in stark contrast to the roots where no obvious inhibition was observed (Fig. 2B). Likewise, the shoot NtNrt1.1 gene in both plants was completely inhibited by $LaCl_3$ or U73122, under either KNO_3 or KCl (Fig. 4C, D), suggesting that the calcium channel and PLC-regulated Ca^{2+} signaling play a significant role in the expression of nitrate transporter genes in shoots. These results also highlight root/shoot differences in nitrate transporter gene expression. This organ differential expression was observed for NtNAR genes as well, which in shoots exhibited almost identical patterns as NtNrt2.1 but was quite different from the roots (Fig. 4A, B; Additional file 1: Figure S8).

Unlike in roots, BT2 gene expression in shoots of the wild type plants under KNO_3 treatment was significantly inhibited by both $LaCl_3$ and U73122, whereas for the ZmN plants this inhibition was either not observed (for $LaCl_3$) or much less severe than the wild type (for U73122) (Fig. 5A). Without nitrate, on the other hand, BT2 expression was nearly completely abolished by these two inhibitors for both plants (Fig. 5B). Similarly, the TGA1 expression in shoots was significantly inhibited by both $LaCl_3$ and U73122 regardless of whether KNO_3 or KCl was present, although subtle difference can be detected between these two plants (Fig. 5C, D). All these results implicate the involvement of the Ca^{2+} signaling pathway in nitrate-related gene expression, thus nitrate sensing. Meanwhile, compared to the wild type plants, the ZmNrt2.1-expressing plants exhibited certain altered patterns of nitrate-related gene expression in response to nitrate availability and Ca^{2+} signaling inhibitors, reflecting the ectopic effect of the maize nitrate transporter on nitrate sensing in the transgenic plants.

Discussion

A main goal for this study was to investigate what would happen to the host plant's nitrate signaling if a foreign nitrate transporter gene, a member of the NO_3^- inducible high-affinity transport system (iHATS), was introduced and expressed. Our results show that the plants expressing the maize high-affinity nitrate transporter ZmNrt2.1 had an overall better primary root growth and higher biomass than the wild type when grown under high nitrate and K^+ but Ca^{2+} deficient conditions,
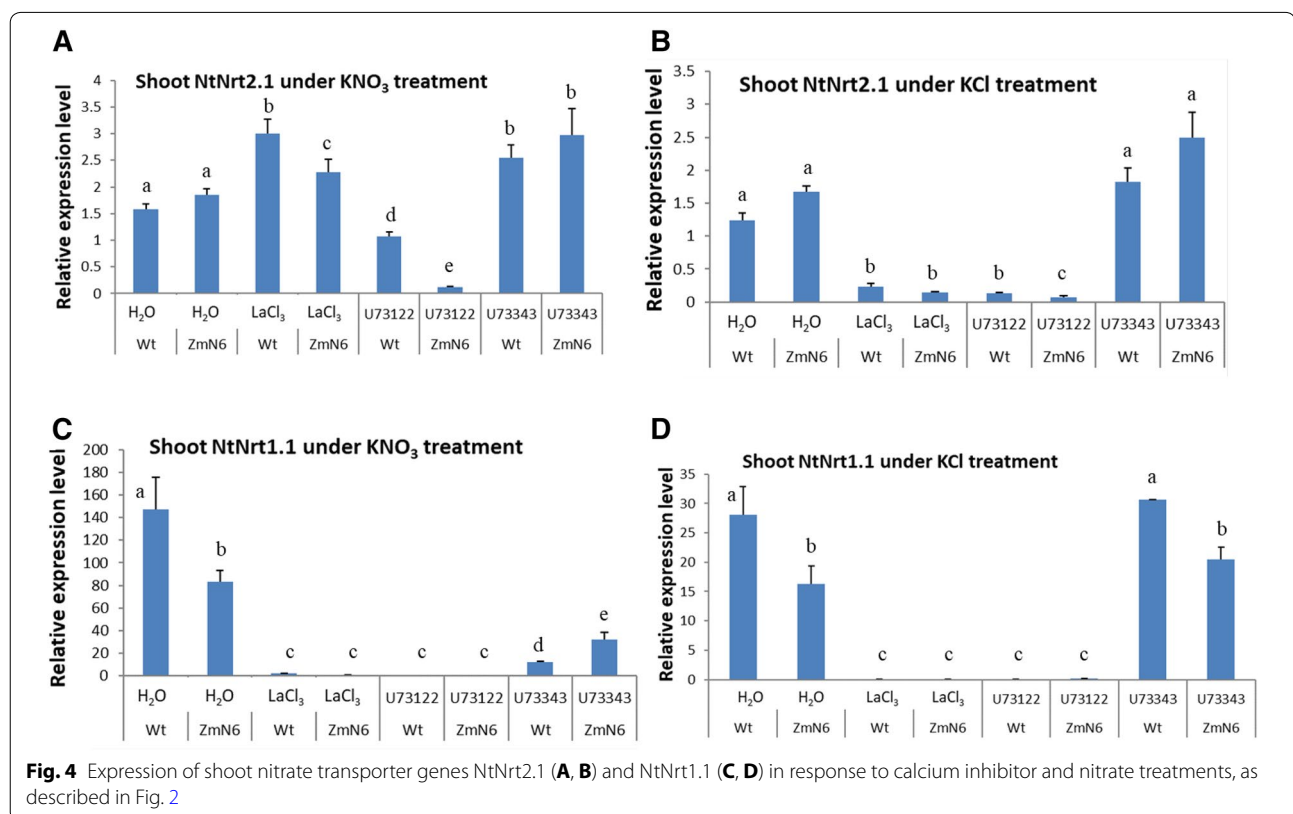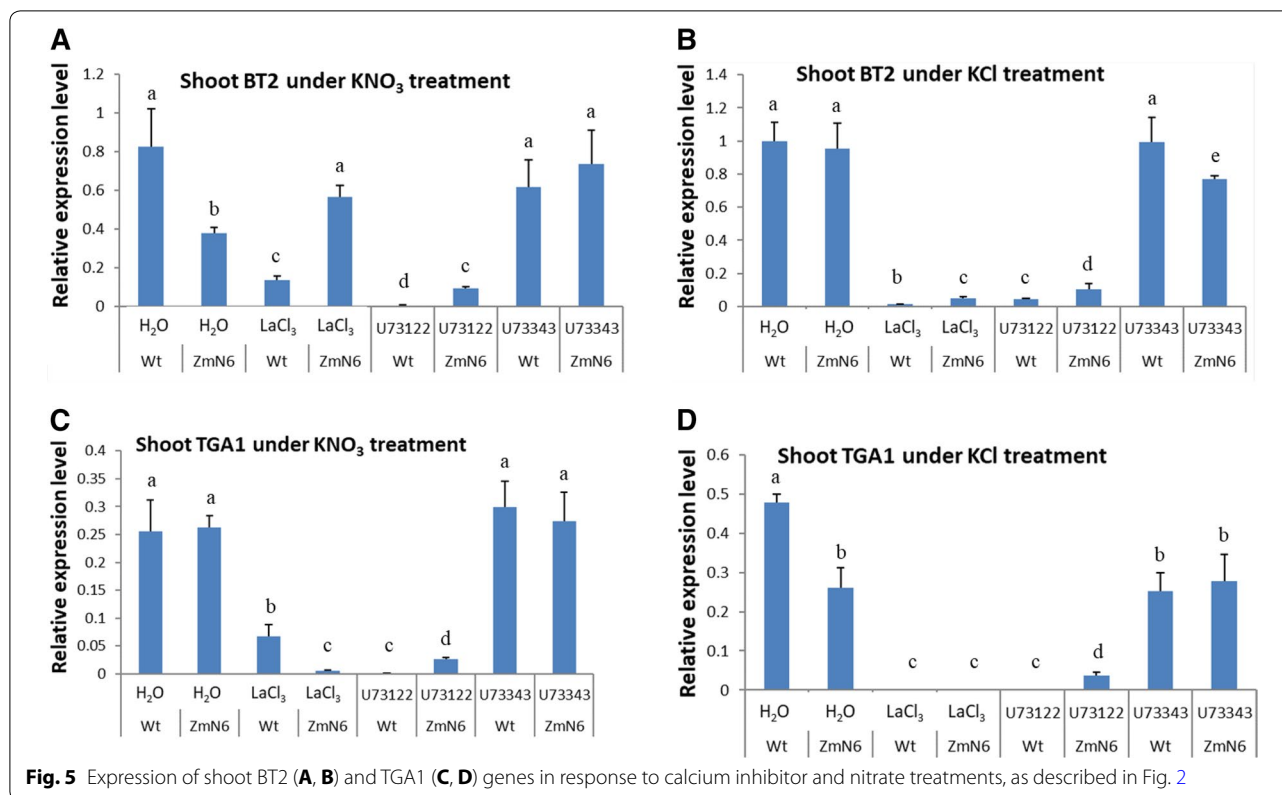

Fig. 4 Expression of shoot nitrate transporter genes NtNrt2.1 (**A**, **B**) and NtNrt1.1 (**C**, **D**) in response to calcium inhibitor and nitrate treatments, as described in Fig. 2

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 9 of 12

Fig. 5 Expression of shoot BT2 (**A**, **B**) and TGA1 (**C**, **D**) genes in response to calcium inhibitor and nitrate treatments, as described in Fig. 2

whereas there was no significant difference when nitrate was deficient (Fig. 1). One explanation may be that the additional nitrate transporter could somewhat enhance plants' capacity for nitrate uptake and assimilation, particularly under nitrogen eutrophication where plant's endogenous iHATS tends to be suppressed by high levels of nitrate. Plant growth responds to soil environmental cues such as the availability of minerals and nutrients. For example, root growth is generally enhanced by nutrient-poor conditions and inhibited by nutrient-rich conditions (Ericsson 1995). Root architecture is affected by nitrate transporters, such as the repression of lateral root initiation by NRT2.1 in Arabidopsis (Little et al. 2005). Therefore, lateral roots and primary roots may respond differently to NO_3^- ion as a signaling molecule (Zhang and Forde 2000; Little et al. 2005). We observed an enhanced primary root growth, particularly in our ZmN plants, when Ca^{2+} was low but K^+ was high (Fig. 1), suggesting that Ca^{2+} and K^+ may be just as important as nitrate for root development. On the other hand, other nitrate and nitrite transporters such as Nrt1.1 are shown to be important for K^+ translocation in Arabidopsis (Drechsler et al. 2015), reflecting an overlapping role for these genes and a complex network of $NO_3^-/Ca^{2+}/K^+$ interactions. Any phenotypic changes observed under different nitrate nutrient conditions must be the results

of interactions among these nitrogen-responsive genes, including ZmNrt2.1 in the ZmN plants.

It is somewhat puzzling that overexpression of Nrt2.1 does not seem to significantly change NO_3^- contents in plants regardless of nitrate provision, as reported by Fraisier et al. (2000) and observed by us (Additional file 1: Figure S6). It is possible that in our study expression of ZmNrt2.1 may not be functionally compatible with the native NtNAR2, therefore limiting its nitrate transportation activity. However, Fraisier et al. (2000) overexpressed a copy of native NpNrt2.1 gene in *Nicotiana plumbagini-folia* plants, therefore Nrt2.1-NAR2 compatibility is not an issue. Lack of clear cause-effect association between Nrt2.1 expression and cellular NO_3^- contents under various NO_3^- availabilities reflects the complex regulation of nitrate homeostasis and nitrogen metabolism during plant growth and development.

It should be pointed out that our study only examined the young plants (up to 3 weeks old) grown in the nutrient-defined medium. It remains to assess how our transgenic plants would compare to the wild type plants when grown under nitrate-challenging (too much or too little) conditions in soil throughout a life cycle. Nonetheless, our study demonstrates a feasibility that elevated high-affinity nitrate transporter transcription that does not respond to the typical suppression by high nitrate levels

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 10 of 12

could be used as a strategy for enhanced nitrate sequestration by plants from the nitrate-polluting environment.

It is also worth noting that in our study, and in literature (e.g. Fraisier et al. 2000; Quaggiotti et al. 2003; Okamoto et al. 2003; Wirth et al. 2007), 1 mM was used as "low" nitrate treatment, which appears to exceed the NRT2's operation limit of < 1 mM (Crawford and Glass 1998). In fact, due to plant growth, the nitrate content in the medium decreases rapidly. For example, in our experiments, the nitrate level on the 1 mM medium dropped below 0.5 mM after 1 week and was near 0.1–0.2 mM after 3 weeks (data not shown), with relatively healthy and reasonable-sized plants. Therefore, 1 mM nitrate should be an acceptable treatment for moderately low nitrate in nitrate transporter studies.

Calcium is a well-known important signal for a wide variety of physiological and biochemical processes in the cell (Dodd et al. 2010; Carafoli and Krebs 2016), as is an essential element for plant growth. However, its importance for nitrate signaling and nitrate-responsive gene regulation has not been fully understood or appreciated. Our results demonstrated a Ca^{2+} requirement for several nitrate-responsive genes, so that their transcription in shoots could be severely inhibited by inhibitors for calcium channel or Ca^{2+} level-regulating PLC, particularly when nitrate was limiting (Figs. 4 and 5). Thus, it is likely that Ca^{2+} and NO_3^-, perhaps K^+ as well, are all important part of the transcriptional regulation network for many nitrate-responsive genes. Overall, our studies are consistent with the notion that Ca^{2+} acts as a second messenger, beside NO_3^-, in the nitrate signaling pathway (Riveras et al. 2015; Undurraga et al. 2017). Regardless of the role of Ca^{2+} or K^+, nitrate availability seems to be a primary and essential signal for the cascade of gene expression for nitrogen nutrition in plants.

Using a combination of transgenic plants, inhibitors and nutrient variation, we were able to begin to dissecting, to a certain extent, the effect of a maize nitrate transporter gene on the expression of several native nitrate-responsive genes. The ZmNrt2.1 is moderately similar to the tobacco NtNrt2.1 with a similar codon usage (Additional file 1: Figure S2 and Table S3). It is not known how post-transcriptional and/or post-translational processing occurs on NRT2.1 protein in either maize or tobacco plants, as reported to take place for the Arabidopsis NRT2.1 (Wirth et al. 2007; Laugier et al. 2012). It is not clear how much, if any, ZmNrt2.1 could oligomerize with NtNAR2 to form an active nitrate transporter complex, as suggested for other plants (Tong et al. 2005; Yong et al. 2010; Pii et al. 2016). It will be important to investigate the genetic or biochemical mechanisms of how the ZmNrt2.1 interacts (or interferes) with the tobacco nitrate transporters and other native nitrate-involving genes/proteins in the

host plant grown under various NO_3^- or K^+ conditions. However, regardless of whether ZmNrt2.1 was present as a monomer or functionally complexed with an accessory partner, this study clearly showed that compared to the untransformed plants, the ZmNrt2.1-expressing plants exhibited a different response in gene transcription when nitrate was limited or the Ca^{2+} signal system was impaired. For example, the fact that ZmN plants were less sensitive to $LaCl_3$ inhibition for BT2 and TGA1 transcription than the wild type (Figs. 3 and 5) suggests that being part of the host's genetic system, the maize ZmNrt2.1 somehow altered or interfered with the endogenous gene regulatory network for nitrogen nutrition in the host plant. Further exploration of this type of ectopic effects imposed by a foreign gene may be worthwhile not only for better understanding plants' nitrogen homeostasis, but also for creating desirable plants suitable for various nitrate environments.

In summary, the transgenic tobacco plants expressing the maize nitrate transporter gene ZmNrt2.1 exhibited altered expression patterns of a set of genes that are modulated not only by nitrate, but also by calcium. Introduction of selective foreign nitrate transporter genes into crop plants, along with managing Ca^{2+} or K^+ availability in soil, may prove to be a feasible approach to improving nitrogen use efficiency and nitrate uptake capacity, and dealing with environmental eutrophication caused by fertilizer overuse.

Additional file

Additional file 1: Table S1. Sequences of primers used in this study. **Table S2.** Medium composition. **Table S3.** Comparison of codon usage. **Figure S1.** Structure of the vector p35S-ZmNrt used for tobacco transformation. **Figure S2.** Comparison of the amino acid sequences of the maize high-affinity nitrate transporter ZmNrt2.1 and the tobacco high-affinity nitrate transporter NtNrt2.1. **Figure S3.** PCR analysis of genomic DNA extracted from leaves of wild type and putative transgenic tobacco plants. **Figure S4.** RT-PCR screening for transgenic plants. **Figure S5.** Gene expression analysis. **Figure S6.** Soluble nitrate contents in plant shoots and roots. **Figure S7.** Representative photographs of plant growth with various levels of nitrate, Ca^{2+} and K^+. **Figure S8.** NtNAR gene expression analysis.

Authors' contributions
XHZ, AI and NE conceived and designed the experiments. AI, XLJ, JC, PV and XHZ performed the experiments. XLJ, YBZ and XHZ analyzed the data. XHZ wrote the manuscript. All commented on the manuscript. All authors read and approved the final manuscript.

Author details
[1] Department of Biological Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA. [2] Department of Soil Sciences, Modibbo Adama University of Technology, Yola, Nigeria. [3] Monitoring Center for Marine Resources and Environments, Guangdong Ocean University, Zhanjiang 524088, China.

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 11 of 12

Publisher's Note

References
Albrecht V, Ritz O, Linder S, Harter K, Kudla J (2001) The NAF domain defines a novel protein-protein interaction module conserved in Ca^{2+}-regulated kinases. EMBO J 20:1051–1063

Alvarez JM, Riveras E, Vidal EA, Gras DE, Contreras-López O, Tamayo KP, Aceituno F, Gómez I, Ruffel S, Lejay L, Jordana X, Gutiérrez RA (2014) Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of *Arabidopsis thaliana* roots. Plant J 80:1–13

Araus V, Vidal EA, Puelma T, Alamos S, Mieulet D, Guiderdoni E, Gutiérrez RA (2016) Members of BTB gene family of scaffold proteins suppress nitrate uptake and nitrogen use efficiency. Plant Physiol 171:1523–1532

Carafoli E, Krebs J (2016) Why calcium? How calcium became the best communicator. J Biol Chem 291:20849–20857

Chiba Y, Shimizu Y, Miyakawa S, Kanno Y, Koshiba T, Kamiya Y, Seo M (2015) Identification of *Arabidopsis thaliana* NRT1/PTR FAMILY (NPF) proteins capable of transporting plant hormones. J Plant Res 128:679–686

Crawford NM, Glass AD (1998) Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci 3:389–395

de Jong F, Thodey K, Lejay LV, Bevan MW (2014) Glucose elevates NITRATE TRANSPORTER2.1 protein levels and nitrate transport activity independently of its hexokinase1-mediated stimulation of *NITRATE TRANSPORTER2.1* expression. Plant Physiol 164:308–320

Dodd AN, Kudla J, Sanders D (2010) The language of calcium signaling. Annu Rev Plant Biol 61:593–620

Drechsler N, Zheng Y, Bohner A, Nobmann B, von Wirén N, Kunze R, Rausch C (2015) Nitrate-dependent control of shoot K homeostasis by the nitrate transporter1/peptide transporter family member NPF7.3/NRT1.5 and the Stelar K$^+$ Outward Rectifier SKOR in Arabidopsis. Plant Physiol 169:2832–2847

Ericsson T (1995) Growth and shoot:root ration of seedlings in relation to nutrient availability. Plant Soil 168–169:205–214

FAO, Food and Agriculture Organization of the United Nations (2015) World fertilizer trends and outlook to 2018. Rome. ISBN 978-92-5-108692-6

Forde BG (2000) Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta 1465:219–235

Forde BG, Clarkson DT (1999) Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. Adv Bot Res 30:1–90

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F (2000) Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J 23:489–496

Franklin-Tong VE, Drobak BK, Allan AC, Watkins P, Trewavas AJ (1996) Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. Plant Cell 8:1305–1321

Good AG, Shrawart AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9:597–605

Guerrero MG, Vega JM, Losada M (1981) The assimilatory nitrate-reducing system and its regulation. Annu Rev Plant Physiol 32:169–204

Hill W, Jin X-L, Zhang X-H (2016) Expression of an arctic chickweed dehydrin, CarDHN, enhances tolerance to abiotic stress in tobacco plants. Plant Growth Regul 80:323–334

Ho CH, Tsay YF (2010) Nitrate, ammonium, and potassium sensing and signaling. Curr Opin Plant Biol 13:604–610

Howarth RW, Marino R (2006) Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. Limnol Oceanography 51:364–376

Hu HC, Wang YY, Tsay YF (2009) AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. Plant J 57:264–278

Hunt L, Otterhag L, Lee JC, Lasheen T, Hunt J, Seki M, Shinozaki K, Sommarin MD, Gilmour DJ, Pical C, Gray JE (2004) Gene-specific expression and calcium activation of *Arabidopsis thaliana* phospholipase C isoforms. New Phytol 162:643–654

Imsande J, Touraine B (1994) N demand and the regulation of nitrate uptake. Plant Physiol 105:3–7

Katayama H, Mori M, Kawamura Y, Tanaka T, Mori M, Hasegawa H (2009) Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (OsNRT2. 1). Breed Sci 59:237–243

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince A-S, Chaillou S, Ferrario-Méry S, Meyer C, Daniel-Vedele F (2014) Nitrate transport and signalling in *Arabidopsis*. J Exp Bot 65:789–798

Kronzucker HJ, Siddiqi MY, Glass A (1996) Kinetics of NH$_4$$^+$ influx in spruce. Plant Physiol 110:773–779

Laugier E, Bouguyon E, Mauries A, Tillard P, Gojon A, Lejay L (2012) Regulation of high-affinity nitrate uptake in roots of Arabidopsis depends predominantly on posttranscriptional control of the NRT2.1/NAR2.1 transport system. Plant Physiol 158:1067–1078

Lejay L, Tillard P, Lepetit M, Olive F, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO$_3$$^−$ uptake systems by N- and C-status of *Arabidopsis* plants. Plant J 18:509–519

Little D, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci USA 102:13693–13698

Liu C, Wang Y, Pan K, Zhu T, Li W, Zhang L (2014) Carbon and nitrogen metabolism in leaves and roots of dwarf bamboo (*Fargesia denudata* Yi) subjected to drought for two consecutive years during sprouting period. J Plant Growth Regul 33:243–255

Loqué D, Tillard P, Gojon A, Lepetit M (2003) Gene expression of the NO$_3$$^−$ transporter NRT1.1 and the nitrate reductase NIA1 is repressed in Arabidopsis roots by NO$_2$$^−$, the product of NO$_3$$^−$ reduction. Plant Physiol 132:958–967

Mandadi KK, Misra A, Ren S, McKnight TD (2009) BT2, a BTB protein, mediates multiple responses to nutrients, stresses, and hormones in Arabidopsis. Plant Physiol 150:1930–1939

Medici A, Krouk G (2014) The primary nitrate response: a multifaceted signalling pathway. J Exp Bot 65:5567–5576

Meharg AA, Blatt MR (1995) NO$_3$$^−$ transport across the plasma membrane of *Arabidopsis thaliana* root hairs: kinetic control by pH and membrane voltage. J Membrane Biol 145:49–66

Muños S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A (2004) Transcript profiling in the *chl1-5* mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. Plant Cell 16:2433–2447

Okamoto M, Vidmar JJ, Glass ADM (2003) Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Physiol 44:304–317

Orsel M, Chopin F, Leleu O, Smith SJ, Krapp A, Daniel-Vedele F, Miller AJ (2006) Characterization of a two-component high-affinity nitrate uptake system in *Arabidopsis*. Physiology and protein-protein interaction. Plant Physiol 142:1304–1317

Pao SS, Paulsen IT, Saier MH (1998) Major facilitator superfamily. Microbiol Mol Biol Rev 62:1–34

Peoples MB, Freney JR, Mosier AR, Bacon PE (1995) Minimizing gaseous losses of nitrogen. In: Bacon PE (ed) Nitrogen Fertilization in the Environment. Marcel Dekker, Inc, New York, pp 565–602

Pii Y, Alessandrini M, Dall'Osto L, Guardini K, Prinsi B, Espen L, Zamboni A, Varanini Z (2016) Time-resolved investigation of molecular components

Ibrahim *et al. Bot Stud* (2017) 58:51

Page 12 of 12

involved in the induction of NO_3^- high affinity transport system in maize roots. Front Plant Sci 7:1657. https://doi.org/10.3389/fpls.2016.01657

Plett D, Toubia J, Garnett T, Tester M, Kaiser BN, Baumann U (2010) Dichotomy in the NRT gene families of dicots and grass species. PLoS ONE 5:e15289

Quaggiotti S, Ruperti B, Borsa P, Destro T, Malagoli M (2003) Expression of a putative high-affinity NO_3^- transporter and of an H^+-ATPase in relation to whole plant nitrate transport physiology in two maize genotypes differently responsive to low nitrogen availability. J Exp Bot 54:1023–1031

Quesada A, Galvan A, Fernandez E (1994) Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. Plant J 5:407–419

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A (2006) The *Arabidopsis* NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci USA 103:19206–19211

Riveras E, Alvarez JM, Vidal EA, Oses C, Vega A, Gutiérrez RA (2015) The calcium ion is a second messenger in the nitrate signaling pathway of Arabidopsis. Plant Physiol 169:1397–1404

Sakakibara H, Kobayashi K, Deji A, Sugiyama T (1997) Partial characterization of the signaling pathway for the nitrate-dependent expression of genes for nitrogen-assimilatory enzymes using detached maize leaves. Plant Cell Physiol 38:837–843

Schmidt GW, Delaney SK (2010) Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. Mol Genet Genomics 283:233–241

Streb H, Irvine RF, Berridge MJ, Schulz I (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306:67–69

Tong Y, Zhou JJ, Li Z, Miller AJ (2005) A two-component high-affinity nitrate uptake system in barley. Plant J 41:442–450

Tsai F-Y, Zhang X-H, Ulanov A, Widholm JM (2010) The application of the yeast N-acetyltransferase MPR1 gene and the proline analogue L-azetidine-2-carboxylic acid as a selectable marker system for plant transformation. J Exp Bot 61:2561–2573

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK (2007) Nitrate transporters and peptide transporters. FEBS Lett 581:2290–2300

Undurraga SF, Ibarra-Henríquez C, Fredes I, Miguel Álvarez JM, Gutiérrez RA (2017) Nitrate signaling and early responses in Arabidopsis roots. J Exp Bot 68:2541–2551

Unkles SE, Rouch D, Wang Y, Siddiqi MY, Glass ADM, Kinghorn JR (2004) Two perfectly conserved arginine residues are required for substrate binding in a high-affinity nitrate transporter. Proc Natl Acad Sci USA 101:17549–17554

Wang R, Tischner R, Gutiérrez RA, Hoffman M, Xing X, Chen M, Coruzzi G, Crawford NM (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of Arabidopsis. Plant Physiol 136:2512–2522

Wang YY, Hsu PK, Tsay YF (2012) Uptake, allocation and signaling of nitrate. Trends Plant Sci 17:458–467

White PJ (2000) Calcium channels in higher plants. Biochim Biophys Acta 1465:171–189

Wirth J, Chopin F, Santoni V, Viennois G, Tillard P, Krapp A, Gojon A (2007) Regulation of root nitrate uptake at the NRT2.1 protein level in *Arabidopsis thaliana*. J Biol Chem 282:23541–23552

Yan M, Fan X, Feng H, Miller AJ, Shen Q, Xu G (2011) Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. Plant Cell Environ 34:1360–1372

Yong Z, Kotur Z, Glass AD (2010) Characterization of an intact two-component high-affinity nitrate transporter from *Arabidopsis* roots. Plant J 63:739–748

Zhang H, Forde BG (2000) Regulation of *Arabidopsis* root development by nitrate availability. J Exp Bot 51:51–59

Zhang X-H, Takagi H, Widholm JM (2004) Expression of a novel yeast gene that detoxifies the proline analog azetidine-2-carboxylate confers resistance during tobacco seed germination, callus and shoot formation. Plant Cell Rep 22:615–622

Zhang X-H, Webb J, Huang Y-H, Lin L, Tang R-S, Liu A (2011) Hybrid Rubisco of tomato large subunits and tobacco small subunits is functional in tobacco plants. Plant Sci 180:480–488

Zhang X-H, Keating P, Wang X-W, Huang Y-H, Martin J, Hartmann JX, Liu A (2014) Production of functional native human interleukin-2 in tobacco chloroplasts. Mol Biotechnol 56:369–379

Zhou JJ, Fernández E, Galván A, Miller AJ (2000) A high affinity nitrate transport system from *Chlamydomonas* requires two gene products. FEBS Lett 466:225–227