Running head: Nitrate Regulation

Nigel M. Crawford
Section of Cell and Developmental Biology
Division of Biological Sciences
University of California at San Diego
9500 Gilman Drive
La Jolla, CA 92093-0116

Phone/FAX: 858-534-1637
e-mail: ncrawford@ucsd.edu

Research area: Systems biology, molecular biology, and gene regulation.
A Genetic Screen for Nitrate-Regulatory Mutants Captures the Nitrate Transporter Gene
\textit{NRT1.1}

Rongchen Wang, Xiujuan Xing, Yong Wang, Amy Tran and Nigel M. Crawford*

Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116 (R.W., X.X, Y.W., A.T., N.M.C.)
This work was supported by a grant from the National Science Foundation: IOB-0519985.

* Corresponding author: email ncrawford@ucsd.edu; Fax (858) 534-1637.

Abbreviations: NIA, nitrate reductase; NiR, nitrite reductase; NRG, nitrate regulation; NRT, nitrate transporter; NLP, nodule inception-like.
ABSTRACT

Nitrate regulatory mutants (nrg) of Arabidopsis were sought using a genetic screen that employed a nitrate-inducible promoter fused to the yellow fluorescent protein marker gene YFP. A mutation was identified that impaired nitrate induction, and it was localized to the nitrate regulatory gene NLP7, demonstrating the validity of this screen. A second, independent mutation (nrg1) mapped to a region containing the NRT1.1 (CHL1) nitrate transporter gene on chromosome 1. Sequence analysis of NRT1.1 in the mutant revealed a nonsense mutation that truncated the NRT1.1 protein at amino acid 301. The nrg1 mutation disrupted nitrate regulation of several endogenous genes as induction of three nitrate-responsive genes (NIA1, NiR and NRT2.1) was dramatically reduced in roots of the mutant after 2 hr treatment using nitrate concentrations from 0.25 mM to 20 mM. Another nrt1.1 mutant (deletion mutant chl1-5) showed a similar phenotype. The loss of nitrate induction in the two nrt1.1 mutants (nrg1 and chl1-5) was not explained by reduced nitrate uptake and was reversed by nitrogen deprivation. Microarray analysis showed that nitrate induction of 111 genes was reduced and of 3 genes increased 2-fold or more in the nrg1 mutant. Genes involved in nitrate assimilation, energy metabolism and pentose-phosphate pathway were most affected. These results strongly support the model that NRT1.1 acts as a nitrate regulator or sensor in Arabidopsis.
INTRODUCTION

Inorganic nitrogen is a vital nutrient for plants. Plants take up and assimilate both nitrate and ammonium with nitrate being the predominant form in most agricultural soils (Crawford and Glass, 1998). Nitrate is taken up by roots then transported into cells via transporters from the \textit{NRT1} and \textit{NRT2} family of nitrate transporters (Forde, 2000; Tsay et al., 2007). Once inside the cell, nitrate is reduced to nitrite by nitrate reductase (NIA) then to ammonium by nitrite reductase (NiR). Ammonium is then assimilated into amino acids.

In addition to serving as a nutrient, nitrate also acts as a signal. When plants are first exposed to nitrate, genes in the nitrate assimilation pathway (\textit{NRT}, \textit{NIA}, \textit{NiR}) are rapidly induced (Wang et al., 2007). Other genes, which are required for reprogramming carbon metabolism and providing chemical energy for reduction and assimilation, are also induced (Stitt, 1999; Wang et al., 2000; Stitt et al., 2002; Wang et al., 2003; Scheible et al., 2004; Wang et al., 2004; Fritz et al., 2006). Transcriptome analyses have shown that over 1500 genes are induced or repressed by nitrate within 20-180 min of treatment (Wang et al., 2003; Scheible et al., 2004; Wang et al., 2004; Gutierrez et al., 2007; Wang et al., 2007). Longer-term responses to nitrate include changes in root growth, development and architecture, in root to shoot ratios and in germination rates (Forde, 2002; Alboresi et al., 2005; Filleur et al., 2005; Walch-Liu et al., 2005; Walch-Liu et al., 2006; Forde and Walch-Liu, 2009).

The regulatory mechanisms and genes responsible for nitrogen responses in plants have been investigated using genetics (for early examples see (Leydecker et al., 2000; Zhang and Forde, 2000)) and systems analysis (Gutierrez et al., 2005; Gutierrez et al., 2007). The \textit{ANRI} MADS box transcription factor, which controls lateral root branching in response to nitrate and is induced by nitrogen deprivation, was the first to be identified (Zhang and Forde, 1998; Gan et al., 2005). A Dof transcription factor was discovered that improves nitrogen use efficiency at low nitrogen (Yanagisawa et al., 2004). More recent discoveries were the master clock control gene \textit{CCA1}, which links organic nitrogen regulation and circadian rhythms (Gutierrez et al., 2008), and microRNA167, which mediates cell-specific control of root development in response to nitrogen (Gifford et al., 2008). Most recently, a protein kinase AtCIPK8 was identified that is needed for nitrate responses at high but not low nitrate concentrations (Hu et al., 2009), and a DNA-binding protein AtNLP7 was found to function in nitrate regulation of nitrate assimilation (Castaings et al., 2009). \textit{AtNLP7} encodes the NIN-like protein 7 (NLP7). \textit{NIN} (nodule
inception) mutants were originally identified in Lotus as being defective in bacterial recognition, infection thread formation and nodule primordia initiation (Schauser et al., 1999). *NIN* genes encode nuclear-targeted DNA binding proteins with bZIP domains containing a signature RWPxRK sequence. The Arabidopsis *NLP7* gene was recently shown to encode a nuclear-targeted protein that is needed for full nitrate induction of several nitrate-responsive genes (Castaings et al., 2009). *NLP7* mutants have altered root growth (longer primary roots and more lateral roots) typical of N-starved plants and are more resistant to water stress.

The nitrate transporter gene *NRT1.1* has also been implicated in nitrogen regulation. A transcriptome analysis using serial analysis of gene expression (SAGE) showed that about 300 genes were miss regulated in *nrt1.1* mutant roots, and in particular, the *NRT2.1* high-affinity transporter gene showed reduced ammonium repression in the *nrt1.1* mutant (Munos et al., 2004). This result is consistent with the report that *NRT1.1* mediates nitrate demand regulation of high-affinity nitrate uptake (Krouk et al., 2006). *NRT1.1* also controls root colonization of nitrate-rich patches by a signaling pathway that may include *ANR1* as both genes are expressed in similar tissues (especially root tips) and *ANR1* derepression requires *NRT1.1* function (Remans et al., 2006). A signaling role for *NRT1.1* is also supported by the finding that nitrate reversal of glutamate inhibition of primary root growth requires *NRT1.1* function (Walch-Liu and Forde, 2008; Forde and Walch-Liu, 2009). However, because NRT1.1 functions as a nitrate transporter, making it difficult to distinguish between regulatory and transport functions, it is still controversial whether NRT1.1 is a nitrate sensor or not.

To identify additional nitrate regulatory genes and mechanisms, we performed a forward genetic screen using a nitrate-regulated promoter fused to a YFP marker. Putative mutants that showed reduced nitrate induction of the marker gene were isolated and examined. Two independent mutations were mapped and sequenced and found to reside in the *NRT1.1* and the *NLP7* genes. Finding the *NLP7* mutant, demonstrated that this screen could identify nitrate regulatory mutants. The *NRT1.1* mutant (*nrg1*) and the characterization detailed below provide strong support that indeed NRT1.1 is acting as a nitrate regulator.

**RESULTS**

Identification of two nitrate-nonresponding mutants.
A nitrate-inducible promoter (NRP) was fused to DNA encoding the yellow fluorescence protein (YFP) and transformed into Arabidopsis. Homozygous transgenic plants were generated and tested for nitrate-responsive YFP expression using fluorescence microscopy. Seedlings grown four days with 2.5 mM NH₄-succinate (on agarose plates with no nitrate) were treated with 20 mM KNO₃ or 20 mM KCl (both with 2.5 mM ammonium succinate) for 16 hr then examined for YFP fluorescence. The nitrate-treated seedlings had much stronger root fluorescence than the chloride-treated controls (Fig. 1A) indicating that YFP expression was induced by nitrate in these plants.

Homozygous transgenic plants were then EMS-mutagenized to produce M2 seedlings, of which approximately 35,000 were screened for low YFP fluorescence after nitrate treatment. Initially 68 seedlings with low fluorescence were identified. Retesting in the next generation recovered 6 seedlings. Two mutants Mut21 (nrg1) and Mut164 were selected for further analysis. An example of the reduced fluorescence phenotype observed in the mutants is shown for Mut21 (Fig. 1C-D).

**Identification of Mut164 as an allele of NLP7.**

The Mut164 mutation was mapped to a 55 kb fragment demarcated by the genes At4G23930 and At4g24040. All 15 genes within this region were sequenced from the mutant. This analysis revealed a mutation (C to T) in the second exon of At4g24020 (NLP7) that converted proline at position 223 to a serine. Because NLP7 has been identified as a nitrate regulatory gene (Castaings et al., 2009), identification of Mut164 in our screen demonstrated that our strategy for identifying nitrate regulatory mutants was working.

**Identification of Mut21 as an allele of NRT1.1.**

The nrg1 mutation responsible for the Mut21 phenotype was mapped to chromosome 1 in a region encompassed by BAC clones F12K11 and F20D23 (Fig. 2). This region contained the NRT1.1 (CHL1) gene. RNA transcript analysis by quantitative polymerase chain reaction (qPCR) using oligonucleotide primers to the 3’ end of the transcript revealed that there was almost no detectable NRT1.1 transcript in the nrg1 mutant (data not shown). NRT1.1 genomic DNA was amplified and sequenced from nrg1. A mutation
was found that converted codon Q301 to a stop codon (Fig. 2). Thus, \textit{nrg1} is allelic to \textit{NRT1.1}.

\textbf{Nitrate induction of gene expression is defective in \textit{nrg1}.}

Our analysis of \textit{nrg1} showed that nitrate induction of the NRP-YFP transgene was greatly diminished. To determine if regulation of endogenous genes was similarly affected, nitrate regulation of several nitrate-inducible genes (\textit{NiR}, \textit{NIA1}, \textit{NRT2.1}) was examined. A well-characterized \textit{nrt1.1} mutant (deletion mutant \textit{chl1-5}, (Tsay et al., 1993; Munos et al., 2004)) was included in these experiments to verify that the Mut21 phenotype was due to the mutation in \textit{NRT1.1}. Plants were grown for 5 days on agarose plates with 2.5 mM NH$_4$-succinate as the sole nitrogen source then treated with 20 mM KNO$_3$ or 20 mM KCl in the presence of 2.5 mM ammonium succinate for two hours. Root mRNA was prepared then analyzed by qPCR. Data in Fig. 3 show that nitrate induction of \textit{NiR}, \textit{NIA1} and \textit{NRT2.1} in both \textit{nrg1} and \textit{chl1-5} was significantly reduced (by greater than 80%) compared to WT. Note that mM ammonium was present during these treatments, which explains the low level of nitrate induction of \textit{NRT2.1}.

\textbf{Nitrate induction of gene expression is restored by N deprivation in \textit{nrg1}.}

The virtual loss of nitrate-induced gene expression by \textit{nrt1.1} mutations was a surprise. We have tested for such phenotypes in the past and found little difference between WT and \textit{nrt1.1} mutants (unpublished data). Recently, Hu et al., reported a 1.7-2.2 decrease in nitrate induction of \textit{NiR}, \textit{NIA1} and \textit{NRT2.1} in \textit{chl1-5} mutants compared with WT (Hu et al., 2009), which is much less than what we observed (see Fig. 3). Upon comparison of experimental protocols, we noticed that our previous conditions included a N starvation pretreatment to enhance the nitrate response, which was not done in our current experiments with Mut21. To determine if the Mut21 phenotype is affected by N deprivation, the previous nitrate induction experiment, in which plants were exposed continuously to N in the form of ammonium (Fig. 3), was repeated except that seedlings were first N-deprived for 24 h before nitrate treatment. The results show almost no loss of nitrate induction in mutant plants (Fig. 4) indicating that N starvation for 24 hr had restored nitrate induction in Mut21 and thus rendered the nitrate response \textit{NRT1.1}-independent.
To determine how long it takes to lose \textit{NRT1.1}-dependent induction upon N starvation, a time course experiment was performed (Supplemental Figures 1-3). Plants were grown hydroponically on 2.5 mM ammonium succinate for seven days then N-starved by transfer to the same media with no ammonium succinate. Plants were then treated with 1 mM KCl or KNO\textsubscript{3} for 30 min. Root were harvested, mRNA prepared and analyzed by qPCR. The data show that for all three genes tested (\textit{NiR}, \textit{NIA1} and \textit{NRT2.1}), nitrate induction began to recover in the mutant after 1-2 hr of N-starvation. After 24 hr, nitrate induction in the mutant was almost as high as for wildtype plants.

The effect of N starvation on \textit{NRT1.1} expression was measured to determine if the loss of the Mut21 phenotype could be accounted for by a loss of \textit{NRT1.1} mRNA. Over the first 8 hours of N starvation, the level of \textit{NRT1.1} mRNA increased about 1.6-fold (Supplemental Figure 4). However, after 24 hr, the level dropped 4-fold. These results indicate that the loss of \textit{NRT1.1}-dependent regulation during the first 8 hours of N starvation is not due to the loss of \textit{NRT1.1} expression (i.e. mRNA) and may be due to a post-transcriptional modification. At 24 hr, the drop in \textit{NRT1.1} mRNA was sufficiently large that it should contribute to the loss of the Mut21 phenotype.

The experiments described above cannot determine where it is the N deprivation in general or the loss of ammonium in particular that is responsible for the loss of the Mut21 phenotype. Including 5 mM ammonium during the 2 hr nitrate induction treatment of N-starved seedlings did not restore the Mut21 phenotype (data not shown). Further experiments are needed to resolve this issue.

\textbf{Loss of nitrate induction in \textit{nrg1} is not accounted for by impaired nitrate uptake.} Since \textit{NRT1.1} encodes a nitrate transporter, it is possible that the loss of nitrate induction in the \textit{nrt1.1} mutants is due to reduced nitrate uptake. To test this idea, nitrate induction of \textit{NiR} in WT and the two \textit{nrt1.1} mutants were assayed at various concentrations of nitrate (0.25 – 20 mM) in the presence of ammonium (Fig. 5). Nitrate induction was virtually abolished in both mutants at all nitrate concentrations tested under these conditions. Next, nitrate accumulation in whole seedlings was also measured after the same 2 hr treatments (Fig. 6) under the same conditions. Nitrate accumulation was lower in the mutants than the WT at all the concentrations of nitrate tested; however, the amount of accumulation was still substantial enough in the mutants (36-77\% of WT) to
support nitrate induction. For example, nitrate accumulation at 20 mM nitrate in the
mutants is as much or more than in WT plants treated with 0.25 mM to 5 mM nitrate, yet
nitrate induction is vanishing small in the mutants at 20 mM nitrate (Fig. 6). In fact, the
amount of nitrate entering the plants under all concentrations tested is more than
sufficient for induction, as uptake from solutions with only 2-5 μM nitrate is needed for
strong induction (Wang et al., 2007). Thus, reduction in nitrate uptake cannot explain
the loss of nitrate induction in the mutants.

Microarray analysis of nitrate-response in nrt1.1 mutants.

Several transcriptome analyses have been reported for nrt1.1 mutants. In addition to the
SAGE experiments for plants grown on ammonium nitrate (Munos et al., 2004), a
microarray analysis using ATH1 chips of nitrate-treated (30 min at 25 mM) roots found
that 42 genes had absolute transcript levels that were lower in the chl1-5 mutant by 1.7-
fold or more (or 17 genes reduced by 2-fold or more) compared with WT (Hu et al.,
2009). We performed microarray analyses in a different way: using both control and
nitrate-treated WT and nrg1 plants that had been grown without N-starvation (i.e. with
continuous ammonium supply) to determine the effect of nrg1 on nitrate induction ratios
under these conditions. Seven-day old plants grown under hydroponic conditions with
ammonium were treated with 1 mM KCl or KNO3 in the presence of ammonium for 30
min. Root mRNA was isolated and analyzed using ATH1 chips (total data set is shown
in Supplemental Table 1).

The microarray data showed that 111 genes had lower induction ratios of 2-fold
or more in nrg1 plants and only 3 genes had higher induction ratios of 2-fold or more in
nrg1 plants (Supplemental Table 2). Many known nitrate-inducible genes including NiR
(induction ratio reduced 5.1-fold in mutant), NIA1 (reduced 4.0-fold), UPM1 (reduced
3.8-fold), NIA2 (reduced 2.5-fold) and NRT2.4 (reduced 2.0-fold) showed reduced nitrate
induction ratios in the mutant. CIPK1 and CIPK3 were also on this list consistent with
the findings of Hu et al. (Hu et al., 2009). Biomaps analysis using the MIPS database
(www.virtualplant.org) revealed that genes most affected by the nrg1 mutation were over
represented in Gene Ontology groups: energy, photosynthesis, pentose-phosphate
pathway, detoxification and light absorption (Supplemental Table 3).
DISCUSSION

There has been mounting evidence that NRT1.1 functions not only as a nitrate transporter but also as a regulator. *NRT1.1* expression is atypical for a root uptake transporter, being targeted to root tips, lateral root primordia and nascent shoot organs (Guo et al., 2001) and being upregulated by acidic pH (Tsay et al., 1993) and auxin (Guo et al., 2002). *NRT1.1* function is required for high nitrate repression of *NRT2.1* and high affinity uptake in the presence of high ammonium (Krouk et al., 2006). *nrt1.1* mutants are defective in lateral root proliferation in nitrate-rich zones and have reduced expression of the lateral root regulatory gene *ANR1* (Remans et al., 2006). *nrt1.1* mutants are also defective in the nitrate reversal of glutamate inhibition of primary root growth (Walch-Liu and Forde, 2008). It was concluded from these studies that NRT1.1 may be playing a signaling role as a nitrate sensor. Our findings that a *nrt1.1* mutant can be captured in a genetic screen for nitrate regulatory mutants and that *nrt1.1* mutants are impaired in nitrate regulation of gene expression over a wide variation of nitrate concentrations are certainly consistent with and support this proposal.

Because *NRT1.1* functions as a nitrate transporter, the signaling defects described above could be explained by reduction of nitrate uptake into cells in which the nitrate sensor resides. In the reports described above, inhibition of nitrate uptake was found not to explain the *nrt1.1* mutant phenotypes; however, in the earlier reports where bulk uptake into roots was measured, it was difficult to rule out the possibility that reduced nitrate uptake into select sensing cells in root tips could account for the effects. Results from Walch-Liu and Forde (Walch-Liu and Forde, 2008) provide additional insights because they found that a nonphosphorylatable form of NRT1.1, which retains low affinity but not high affinity uptake activity (Liu and Tsay, 2003), was not capable of nitrate reversal of glutamate inhibition of root growth (Walch-Liu and Forde, 2008). In our experiments, we measured gene expression in whole roots, which is not restricted to a small number of select cells in the root, so that measurements of nitrate uptake into seedlings should be more indicative of nitrate availability for induction. In our system, nitrate uptake in the mutants was more than sufficient to induce a nitrate response, yet induction was clearly impaired.
The most consistent model to explain all the published results and our findings is that NRT1.1 is sensing nitrate directly and thus serves as a nitrate transceptor. Transceptors, which are transporters that also act as sensors, have been described in yeast (Holsbeeks et al., 2004). NRT1.1 can transport nitrate yet it appears to play a regulatory role as well. If NRT1.1 is in fact a transceptor, it should be possible to isolate mutants that separate the transport from sensing functions. The T101A mutation that is defective in a sensing function (nitrate reversal of glutamate inhibition) (Walch-Liu and Forde, 2008) but retains partial transport activity (Liu and Tsay, 2003) provides support for this idea. However, there are many questions still unanswered by this model. Is the regulation of NRT1.1 by nitrate, acidic pH and auxin, important for controlling nitrate uptake or regulation? How does the switch between high and low affinity states by phosphorylation of T101 modulate nitrate regulation? Also, there are several phenotypes for nrt1.1 mutants that are difficult to explain simply by loss of nitrate sensing. nrt1.1 mutants are defective in root growth in young seedlings even in the absence of nitrate in the medium (Guo et al., 2001). The altered regulation of NRT2.1 in the nrt1.1 mutant results from reduced ammonium repression (Munos et al., 2004). Lastly, NRT1.1-dependent regulation is lost during N deprivation even though nitrate induction of endogenous genes still occurs (Fig. 4). Thus, other nitrate sensing systems must be present. These questions require further analysis before we can achieve a full understanding of nitrate sensing and the role of NRT1.1.

MATERIALS AND METHODS

Plant materials and growth conditions
Mutagenesis: Homozygous backcrossed transgenic seeds containing the NRP-YFP construct (1.2 g in 20 ml of water) were treated with EMS (methanesulfonic acid ethyl ester) at 15 mM for 16 hr with agitation (30 rpm). M2 seeds were produced and pooled into families for screening. NRP contained promoter fragments from the NIA1 and NiR promoters fused to the 35S minimal promoter (see Genbank Accession # GQ374175).

Mutant screen: M2 seedlings were screened on vertical 100 x 100 mm square plates containing 25 ml of 0.6% agarose media. Surface sterilized seeds were aligned horizontally on the plate surface at a density of about 100 seeds per row. Three rows of seeds were placed on each plate. The initial medium (described in (Wang et al., 2004))
was nitrate-free with 2.5 mM ammonium succinate as the nitrogen source. After incubation at 4°C for two days, seedlings were grown at 25°C with 24 hr light. Four-day old seedlings were then flooded with 12.5 ml of medium containing 20 mM KNO₃ and 2.5 mM ammonium succinate for 16 hr. Seedlings were screened under a fluorescence microscope (Nikon Eclipse TE2000-U) and rescued. Putative mutants were selfed then rescreened. Confirmed mutants were backcrossed to the transgenic WT and made homozygous before analysis.

Growth and treatment conditions: For qPCR analyses and nitrate accumulation assays, seedlings were grown on vertical agarose plates as described above for 5 days with 2.5 mM ammonium succinate as the sole nitrogen source in plant growth medium (Wang et al., 2004). The seedlings were then flooded with 12.5 ml of plant growth medium (with 2.5 mM ammonium succinate) plus KNO₃ at various concentrations for 2 hr with agitation (60 rpm) under light. Roots were then collected for total RNA preparation (as described (Wang et al., 2003)). Control samples were prepared at the same time with the same concentration of KCl in place of KNO₃.

For nitrate treatments without ammonia, seedlings were grown on plant growth medium with 2.5 mM ammonium succinate for 4 days then transferred to fresh agarose plates without nitrogen for 24 hr followed by flooding with nitrate containing plant growth media as described above except that there was no ammonium succinate in the liquid medium.

For the microarray analysis, plants were grown in aseptic hydroponics as described (Wang et al., 2007) for 7 days with modifications as follows: Seedlings were transferred to 100 ml of fresh medium with 2.5 mM ammonium succinate after 6 days of growth and continued incubation for 24 hr. Nitrate and control chloride treatments were initiated by adding KNO₃ or KCl to the growth media to yield 1 mM concentration then incubated for 30 min before harvesting roots.

**Gene expression and nitrate analysis**

qPCR analysis: RNA samples were prepared from roots as described (Wang et al., 2007). Real-time quantitative PCR was performed as described (Wang et al., 2004). Relative
expression levels of $NRT1.1$ were compared with the internal reference gene, UBQ-associated protein gene (At5g12120).

Microarray analysis: Roots were collected for total RNA preparation as described (Wang et al., 2007). Experiments were done in duplicate then averaged to generate induction ratios using the Affymetrix software as described (Wang et al., 2003). In all cases, data were filtered to require that signal levels were detectable in both replicates for at least one of the treatments (indicated as “P” by the Affymetrix software) and had an absolute value of 100 or more.

Nitrate accumulation: Nitrate in seedlings of WT and mutants were measured using the hydrazine-sulfate method as described (Wang et al., 2004).

Positional cloning of $nrg1$ was performed on individual F2 recombinants using simple sequence length polymorphisms as described (Lukowitz et al., 2000).

ACKNOWLEDGMENTS

This work was funded by a grant from the National Science Foundation IOB-0519985. We thank Dr. Mamoru Okamoto and Kati Wu for valuable assistance and advice.
LITERATURE CITED

Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environ 28: 500-512

Castaings L, Camargo A, Pocholle D, Gaudon V, Texier Y, Boutet-Mercey S, Taconnat L, Renou JP, Daniel-Vedele F, Fernandez E, Meyer C, Krapp A (2009) The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. Plant J 57: 426-435

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

Filleur S, Walch-Liu P, Gan Y, Forde BG (2005) Nitrate and glutamate sensing by plant roots. Biochem Soc Trans 33: 283-286

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

Forde BG (2002) Local and long-range signaling pathways regulating plant responses to nitrate. Annu. Rev. Plant Biol. 53: 203-224

Forde BG, Walch-Liu P (2009) Nitrate and glutamate as environmental cues for behavioural responses in plant roots. Plant Cell Environ 32: 682-693

Fritz C, Palacios-Rojas N, Feil R, Stitt M (2006) Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. Plant J 46: 533-548

Gan Y, Filleur S, Rahman A, Gotensparre S, Forde BG (2005) Nutritional regulation of ANR1 and other root-expressed MADS-box genes in Arabidopsis thaliana. Planta 222: 730-742

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci U S A 105: 803-808

Guo FQ, Wang R, Chen M, Crawford NM (2001) The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is activated and functions in nascent organ development during vegetative and reproductive growth. Plant Cell 13: 1761-1777
Guo FQ, Wang R, Crawford NM (2002) The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is regulated by auxin in both shoots and roots. J Exp Bot 53: 835-844

Gutierrez RA, Gifford ML, Poultnery C, Wang R, Shasha DE, Coruzzi GM, Crawford NM (2007) Insights into the genomic nitrate response using genetics and the Sungear Software System. J Exp Bot 58: 2359-2367

Gutierrez RA, Lejay LV, Dean A, Chiaromonte F, Shasha DE, Coruzzi GM (2007) Qualitative network models and genome-wide expression data define carbon/nitrogen-responsive molecular machines in Arabidopsis. Genome Biol 8: R7

Gutierrez RA, Shasha DE, Coruzzi GM (2005) Systems biology for the virtual plant. Plant Physiol 138: 550-554

Gutierrez RA, Stokes TL, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero DC, McClung CR, Coruzzi GM (2008) Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. Proc Natl Acad Sci U S A 105: 4939-4944

Holsbeeks I, Lagatie O, Van Nuland A, Van de Velde S, Thevelein JM (2004) The eukaryotic plasma membrane as a nutrient-sensing device. Trends Biochem Sci 29: 556-564

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

Krouk G, Tillard P, Gojon A (2006) Regulation of the high-affinity NO3- uptake system by NRT1.1-mediated NO3- demand signaling in Arabidopsis. Plant Physiol 142: 1075-1086

Leydecker MT, Camus I, Daniel-Vedele F, Truong HN (2000) Screening for Arabidopsis mutants affected in the Nii gene expression using the Gus reporter gene. Physiol. Plant. 108: 161-170

Liu KH, Tsay YF (2003) Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J. 22: 1005-1013
Lukowitz W, Gillmor CS, Scheible WR (2000) Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. Plant Physiol 123: 795-805

Munos 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

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 U S A 103: 19206-19211

Schauer L, Roussis A, Stiller J, Stougaard J (1999) A plant regulator controlling development of symbiotic root nodules. Nature 402: 191-195

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol 136: 2483-2499

Stitt M (1999) Nitrate regulation of metabolism and growth. Curr. Opin. Plant Biol. 2: 178-186

Stitt M, Muller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot 53: 959-970

Tsay Y-F, Schroeder JI, Feldmann KA, Crawford NM (1993) A herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72: 705-713

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

Walch-Liu P, Filleur S, Gan Y, Forde BG (2005) Signaling mechanisms integrating root and shoot responses to changes in the nitrogen supply. Photosynth Res 83: 239-250
Walch-Liu P, Forde BG (2008) Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. Plant J 54: 820-828

Walch-Liu P, Ivanov, II, Filleur S, Gan Y, Remans T, Forde BG (2006) Nitrogen regulation of root branching. Ann Bot (Lond) 97: 875-881

Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. Plant Cell 12: 1491-1509

Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132: 556-567

Wang R, Tischner R, Gutierrez 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 R, Xing X, Crawford N (2007) Nitrite acts as a transcriptome signal at micromolar concentrations in Arabidopsis roots. Plant Physiol 145: 1735-1745

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Natl Acad Sci U S A 101: 7833-7838

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

Zhang HM, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279: 407-409
FIGURE LEGENDS

**Figure 1.** Nitrate induction of NRP-YFP in wildtype and Mut21 roots.

Transgenic seedlings (containing the NRP-YFP construct) grown with ammonium but no nitrate for four days were treated with either 20 mM KNO₃ or 20 mM KCl in the presence of 2.5 mM ammonium succinate for 16 hours. Fluorescent (Panels A & C) and visible light (panels B & D) images were captured with a fluorescent microscope to visualize YFP expression.

**Figure 2.** Mapping of nrg1 (Mut21).

Shows schematic diagrams of the Arabidopsis chromosome 1 showing where nrg1 mapped. Exons are shown in large black boxes. Amino acid and nucleotide changes found in Mut21 are also shown.

**Figure 3.** Nitrate induction of endogenous genes.

Wildtype and two nrt1.1 mutant seedlings (nrg1 and chl1-5) were grown on 2.5 mM ammonium succinate for 5 days on agarose plates then treated with either 20 mM KNO₃ or 20 mM KCl in the presence of 2.5 mM ammonium succinate for two hours. Root mRNA levels were determined by qPCR. Error bars represent standard deviation of biological replicates (n=3).

**Figure 4.** Nitrate induction of endogenous genes after 24 hr N-deprivation.

Plants were grown and treated as described in legend to Fig. 3 except at day 4, plants were transferred to N-free medium for 24 hrs then treated with 20 mM nitrate or chloride for 2 hr with no added ammonium succinate. Root mRNA levels were determined by qPCR. Error bars represent standard deviation (n=3).

**Figure 5.** Titration of the nitrate induction response.

Seedlings were grown 5 days on agarose plates with 2.5 mM ammonium succinate (same as Fig. 3) then treated with various concentrations of KNO₃ or KCl for 2 hours in the presence of 2.5 mM ammonium succinate before roots were collected for RNA
preparation. NiR mRNA levels were determined by qPCR. Error bars represent standard deviation (n=3).

**Figure 6.** Nitrate accumulation.

Seedlings grown 5 days with 2.5 mM ammonium succinate were treated with various concentrations of KNO$_3$ (same as for Fig. 5) in the presence of 2.5 mM ammonium succinate for 2 h. Whole seedlings were then collected for nitrate assays as described in Materials and Methods. Error bars represent standard deviation (n=3).
Figure 1. Nitrate induction of NRP-YFP in wildtype and Mut21 roots.

Seedlings (containing the NRP-YFP construct) grown with ammonium but no nitrate for four days were treated with either 20 mM KNO$_3$ or 20 mM KCl in the presence of 2.5 mM ammonium succinate for 16 hours. Fluorescent (Panels A & C) and visible light (panels B & D) images were captured with a fluorescent microscope to visualize YFP expression.
Figure 2. Mapping of nrg1 (Mut21).
Shows schematic diagrams of the Arabidopsis chromosome 1 showing where nrg1 mapped. Exons are shown in large black boxes. Amino acid and nucleotide changes found in Mut21 are also shown.
Figure 3. Nitrate induction of endogenous genes.

Wildtype and two nrt1.1 mutant seedlings (nrg1 and chl1-5) were grown on 2.5 mM ammonium succinate for 5 days on agarose plates then treated with either 20 mM KNO3 or 20 mM KCl in the presence of 2.5 mM ammonium succinate for two hours. Root mRNA levels were determined by qPCR. Error bars represent standard deviation (n=3).
**Figure 4.** Nitrate induction of endogenous genes after 24 hr N-deprivation.

Plants were grown and treated as described in legend to Fig. 3 except at day 4, plants were transferred to N-free medium for 24 hrs then treated with 20 mM nitrate or chloride for 2 hr with no added ammonium succinate. Root mRNA levels were determined by qPCR. Error bars represent standard deviation (n=3).
Figure 5. Titration of the nitrate induction response.

Seedlings were grown 5 days on agarose plates with 2.5 mM ammonium succinate then treated with various concentrations of KNO₃ or KCl for 2 hours in the presence of 2.5 mM ammonium succinate before roots were collected for RNA preparation. NiR mRNA levels were determined by qPCR. Error bars represent standard deviation (n=3).
Figure 6. Nitrate accumulation.

Seedlings grown 5 days with 2.5 mM ammonium succinate were treated with various concentrations of KNO₃ (same as for Fig. 5) in the presence of 2.5 mM ammonium succinate for 2 h. Whole seedlings were then collected for nitrate assays as described in Materials and Methods. Error bars represent standard deviation (n=3).