Study of nitrate regulatory elements and their response to nitrite in Arabidopsis thaliana

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Study of nitrate regulatory elements and their response to nitrite in Arabidopsis thaliana

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Abraham H. Tang

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Nitrogen is a critical nutrient that has important functions in plants. It supports DNA and protein synthesis and serves as an important signal that regulates biochemical pathways and gene expression. Nitrate is the form most often used by plants and is often provided as a fertilizer; however, excess fertilizer application has lead to significant damage to the environment. The elucidation of nitrate metabolism and regulation has thus been an important endeavor that could help to reduce fertilizer use and improve the health of the environment. During the second step of nitrate assimilation, nitrate is converted to nitrite. While nitrate is a nutrient to plants, nitrite is toxic to plants upon accumulation. Yet, it has been previously shown that many nitrate-inducible genes also respond to nitrite. Recently, the Crawford lab identified three specific nitrate enhancer elements that are present within the NIA1 nitrate reductase promoter. In this work, these nitrate enhancer elements were found to mediate nitrite induction. A screen for nitrate enhancer
elements within the NiR promoter was also performed. The sequence between Taq1 to RsaI in the NiR promoter is important for nitrate induction, and the sequence just downstream of the start of translation (to the Xho site) is important for high constitutive expression. The work presented here shows that nitrite responses in plants can be mediated by the same DNA enhancer elements that serve as nitrate elements, and may provide new tools for the search for new nitrate enhancer elements.
INTRODUCTION

Arabidopsis thaliana, a model organism in plant science

Arabidopsis thaliana is considered one of the best-studied model organisms for a variety of plant sciences, and it has even been referred to as "the botanical Drosophila" (Leonelli, 2007). Arabidopsis thaliana has several properties that allow it to serve as a model organism. These properties include its small genome and physical size, fast life cycle, and ease of transformation using Agrobacterium tumefaciens. The genome of Arabidopsis thaliana contains five chromosomes, composed of approximately 157 million base pairs (Bennett et al., 2003). In the year 2000, the small genome of Arabidopsis thaliana was sequenced and published, making Arabidopsis thaliana the first plant to ever be completely sequenced (Schoof et al., 2002).

Nitrate as a nutrient and as a regulatory signal

Nitrogen is an element that is needed by all plants to make amino acids, proteins, and DNA. The majority of nitrogen absorbed by plants comes in the form of inorganic nitrate in soils, although legume plants are also able to utilize the nitrogen in the atmosphere through nitrogen fixation (Crawford et al., 1998). As a result, nitrate is an important nutrient for the plant to grow and reproduce. Nitrate exists as a natural material in soil, yet the amount of nitrate present in the soil is not enough to sustain the large multitude of crops grown in an effort to feed the world’s populations. Chemical fertilizers are used to help address this issue and supply nitrogen in the form of nitrate or
ammonium, which is rapidly converted to nitrate by nitrifying bacteria in the soil (Payne, 1976).

While nitrate plays a key role as a nutrient in plants, it also serves as a signal to regulate plant metabolism. For example, there are over one thousand genes in Arabidopsis that are either induced or repressed by nitrate. Numerous nitrate-responsive genes include nitrate transporters (NRT), nitrate reductase (NIA), nitrite reductase (NiR), genes affecting iron metabolism, and genes affecting sulfate uptake (Wang et al., 2003). Nitrate also serves as a signal in regulating plant growth and development. Studies of mutant plants with gene disruptions in certain nitrate transporters showed that the local availability of nitrate promotes lateral root development (Bao et al., 2007). Nitrate signaling also has been shown to break seed dormancy in Arabidopsis plants (Alboresi et al., 2005).

**Nitrate uptake**

Nitrate first reaches the roots via bulk flow, where the molecule is then actively transported into the symplasm. The initial uptake of nitrate into root cells needs energy in the form of proton gradients. As such, nitrate uptake is thought to be mediated by proton symporters (Crawford et al., 1998). During nitrate uptake, the plasma membrane depolarizes, meaning an increasing positive charge within the cell. This is due to the symporter having a stoichiometric ratio of 2:1 H⁺:NO₃⁻ as a 1:1 ratio would not result in depolarization (Miller et al., 1996).
There exists two nitrate transporters gene families identified in *Arabidopsis* that are involved in nitrate uptake: AtNRT1 and AtNRT2, which include the genes *AtNRT1.1, AtNRT1.2, AtNRT2.1*, and *AtNRT2.2*. AtNRT1.1 is also referred to as *CHL1*. Due to the necessity of plants to adapt to various concentrations of nitrate in the soil, plants have developed both a low-affinity uptake system (for when the external concentration is above 1 mM), and a high-affinity uptake system (for when the external concentration is 1 μM–1 mM). AtNRT1.2 is involved in low-affinity uptake while AtNRT2.1 and AtNRT2.2 are both involved in high-affinity uptake (Huang et al., 1999; Li et al., 2006; Little et al., 2005). Of the transporters mentioned above, CHL1 functions uniquely as both a low-affinity transporter as well as a high-affinity transporter depending on the phosphorylation of CHL1 at T101 which is triggered by external nitrate concentrations (Liu et al., 2003).

**Nitrate assimilation**

In plants, nitrate is first reduced to nitrite, subsequently nitrite is further reduced to ammonia. The rate-limiting step is the conversion of nitrate to nitrite, which is catalyzed by the enzyme nitrate reductase (NR), while the conversion of nitrite to ammonia is done via nitrite reductase (NiR) (Solomonson et al., 1977). Nitrate reductase consists of 3 functional domains, including a MoCo-binding domain, a heme-binding domain, and a FAD-binding domain. The MoCo-binding domain is located on the N-terminus, the heme-binding is located in the middle of the structure, and the FAD-binding domain is located at the C terminus (Crawford et al., 1993).
There are two NIA genes that have been identified in Arabidopsis: NIA1 and NIA2. Both of these genes are expressed in shoots and roots, with NIA2 encoding 90% of the NR activity in Arabidopsis shoots. Interestingly, the level of NR activity in shoots is tenfold higher than the basal level needed for normal vegetative growth (Crawford et al., 1993). Mutations in the NIA1 gene merely reduce NR activity levels by 10-15% when compared to wild-type genetic backgrounds, thus it would be difficult to identify single nia1 mutants. However, such mutants can be found by screening for nia1nia2 double mutants that are more chlorate resistant than single nia2 mutants. One such double mutant displayed merely 0.5% of the wild-type level of NR activity. The mutation in the NIA1 gene converted an alanine to a threonine in the MoCo domain, and the alanine has been found to be conserved in all eukaryotic NR sequences (Wilkinson et al., 1991).

The nitrate reductase genes can be regulated by nitrate, light, circadian rhythms, phytochrome, plastidic factors, and CO₂ levels. Nitrate is the primary signal that induces nitrate reductase mRNA levels. Even in the presence of cycloheximide to inhibit protein synthesis, mere minutes of nitrate treatment induces an increase in nitrate reductase mRNA levels. In tobacco, the removal of nitrate from growth solution still results in elevated nitrate reductase mRNA levels for more than a week, despite nitrate reductase activity and protein amounts dropping abruptly during the first day that nitrate is removed (Galangau et al., 1988). This finding suggests that in the absence of nitrate, posttranscriptional regulation is involved in reducing nitrate reductase levels. In Arabidopsis, nitrate reductase can be regulated posttranslationally via phosphorylation of serine residues during removal of light or decrease in CO₂ levels. Specifically, Ser-534 is
an essential site conserved among plant nitrate reductases that is essential for post-translational regulation (Su et al., 1996).

**Nitrite as a regulatory signal**

Nitrite reductase is regulated similarly to nitrate reductase as both genes are regulated coordinately (Crawford et al., 1993). Nitrite is considered to be toxic to plants, as harmful effects can be observed upon accumulation of nitrite. For example, NiR-deficient plants may become chlorotic or even experience rapid death due to the accumulation of high amounts of nitrite (Vaucheret et al., 1992). Recently it has been shown that nitrite treatments increase mRNA levels in more than half of the nitrate-induced genes (Wang et al., 2007), which is surprising given nitrite’s toxicity. Response to nitrite can be very rapid and robust, as much as if not more so than to nitrate. The mRNA levels of nitrate-inducible genes such as NiR can be induced within 0.5 to 1 hr after applying nitrite to *Arabidopsis thaliana* roots, with concentrations as low as 100 nM (Wang et al., 2007).

**Search for nitrate enhancers and analyses of responses to nitrite**

An important component of nitrate signaling is the cis-acting nitrate enhancer sequences present in nitrate-responsive gene. Several labs have reported short (~200 bp) promoters that are sufficient to confer nitrate regulation to marker genes; however, until recently, no sequence was identified that displayed nitrate enhancer function (ability to confer nitrate regulation to a minimal core promoter). In 2008, a 150 bp sequence from the NRT1.1 promoter was reported that showed nitrate enhancer function as assayed by
long term nitrate treatments (Girin et al., 2007); however, no elements within this enhancer fragment were reported that were responsible for the enhancer activity. The Crawford lab has identified a second 180 bp nitrate enhancer fragment from the NIA1 promoter (Wang et al., 2009). Together with a 130 bp fragment from the NiR promoter fused to a 35S minimal promoter, this fragment showed strong nitrate responses (>15X) over 20-30 min of nitrate treatment. The Crawford lab went on to identify three nitrate enhancer elements within the 180 bp NIA1 fragment that were necessary and sufficient for the nitrate enhancer function (Crawford, unpublished data). In my work, I investigated the effects of nitrite, as an alternative inducer, on various nitrate enhancer constructs in direct comparisons to the responses of such constructs to nitrate. While it has been previously shown that nitrite can induce more than half of the nitrate-induced genes, little is known about the ability of nitrite to induce specific nitrate enhancer elements (Wang et al., 2007). My goal was to determine if specific nitrate enhancer elements would respond similarly to nitrite treatment as they would with nitrate treatment. In addition, I analyzed the NiR promoter, which is critical for nitrite assimilation, and searched for any potential nitrate enhancer elements in the NiR promoter.
MATERIALS AND METHODS

Strategy for Cloning

To create transcriptional beta-glucuronidase (GUS) reporters, promoter fragments of the gene At2G15620 (NiR1) were cloned into the vector pDW294, which contains a minimal CaMV 35S promoter used to drive transcription of the GUS gene (Hong et al., 2003). The promoter fragment of each gene was amplified and isolated using Expand High Fidelity PCR (Roche), with restriction sites added to the ends of both the 5’ and 3’ primers (Table 1 for sequence of oligonucleotides). PCR products were cloned into the pGEM-T Easy vector (Promega), and then cut out with the relevant restriction enzymes and ligated into the final vector, pDW294.

Growth and treatment conditions

For the analysis of nitrite effects upon constructs containing NIA1 nitrate enhancer elements, Arabidopsis seedlings were grown as described previously (Rongchen Wang et al., 2003). Seedlings were grown on vertical plates containing 25 ml of agarose medium with 2.5 mM ammonium-succinate as the sole nitrogen source for 7 days under continuous light. Seedlings were flooded with 12.5 ml of liquid medium containing 2.5 mM of ammonium-succinate plus 1 mM KCl (control), 1 mM KNO₂ (nitrite-treated) or 10 mM KNO₃ (nitrate-treated) for 30 min with shaking (60 rpm). Roots were collected at end of treatments for total RNA. Relative messenger levels were estimated by qPCR with reference gene At5G12120 (ubiquitin-associated protein) as described (Wang et al., 2007).
For the nitrate enhancer screen, experiments were done using the transient expression system (developed by Dr. Peizhu Guan in the Crawford lab). *Nicotiana benthamiana* seeds were germinated in soil and transferred to perlite when leaves were between three to four-leaf stage. Seedlings were grown in perlite with hydroponic solution (10 mM KPO₄, 2.5 mM (NH₄)₂SO₄, 2 mM MgSO₄, 1 mM CaCl₂, 0.1 mM FeNa₂EDTA, and micronutrients) for approximately three weeks before infiltrating with *Agrobacteria*. The *Nicotiana benthamiana* plants were irrigated with 10 mM KCl or 10 mM KNO₃ approximately 30 minutes prior to injection. Upon injection, a 1-mL syringe (sans needle) was used to inject the infiltrate into the leaves. Leaves chosen for injection were young and fully expanded, and the infiltrate encompassed the entire leaf. Two to three days after infiltration, plants were irrigated again with hydroponic solution containing either 10 mM KCl or 10 mM KNO₃. The 2nd injected leaf from the top was collected for GUS assays.

For the analysis of nonresponding mutants and their response to nitrite in the presence or absence of ammonium-succinate, Arabidopsis seedlings containing NRP-YFP constructs were grown in hydroponic conditions on vertical plates containing 25 ml of agarose medium with 2.5 mM ammonium-succinate as the sole nitrogen source for 5 days under continuous light. Seedlings were then transferred to plates of agarose medium containing 2.5 mM of ammonium-succinate or no ammonium-succinate plus 5 mM KCl (control), 1 mM KNO₂ (nitrite-treated) or 5 mM KNO₃ (nitrate-treated) for 20 hours. Seedlings were isolated and analyzed for fluorescence under a microscope. Quantification was done using ImageJ software (http://rsbweb.nih.gov/ij/).
Transient expression *Agrobacteria* preparation

Constructs were transformed into *Agrobacterium tumefaciens* strains C1C58. Single colonies were picked and grown in 5-mL of Luria broth (50 µg/mL rifampcin, 5 µg/mL tetracycline, 25 µg/mL gentamycin) for approximately 20 hours at 28 degrees Celsius. 0.5 mL of the 5-mL culture was then used to inoculate 50-mL Luria broth culture (20 µM acetosyringone, 5 µg/mL tetracycline, 10mM MES) for another 20 hours at 28 degrees Celsius. Bacteria were then spun down, pelleted, and resuspended in infiltration buffer (150 µM acetosyringone, 10 mM MES, 10 M MgCl$_2$) to an OD$_{600}$ of 0.5. The resuspended bacteria were then incubated at room temperature for 3 hours before injecting (sans needle) into *Nicotiana benthamniana* leaves (Llave et al., 2000).

**GUS assay**

For the transient system, *Nicotiana benthamiana* leaves were frozen in -80 degrees Celsius after 3 days post infiltration. Leaves were then ground and lysed in 1 mL of GUS extraction buffer (0.1 M KPO$_4$, 2 mM EDTA, 5% glycerol, 2 mM DTT). GUS assays were performed in GUS assay buffers (50 mM NaPO$_4$, 10 mM EDTA, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, and 1 mM $p$-nitrophenyl β-D-Glucoronide) as described (Jefferson et al., 1986). OD reading was measured at 415 nm after 3 hours.

**Bioinformatics promoter analysis**

Potential transcription factor binding sites were predicted using AthaMap database (http://www.athamap.de/).
RESULTS

Nitrite induces Nia1 nitrate enhancer elements

The Crawford lab has searched for nitrate enhancer fragments within promoters of nitrate-regulated genes by fusing fragments from promoters of nitrate-regulated genes to a 35S minimal promoter-GUS construct. Any fragment with nitrate enhancer function would confer nitrate inducibility to the construct. A 180 bp fragment within the NIA1 promoter was identified, but it had low activity. It was found that a second 130 bp fragment from the NiR promoter, which had almost no nitrate enhancer activity by itself, greatly boosted the nitrate enhancer function of the 180 bp NIA1 fragment (by 15X). These two fragments with the 35S minimal promoter define what we call the “Tripartite promoter” labeled R47 (Figure 1). Subsequently it was shown that the 180 bp fragment could be reduced to 109 bp and still retain full enhancer function. The tripartite promoter containing this 109 bp Nia1 fragment was labeled R56 (Figure 1). Site-directed mutagenesis of the 109 bp fragment revealed three nitrate enhancer elements that are necessary and sufficient for nitrate induction with sufficient spacing. These elements correspond to potential Alfin1, HVH21, and Myb2 binding sites (Crawford, unpublished data).

I wished to determine if nitrite would similarly induce expression from these three specific nitrate enhancer elements. Transgenic seedlings containing the tripartite promoter fused to GUS with various mutations were used. Four key constructs were tested, including AT2 (+HVH21 +Myb2 +Alfin1), JT1 (-HVH21 -Myb2), AV2 (-Alfin1),
and R56 (tripartite NRP-GUS construct) (Figure 1A). Roots were collected at the end of treatments for total RNA, and relative mRNA levels were estimated by qPCR compared to the reference gene At5G12120 (ubiquitin-associated protein). Treatment conditions were at 10 mM KCl, 10 mM KNO₃, and 5 mM KNO₂ in the presence of ammonium-succinate.

The GUS response of the four tested constructs showed that nitrite can induce constructs containing the three specific nitrate enhancer elements Alfin1, HVH21, and Myb2 3.5-fold. Furthermore, there were significantly higher folds of induction in the response to nitrite than the response to nitrate in all four of the tested constructs. The R56 positive control displayed 8.3-fold induction in response to nitrate yet displayed a 14.5-fold induction in response to nitrite. AT2, the construct containing all three unmodified enhancer elements, displayed a 5.4-fold induction in response to nitrate and a 10.3-fold induction in response to nitrite. Both JT1 (missing HVH21 and Myb2 sites) and AV2 (missing the Alfin1 site) constructs showed dramatically decreased responses to both nitrate and nitrite. While nitrite was revealed to be a much stronger inducer for these constructs, the relative folds of induction amongst constructs remained comparable between nitrite and nitrate treatments. R56 consistently showed the highest fold of induction, AT2 ranked second still displaying strong levels of induction, AV2 had much weaker responses ranking third strongest response, and JT1 showed the lowest levels of induction in response to both nitrite and nitrate (Figure 1B). These results show that the same elements needed for nitrate induction in the 109 bp fragment are necessary and sufficient for nitrite reduction.
**Bioinformatics analysis of NiR promoter compared to Nia1 180 bp fragment**

The tripartite promoter used in the experiments above is a composite construct made up of fragments from two different promoters (from NIA1 and NiR). The 130 bp fragment from NiR greatly enhanced the activity of the 180 bp (R47) or 109bp (R56) fragment from NIA1. We wondered if the 130 bp NiR fragment would also be useful for identifying enhancers within the NiR promoter, from which it was derived. I first performed a bioinformatics analysis of the NiR promoter with special attention being placed on the 130 bp fragment. The AthaMap algorithm ([www.athamap.de](http://www.athamap.de)) was used for this analysis.

Analysis of the 130 bp NiR fragment revealed multiple potential elements. Two of the predicted elements in the NiR promoter fragment were Alfin 1 and HVH21 transcription factor binding sites, which are similar to the sites found in the 109 bp NIA1 fragment. The Myb2 site of the NIA1 fragment was not found. The general consensus for Alfin1 site is GNGGTG or GTGGNG ([Bastola et al., 1998](#)). The Alfin1 site in the NIA1 fragment matches this description with GTGGCG sequence in the (−) direction. However, in NiR, this sequence exists as GAAGGTG in the (+) direction. For HVH21, a TGAC core has been identified as being important for binding ([Krusell et al., 1997](#)). In NIA1, this site is oriented in the (+) direction upstream of the Alfin1 site. However, in NiR, the TGAC is oriented in the (+) direction downstream of the Alfin1 site. In NIA1, the nitrate enhancer elements are located 77 bp apart oriented in reverse directions, with a critical Myb2 site in between. In NiR, the sites are located merely 43 bp apart and oriented in the same direction (Figure 7).
AthaMap was also used to analyze up to 2KB upstream region from the start of transcription and the downstream region before the start of translation for regions rich in transcription factor binding sites. Regions with a high density of potential sites were found between the start of translation and the Xho1 site.

**Intact promoter activity in transient system**

The process of generating transgenic *Arabidopsis thaliana* plants via Agrobacteria transformation can take several months. For analyzing the expression of proteins of interest, including markers for gene expression, there exists a much faster method that does not require the generation of transgenic plants. This method involves transiently expressing proteins of interest in plant cells via agroinfiltration. While agroinfiltration works poorly when used on *Arabidopsis thaliana*, it works very well with *Nicotiana benthamiana* (Goodin et al., 2008). Agroinfiltration involves injecting suspensions of *Agrobacterium tumefaciens* cells into the intracellular space within leaves. The *Agrobacterium tumefaciens* carry binary plasmid vectors designed such that the genes of interest are inserted within the T-DNA region of the bacterial Ti plasmid. The infiltrated sections may then be sampled and utilized for further assays or analyses (Goodin et al., 2008).

I wished to use the transient system to test constructs using fragments that relied only on fragments from the intact NiR promoter. Based on previous data, a 1.2 kb NiR promoter fragment from the EcoRV site to the Xho1 site (see Figure 2) shows strong inducible activity in transgenic plants (Crawford, unpublished data). To test if this and other promoter fragments could be shown to have inducibility in the transient system, two
different sets of intact NiR promoter deletion constructs previously generated by the Crawford lab were analyzed in the transient system. These constructs included one set that contained transcriptional fusions to GUS, and another set that contained translational fusion to GUS. The transcriptional fusions included NiR promoter DNA ending at 3 bp before the start of translation. The translational fusions included NiR promoter DNA ending approximately 140 bp after the start of translation at the XhoI site. The transcriptional fusion constructs were labeled N89 (EcoRV-ATG), N90 (Taq1-ATG), and N91 (Rsa1-ATG), while the translational fusion constructs were labeled N95 (EcoRV-ATG), N96 (Taq1-ATG), and N97 (Rsa1-ATG) (Figure 2). These constructs were tested in the transient expression system to determine if a full length promoter would properly function and display nitrate enhancer activity in the transient system.

The results showed that there are two important regions that I could identify in the NiR promoter, which includes sequences just downstream of the start of translation. All tested constructs displayed inducible activity with 10 mM \( \text{KNO}_3 \); however, some showed higher levels than others. Specifically, transcriptional fusion constructs N89, N90, and N91 displayed 5.4-fold, 4.4-fold, and 2.5-fold induction, respectively (Figures 2B and 3). Translational fusion constructs N95, N96, and N97 displayed 3.1-fold, 4.7-fold, and 2.5-fold induction, respectively (Figures 2B and 3). While the fold of induction was comparable between transcriptional and translational fusions, it was interesting that the absolute value of the responses between the transcriptional and the translation fusion constructs differed significantly. Transcriptional fusion constructs had relatively lower GUS activities in both constitutive and inducible conditions compared with translational
fusions (Figures 2B and 3A). Therefore, the sequences between the start of translation and the Xho1 site provide strong levels of expression (both constitutive and induced). In addition, sequences between the Taq1 and Rsa1 sites upstream of the start of transcription confer significant nitrate-inducibility.

**Search for nitrate enhancers in NiR promoter region**

Considering that the Taq1-Rsa1 sequences in the NiR promoter were needed for strong inducibility, I searched for nitrate enhancers in this and other sequences in the NiR promoter using the 130 bp NiR-35S minimal promoter fragments used for the tripartite promoter above fused to YFP. The prediction was that any fragment with a nitrate enhancer would show boosted activity when placed upstream of the 130 bp NiR fragment. Five ~500 bp fragments were taken from the NiR promoter up to 2KB upstream (with overlaps) from the start of translation (Figure 4B). These fragments were used to replace the NIA1 180 bp fragment in the NRP-GUS tripartite promoter, thereby forming a construct with two fragments from NiR. The transient system with *Nicotiana benthamiana* was used to test for nitrate response with these constructs, named N1A, N2A, N3A, N4A, and N5A. Another modified construct (70A) was created by fusing the 130 bp NiR fragment with a 70 bp fragment of NiR downstream from the start of transcription that was rich in transcription factor binding sites. Lastly, a construct was created using the 150 bp NRT2.1 fragment previously identified by Girin to test the inducibility of this enhancer sequence with the NiR 130 bp fragment (Girin et al., 2007).

The search for nitrate enhancer elements using 500 bp overlapping fragments did not find any regions containing significant inducible activity (Figure 5). The analysis was
done using the transient system and treating the plants with 10 mM KNO$_3$ treatment. The negative control, 130 bp fragment by itself (130A), displayed some inducible activity at approximately 2-fold. The positive control, containing NRP-GUS construct (R471A, containing 180 bp Nia1, NiR 130 bp, 35S-minimal fused to GUS), displayed a strong 7.5-fold of induction upon 10 mM KNO$_3$ treatment. Constructs N2A, N3A, N4A, and N5A each showed greater levels of constitutive expression, but less fold of induction than 1301A (Figure 5A). N3A in particular displayed very strong constitutive activity, but its fold of induction was the lowest at less than 1-fold. The addition of the 70 bp downstream region rich in predicted transcription factor binding sites did not appear to increase inducibility. Only one fragment, N1A, appeared initially to have slightly more inducible activity compared to 130A. However, upon repeated experiments, this was shown not to be significant enough to indicate consistently strong inducible activity. Interestingly, the 2.1A construct containing the enhancer sequence identified by Girin displayed 3 fold induction, slightly more than 1301A but not nearly as strong as R471A in this promoter system (Figure 5B).

**Expression of nonresponding mutants in response to nitrite and ammonium**

The Crawford lab used the tripartite promoter (R56 or NRP) fused to YFP and transformed into Arabidopsis to search for nitrate regulatory mutants. M2 seedlings were produced after treating the homozygous transgenic plants with ethyl methanesulfonate for mutagenesis. 68 mutants were identified to be non-responding by displaying low fluorescence in response to nitrate (Wang et al., 2009). I analyzed two of these mutants, mutant 5 (Mut5) and mutant 21 (Mut21 which is a point mutation of Chl1), to determine
their response to nitrite in the presence and absence of ammonium-succinate.

In the presence of ammonium-succinate, wildtype seedlings containing the NRP-YFP promoter previously created by the Crawford lab responded strongly to 5 mM NITRATE treatment with approximately 9.4-fold induction on average. Mut5 seedlings showed significant decreases in the level of activity with only 2.6-fold induction on average. Mut21 displayed the least activity, with an average fold of 1.65 (Figure 6A). However, in the absence of ammonium-succinate, mutants 5 and 21 responded strongly to NITRATE, at 12.4-fold and 8.4-fold respectively compared to the 7.6-fold of the wildtype seedlings (Figure 6B). Therefore, the regulatory phenotype of these mutants depends on the presence of ammonium in the media.

When nitrite was used as the inducer, some interesting differences were found in absence of ammonium. In the presence of ammonium-succinate, 1 mM nitrite yielded similar results to the nitrate response (Figure 6). Wildtype displayed induction, while Mut5 and Mut21 displayed 4.2 and 2.5-fold induction, respectively (Figure 6A). However, in the absence of ammonium-succinate, wildtype displayed very weak levels of induction at only 2.6-fold, while Mut5 and 21 displayed strong induction at 6.7-fold and 8.3-fold, respectively (Figure 6B). Thus, to obtain comparable results between nitrite and nitrate treatment, it appeared that the presence of ammonium-succinate would be necessary for future experiments.
DISCUSSION

**Nitrite as a more potent inducer of nitrate enhancer elements**

Previously, it has been shown that there is a significant overlap between nitrate and nitrite induction, as nitrite is able to induce approximately 75% of the genes that are nitrate-inducible. Nitrate reductase (NIA1) and nitrite reductase (NiR) were specifically found to be inducible by both nitrate and nitrite. Three nitrate enhancer elements were recently found within the NIA1 promoter: Alfin1, HVH21, and Myb2 sites. Alfin1 and HVH21 sites were both found to be necessary, but not sufficient for induction. The presence of all three elements with the correct spacing showed strong inducible activity (Crawford, unpublished data). Yet, it remained to be determined if these three specific nitrate enhancer elements found within the NIA1 promoter would respond not only to nitrate but also to nitrite.

Treating AT2 (+HVH21 +Myb2 +Alfin1), JT1 (-HVH21 -Myb2), AV2 (-Alfin1), and R56 (wildtype tripartite NRP-GUS construct) transgenic seedlings to 20 minutes of 1mM nitrite found that these three nitrate enhancer elements respond to similarly to nitrite and nitrate. The ranking of the fold of induction from highest to lowest was comparable between both nitrate and nitrite treatments. For both nitrate and nitrite, wildtype (R56) as expected showed the highest response, followed by the construct containing all three enhancer sites with a 44 bp spacer (AT2). The Afin1 single mutant (AV2) had less activity, followed by the HVH21 Myb2 double mutant (JT1) which displayed the least activity (Figure 1B). These results are consistent with previous findings (Crawford, unpublished data).
The main difference between the nitrite and nitrate treatments was the actual fold of induction. Nitrite treatment (1 mM) leads to higher folds of induction than nitrate at higher concentrations (5 mM). This is consistent with previous findings that for NIA1, 20 minutes of nitrite treatment appeared to be a slightly stronger inducer than nitrate between 2 µM to 5 mM concentrations (Wang et al., 2007). This may be due to several reasons. One possible explanation is that there may have been contamination of the nitrite solutions with nitrate. At greater than 10 µM concentrations, nitrate could contribute to the activity (Wang et al., 2007). However, the fact that nitrite was at a concentration 5x lower than nitrate seems to suggest that nitrate contamination was not the case for such a significant increase in the fold of induction. It may also be possible that nitrite does indeed serve as a more potent inducer than nitrate. Since nitrite is toxic to plants upon accumulation, it may be necessary for plants to develop greater sensitivity to nitrite at lower concentrations compared to nitrate. Therefore, the nitrite can serve as a more potent inducer for nitrate enhancer elements.

**Search for nitrate enhancers within the NiR promoter**

Previously, the Crawford lab identified the 180 Nia1 promoter region containing the three nitrate enhancer elements by fusing the Nia1 promoter fragment together to a NiR 130 bp promoter fragment, 35S-minimal, and GUS. By itself in the transgenic lines, the 180 Nia1 promoter fused to 35S-minimal showed minimal activity (Crawford, unpublished data). However, when placed directly upstream of the NiR 130 bp fragment, the response seemed to be greatly stimulated. Since the NiR 130 bp fragment seemed to strongly stimulate NIA1 response, it became interesting to determine if the NiR 130 bp
fragment would be equally useful in combination with a fragments from within its own promoter to search for new nitrate enhancers.

Several fragments from the NiR promoter were tested intact fused to GUS in the transient system. The fusions were either transcriptional with the 3’ end of the promoter fragment 3 bp upstream from the start of translation, or translational with the 3’ end of the promoter fragment ending 141 bp downstream from the start of translation. Translational fusion constructs displayed much stronger folds of induction, but also showed higher levels of constitutive expression. It appears that within the 144 bp between the transcriptional and the translational fusions, there exists some transcription factor binding sites that could help to enhance the overall activity. AthaMap predicts that between the transcriptional and translational fusions, there are three HVH21 sites and one Alfin1 site, and numerous other potential transcription factor binding sites.

Three different lengths of the NiR promoter were tested, with the 5’ end beginning at the NiR EcoRV, TaqI, and RsaI cutting sites in order from largest to smallest respectively (Figure 2B). In the transcriptional fusion constructs, the induction showed a steady decrease in the fold of induction as the promoter fragments decreased in length (Figure 3B). While transcriptional fusion constructs showed a steady decrease in fold activity from EcoRV to TaqI, the translational fusion constructs showed that the EcoRV fragment had lower fold of induction compared to the translational TaqI fragment. This may be due to either higher constitutive activity relative to the inducibility, or saturation of the GUS assay. If it were due to higher constitutive activity, one might expect to see such constitutive activity in the corresponding transcriptional
fusion construct also. A slight amount of constitutive increase in activity is observed in
the transcriptional fusion, yet the induction response is much greater as well. Saturation
of the GUS assay could potentially explain this discrepancy in that the actual activity
levels in the translational EcoRV construct may have been higher than actually measured.
Nonetheless, the transcriptional and translational fusion constructs both consistently
showed a decrease in activity from the TaqI to the RsaI fragment. Thus, there appeared to
be some important sites between the RsaI and the TaqI sequence in the NiR promoter
region. These results therefore demonstrated that full length promoters can display
inducible activity in the transient system, and that in theory, the NiR 130 bp fragment
could be used to search within its own promoter fragments to display nitrate-inducible
activity.

However, the nitrate enhancer screen did not show significant inducible activity
when five different overlapping ~500 bp NiR promoter fragments were fused to the NiR
130 bp fragment (Figure 4, Figure 5). It is unlikely that the lack of inducible activity is
due to the complete absence of any nitrate enhancer elements, as the intact promoters
display significantly stronger levels of activity compared to the baseline NiR 130 bp by
itself. It may be possible that the enhancers present in the NiR promoter simply are not
strong enough to display significant inducible activity when treated with nitrate.

Interestingly, the first 500 bp fragment immediately upstream of the NiR 130 bp
contained the sequence between the TaqI and the RsaI cut sites, yet it did not consistently
display enhanced activity when inserted upstream of the NiR 130 bp. As recently shown
in the NIA1 promoter, multiple elements may be needed to form a cis-regulatory module
by working together (Crawford, unpublished data). Three nitrate enhancer elements are all needed to be present with the correct spacing in order for nitrate induction to occur for the NIA1 promoter. When the Alfin1 site alone was missing, activity dropped significantly. Additionally, the spacing between enhancer elements was shown to be important (Crawford, unpublished data). If multiple enhancer elements were similarly required for induction in the NiR promoter, then a simple omission of merely one of the enhancers would result in no inducible activity. Furthermore, the proper spacing between the 500 bp NiR fragments and the 130 bp NiR fragment in the designed constructs was not maintained with a spacer relative to the spacing in the intact promoters. The N1A construct contained sequences from the region between Taq1 and RsaI, which appeared to be significant in the intact promoter. However, the spacing between the N1A fragment and the 130 bp fragment in the intact promoter was shorter in the intact promoter compared to the designed construct. The lack of proper spacing and the potential presence of a complexity of regulatory elements may have accounted for the absence of any observable inducible activity.

**Role of ammonium in regulating nitrite and nitrate induction**

Ammonium in the growth media has an effect on gene expression induced by nitrate and nitrite. In the presence of ammonium (provided as ammonium succinate), nitrite induction in transgenic seedlings containing tripartite NRP-YFP constructs mirrored the nitrate response and actually displayed slightly stronger activity. This confirms results published by the Crawford lab that nitrite, at lower concentrations than nitrate, is a more potent inducer than nitrate. However, in the absence of ammonium,
there was a significant difference: nitrite induction was approximately 75% lower than nitrate induction. Ammonia has been previously shown to repress induction of NIA1 and NiR by nitrate (Joy, 1969). Hence, it could be possible that since ammonium may repress induction, uptake of nitrate and nitrite may also be reduced. At first glance, this may suggest that presence of ammonium would yield a nitrite response lower than in the absence of ammonium. However, while nitrite may act as a signal to induce expression, accumulation of nitrite can result in toxicity. A possible explanation for the decreased activity in the absence of ammonium may be that there is too much uptake of nitrite, resulting in toxicity. However, with ammonium present, it may reduce the amount of nitrite taken up to such a level where it would not be toxic yet can still induce activity. Thus, the presence of ammonium may be helpful in regulating nitrite uptake in order to prevent toxicity from occurring.

In the Mut5 mutant, nitrate and nitrite responses were greatly reduced in the presence of ammonium compared with wildtype (Figure 6A). However, in the absence of ammonium, the seedlings still showed strong response to nitrate and nitrite (Figure 6B). The fold of induction of nitrite was about half compared to the nitrate fold of induction. Too much pure nitrite accumulation results in toxicity, however, Mut5 has a defect since it is nonresponding to nitrate in the presence of ammonium. There may be some possibility that this mutation may have another effect: the effect of not taking up nitrite as much as wildtype. By not taking up as much, it would not reach as toxic levels as high as wildtype, but it would still induce some activity. It may be asked why nitrate alone still induces very strong activity. This may mean that this mutation has a nitrate uptake defect
that is strongly dependent on ammonia, but a nitrite uptake defect that is partially
dependent on ammonia.

Mut21 was previously sequenced, and the mutation was found to be in the CHL1
gene (Wang et al., 2009). Mut21 can uptake nitrite and show induction just as well as
with nitrate in the absence of ammonia. CHL1 mutants have been previously shown to
be partially dependent on the presence of ammonium in the growth media to display a
defect in nitrate uptake (Wang et al., 1998). The regulatory defect of this mutant appears
to strongly occur when ammonia is present (Figure 6A). If there is no ammonia, the
defect is not seen. However, the nitrite fold of induction in the absence of ammonia is
significantly higher than wildtype. It may be that the uptake defect is strongly but not
completely dependent on ammonia. Being only strongly dependent in a partial manner,
the defect may exist in the absence of ammonia to a certain extent. There may be a slight
defect in assimilating nitrate or nitrite in the absence of ammonia, thus helping to prevent
toxicity accumulation as seen in the wildtype and allowing for induction to occur. In the
absence of ammonia, there appears to be no significant decrease in nitrate induction in
Mut21 relative to the wildtype (Figure 6B). Given that the Mut21 defect may be strongly
but not completely dependent on ammonia, it would not completely wipe out uptake of
nitrate when no ammonia is present. With a slight decrease in nitrate uptake, there may
not be a significant difference in the induction response. However, it may be a slight
enough decrease to limit the toxicity of nitrite accumulation, therefore allowing strong
nitrate and nitrite induction in the absence of ammonia.
Summary and future experiments

The work presented here demonstrates that specific nitrate enhancer elements are able to respond to nitrite treatment in a similar way to nitrate treatment. For such nitrate enhancer elements, nitrite was shown to be a more potent inducer in the presence of ammonium. However, by testing intact promoters, the sequences downstream of the NiR ATG appear to boost overall expression for induction. In the intact promoters, the region between TaqI to the RsaI was the only region that seems to contribute to nitrate induction. Yet, the method used to search for new nitrate enhancer elements using overlapping ~500 bp fragments did not successfully yield any new enhancer sequences. It may be possible that the enhancers are present, but simply not strong enough to display significant inducible activity. Lastly, mutants seemed to respond more similarly to nitrate when treated with nitrite in the presence of ammonium. Thus, for future experiments, nonresponding mutants should be analyzed in the presence of ammonium if comparing similarities between nitrate to nitrite response.

Overall, this work has shown that there exists extensive overlap between nitrate and nitrite regulation. Three identified nitrate enhancers and a nitrate transporter respond similarly to nitrite as they do to nitrate. The extensive overlap between sensing systems for nitrate and nitrite may provide new tools for studying cis-acting sites and may aid in the search for new nitrate enhancer elements.
Figure 1. Response of nitrate enhancer elements to nitrate and nitrite. (A) Schematic diagram of the tripartite construct, including recently identified nitrate enhancer elements (Crawford, unpublished data). (B) Fold of induction of nitrate and nitrite responses based on qPCR analysis. Error bars are standard deviations over three biological replicates.
Figure 2. Schematic representation of intact NiR transcriptional vs translational fusion constructs used for transient assays. (A) A diagram showing the design of the construct: a NiR intact promoter fragment is fused to GUS. (B) A schematic representation of the different lengths of intact NiR promoters tested. Translational fusions included constructs N95 (EcoRV-Xho1), N96 (Taq1-Xho1), and N97 (Rsa1-Xho1). Transcriptional fusions included N89 (EcoRV-ATG), N90 (Taq1-ATG), and N91 (Rsa1-ATG). Folds of induction are shown to the right of each respective fragment length, in the format of fold of induction, as well as in figure 3 below. The region between Hinf1 and Alu1 contains the 130 bp promoter fragment used in the tripartite constructs.
Figure 3. Intact promoter activity in the transient system. (A) GUS assay with OD reading at 415nm after 3 hours. Error bars are standard deviations over two biological replicates. Transcriptional fusion constructs include 89 (EcoRV-ATG), 90 (TaqI-ATG), 91 (RsaI-ATG). Translational fusion constructs include 95 (EcoRV-XhoI), 96 (TaqI-XhoI), and 97 (RsaI-XhoI). R47 indicates tripartite promoter containing 180 bp Nia1, 130 bp NiR, 35Smin fused to GUS. (B) Folds of induction were comparable between TaqI and RsaI transcriptional and translational fusion constructs.
Figure 4. Schematic design of constructs and promoter fragments used in nitrate enhancer screen. The original tripartite construct contained Nia1 180 bp fragment, NiR 130 bp fragment, and 35S-minimal promoter fused to GUS. This construct was modified by replacing the Nia1 180 bp fragment with a NiR ~500 bp fragment to test if the NiR 130 bp fragment would be useful in searching for new nitrate enhancers within its own promoter. The diagram shows the relative positions of the NiR promoter fragments used in the screen.
Figure 5. Search for nitrate enhancers in NiR promoter. The third promoter fragment upstream (N3A) yielded constitutive activity. Most other fragments did not yield significant inducibility. Nrt2.1 enhancer fragment identified by Girin appeared to have some inducibility, but not as strong as Nia1 fragment as seen in the wildtype (R471A, referring to Nia1 180bp, NiR 130bp, 35S-min fused to GUS). Error bars are standard deviations over three biological replicates.
Figure 6. Nonresponding mutants nitrate and nitrite in the presence or absence of ammonium-succinate. (A) In the absence of ammonia, the folds of induction between nitrate and nitrite appeared to show no correlation. (B) In the presence of ammonia, the folds of induction between nitrate and nitrite appeared to show correlation. Nitrite response yielded higher folds of induction than nitrate. Both mutants appear to have a defect in nitrate and nitrite assimilation that is partially or strongly dependent on the presence of ammonia.
Figure 7. Comparison of Nia1 and NiR promoter fragments containing identified nitrate enhancer elements. Nia1 contains three enhancer elements: HVH21, Myb2, Alfin1. HVH21 and Alfin1 are oriented in reverse directions. NiR contains Alfin1 and HVH21 elements both oriented in the same direction.
**Table 1. NiR nitrate enhancer search constructs.** List of primers and restriction enzymes used to create GUS constructs in the search for nitrate enhancers in the NiR promoter.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Gene</th>
<th>5' primer</th>
<th>3' primer</th>
<th>Restriction Enzyme</th>
</tr>
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<tr>
<td>2.1A</td>
<td>AT1G0 8090 (Nrt2.1)</td>
<td>TATAAGCTTATATC GGCAACCTTTTGGTG ATAAGC</td>
<td>ATACTGCAGTA GGGTTCCTAGC CAGTGTGGA</td>
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<tr>
<td>70A</td>
<td>AT2G1 5620 (NiR)</td>
<td>TATAAGCTTAAGA GCATCATTCTCCC TCTACAA</td>
<td>ATACTGCAGAG TATGGCCGGAA GAAGGAGTTG</td>
<td>HindIII + PstI</td>
</tr>
<tr>
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<td>ATACTGCAGTT TATATTCTATAT GATTCACTTA GTCTATTGACT TCAT</td>
<td>HindIII + PstI</td>
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<tr>
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<tr>
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<td>HindIII + NsiI</td>
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<td>AGAGCTGCAG CGACGTAAAT AATTGACCAC GT</td>
<td>HindIII + PstI</td>
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