UNIVERSITY OF CALIFORNIA, SAN DIEGO

NLP6 and NLP7 Bind to *Cis*-acting Elements of the NIA1 Nitrate Reductase Gene and Activate Gene Expression in a Nitrate-dependent Manner

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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2013

The Thesis of Lam Khanh Vuong is approved and it is accepted in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2013

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Acknowledgements

I would first like to express my appreciation to Dr. Crawford for giving me the opportunity to join his lab. The time I have spent in the lab has not only taught me the skills needed for basic research, but also the ability to think critically through problems. Dr. Crawford has always offered support and, guidance, and future career advice whenever needed.

I would like to thank Dr. Peizhu Guan for being an excellent mentor. Peizhu was always willing to provide advice on experimental designs. Furthermore, she has provided a great deal of help in the preparation of biotin-labeled probes, which is critical for the completion of my project. For her guidance and moral support, I am very grateful.

I would also like to thank Dr. Rongchen Wang for teaching me basic laboratory techniques, when I first joined the lab. He has helped me troubleshoot countless technical problems, and for that, I am grateful.

Lastly, I would like to thank Dr. Martin Yanofsky and Dr. Yunde Zhao for being members of my committee.

ABSTRACT OF THE THESIS

NLP6 and NLP7 Bind to *Cis*-acting Elements of the NIA1 Nitrate Reductase Gene and Activate Gene Expression in a Nitrate-dependent Manner

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University of California, San Diego, 2013

Professor Nigel M. Crawford, Chair

Inorganic nitrogen, in the form of nitrate, is both an important nutrient and signaling molecule in the growth and development of plants. The transcription factor NLP7, a downstream component of the nitrate signaling pathway, along with its homologue NLP6, were shown in this study to participate in DNA-protein interactions with an enhancer sequence from the Nitrate Reductase NIA1 gene in *in vitro* electrophoretic mobility shift assays. Furthermore, this study showed that two binding sites exist within the NIA1 enhancer element for NLP6 and NLP7. *In vivo* studies using the transient expression system in *Nicotiana benthamiana* showed that these factors could activate expression of a nitrate-responsive gene in a nitrate-dependent manner. Results from this study suggest that NLP6 and NLP7 regulate the transcription of target nitrate-responsive genes by directly binding to *cis*-acting elements in the promoter region.

INTRODUCTION

Nitrate uptake and assimilation

Nitrogen is a component of nucleotides and proteins that are essential for life; its availability in the form of nitrate (NO_3^-) is a major factor limiting plant biomass and food production. As sessile organisms living in fluctuating environmental conditions, terrestrial plants have evolved complex mechanisms for the uptake and assimilation of nitrate. In *Arabidopsis thaliana*, two families of proton-coupled nitrate transporters, NRT1 and NRT2, have been identified (Tsay et al., 2007). The NRT1 gene family codes for low affinity transporters, with the exception of the dual-affinity transporter NRT1.1, that operate when nitrate is in the millimolar range, while the NRT2 family codes for high affinity transporters that operate when nitrate concentrations are in the micromolar range (Orsel et al., 2006). Four nitrate transporters, NRT1.1, NRT1.2, NRT2.1, and NRT2.2, have been shown to function in nitrate uptake in roots (Krouk et al., 2010). Once nitrate enters root cortical cells, it travels through the endodermis into the pericycle and is transported into the root vascular system by nitrate transporter NRT1.5 (Dechorgnat et al., 2011). Following long distance transport to the shoot, another nitrate transporter, NRT1.8, facilitates the unloading of nitrate from the xylem into leaf tissues (Wang et al. 2012). Upon entry into leaf cells, nitrate can either enter vacuoles through the $NO_3^-/2H^+$ antiport CLCa for storage, or it can be reduced to nitrite by cytosolic nitrate reductase (Dechorgnat et al., 2011). The next step of nitrate assimilation involves the transport of nitrite into chloroplast, where it is further reduced to ammonium by nitrite reductase. Finally, glutamine synthetase (GS) and glutamate synthase (GOGAT)

incorporate ammonium into organic acids for the synthesis for amino acids (Crawford et al., 1993).

Nitrate signaling

In addition to its role as a vital nutrient in plants, nitrate also acts as a signaling molecule for regulating nitrate assimilation and carbon metabolism. Microarray experiments have revealed that over 555 genes in *Arabidopsis thaliana* roots showed significant increases in their mRNA levels in response to only 20 minutes of nitrate treatment (Wang et al., 2003). The change in gene expression was most notable in nitrate assimilatory genes encoding such proteins as nitrate transporters, nitrate reductase, and nitrite reductase, along with genes involved in glycolysis and the pentose phosphate pathway, since they showed a two or more fold increase in mRNA levels following nitrate treatment (Wang et al., 2003). The importance of nitrate as a signaling molecule warrants further investigations into the nitrate signaling pathway.

Recently, the nitrate transporter NRT1.1 was found to have nitrate regulatory functions (Wang et al., 2009). In addition, it was found that a mutation in the 10^{th} and 11^{th} trans-membrane domain of the NRT1.1 gene prevented nitrate uptake; however, nitrate signaling still occurred because induction was observed for nitrate responsive genes such as NRT, NiA, and NiR (Ho et al., 2009). NRT1.1 was later designated as a transceptor. The activity of the NRT1.1 transceptor is regulated by protein kinase CIPK23, a calcium sensor like-interacting protein kinase. In response to low external nitrate concentration, CIPK23 phosphorylates the NRT1.1 transceptor at threonine 101, which turns NRT1.1 into a high affinity nitrate transporter. The activity of CIPK23

decreases dramatically when external nitrate concentration is high, which reverts NRT1.1 transceptor back to its un-phosphorylated state, resulting in the conversion of NRT1.1 transceptor into a low affinity nitrate transporter (Hu et al 2009). Other components of the nitrate signaling pathway that have been identified so far are transcription factors LBD37/38/39, negative regulators of nitrate assimilatory genes (Krouk et al., 2010), NLP7, a positive regulator of nitrate-responsive genes (Castaings et al., 2009), ANR1, and SPL9. ANR1 is a transcription factor that controls the stimulation of lateral root elongation in response to nitrate, while the exact function of SPL9 is still unknown (Bouguyon et al., 2012). Overall, the only well-characterized components of the nitrate sensing pathway are one sensor, two kinases, and three transcription factors. Potential adaptor proteins, downstream kinases, and phosphatases that could play a role in relaying the message from sensor to transcription factor have yet to be discovered. Furthermore, the mechanism of how transcription factors regulate gene expression of nitrate assimilatory genes is still unknown.

One outcome of a signal transduction cascade is a change in gene expression. The NLP7 transcription factor, a positive regulator of genes that respond to nitrate signaling in plants, is a member of the NIN-like protein family. NIN proteins, which stand for nodule inception proteins, were first discovered in the nodules of *Lotus japonicus*, a wild legume (Schauser et al 1999). NIN proteins control the initiation of the infection thread formed by the coordinated action of both the host plant and the symbiotic nitrogen fixing bacteria (Schauser et al 1999). NLP7 does not play a role in nodule inception; it contains the characteristic RWP-RK domain that is similar to the DNA binding domain of Z9 and

bZIP8 transcription factors (Konishi et al 2011). It is a critical component of the nitrate signaling pathway because a 35% reduction in the mRNA levels of nitrate transporter NRT2.1 and NRT2.2, nitrate reductase NiA1 and NiA2 was seen in *nlp7* knockout *Arabidopsis thaliana* (Castaings et al 2009). Furthermore, nlp7 mutants had higher root density and showed delayed flowering time compared to wild-type Arabidopsis plants (Castaings et al., 2009). Although NLP7 contains a putative DNA binding domain predicted by bioinformatics and biophysics, it is still unclear how NLP7 mediates nitrate signaling at the molecular level.

The goal of this project is to determine if the NLP7 protein and its related homologue NLP6 can interact with known enhancer sequences, also called nitrate-responsive *cis*-elements, to alter the expression of their target genes in response to nitrate signaling. Once binding of nitrate-responsive transcription factor and enhancer have been established, the exact binding site (around 8bp) will be determined. Results from this experiment could provide the evidence showing where NLP7 binds at the molecular level, which could help shed some light on the final steps of nitrate signaling pathway.

RESULTS

In 2010, the Crawford lab identified a 109-bp enhancer fragment from the distal promoter region of the nitrate reductase NIA1 gene in an enhancer screen of transgenic Arabidopsis. In the presence of nitrate, this 109-bp cis-regulatory element was able to stimulate the expression of the GUS reporter gene fused to the minimal 35S promoter. Efforts to subdivide the 109-bp into smaller fragments using overlapping 30-bp fragment triplicates were unsuccessful to further localizing the enhancer activity. However, mutational analysis of predicted transcription factor binding site resulted in the identification of the three regulatory motifs, HVH21, Myb, and Alfin1, which were critical for enhancer function. In addition to the identification of an enhancer fragment in NIA1, the lab also identified NLP7, a transcription factor with predicted basic leucine zipper and helix-turn-helix DNA binding motif, in a forward genetic screen based on reporter gene expression driven by N-regulated promoter. The NLP6 transcription factor, a close homologue of NLP7, was discovered through a yeast 1-hybrid screen. Although *nlp6* mutants does not show any observable phenotypic aberrations, a comparison of amino acid sequences between NLP6 and NLP7 in Arabidopsis showed high similarities in both the primary and secondary protein structure of these proteins (Figure 1) (Schauser et al., 2005). Given a known nitrate inducible enhancer fragment and transcription factors with putative DNA binding domain, my first goal for this project was to determine if the putative DNA binding domain of NLP6 and NLP7 can bind to the 109-bp enhancer fragment of the NIA1 gene.

DNA Binding Activities of NLP6 and NLP7 DNA binding (DB) Domain

To determine DNA-protein interaction between the NIA1 enhancer fragment and the NLP transcription factors, an electrophoretic mobility shift assay (EMSA) using purified NLP6 and NLP7 DNA binding domains (Figure 2a) along with biotin-labeled nia109-bp probe (Figure 2b) was performed. The resulting gel image showed strong shifted bands for both the NLP6 and NLP7 DNA binding domains (Figure 2c), indicating that they both form complexes with the nia109-bp enhancer fragment. Furthermore, when unlabeled nia109-bp probe was used as specific competitors in the binding reactions, binding of NLP6 and NLP7 DB to the enhancer fragment was abolished (Figure 2d). This suggests that the shift in Figure 1c is not an artifact of non-specific protein-DNA interaction.

Localization of NLP6 and NLP7 DB domain activities

After experimental data was obtained for the binding of the putative DNA binding domains of NLP6 and NLP7 to the N-responsive enhancer element of the nitrate assimilatory gene NIA1, I wanted to further localize the binding region of these transcription factors to determine their binding sites. We generated five overlapping probes spanning 35 to 40 base-pair regions of the nia109-bp enhancer fragment (Figure 3a). These truncated overlapping probes were called 5 prime (5’), gap1 (G1), middle region (MR), gap2 (G2), and 3 prime (3’). They were labeled with biotin and used in binding reactions with NLP6 and NLP7 DB for my next set of gel shift assays.

When the five biotin-labeled overlapping probes were used in binding reactions with NLP6 DB purified protein, prominent shifted bands are visible only for the middle region and 3’ region of nia109-bp; a very faint shift is visible for the gap1 region (Figure

3b). Similar results were seen when purified NLP7 DB protein were subjected to gel shift assays with the five truncated biotin probes (Figure 3c). This indicates that the 5 prime region of the nia109-bp enhancer fragment does not interact with transcription factors NLP6 and NLP7, suggesting that the first 40 base-this enhancer fragment does not play a crucial role in regulating gene expression. On the other hand, the middle region and 3' region of nia109-bp enhancer fragment are important for regulation by trans-acting factors.

Determination of NLP6 and NLP7 DB binding sites

Since gel shift assays showed that both NLP6 and NLP7 DB bind to two regions on the nia109-bp enhancer fragment, more specifically the middle and 3' region. We generated biotin labeled mutated versions of these regions, each containing a 4-bp mutation. Five mutated probes were generated for the middle region (Figure 4a), while six mutated probes were generated for the 3 prime region (Figure 5a). Mutants of the middle region were localized in the area that overlaps with gap1. Mutants of the 3 prime region were localized to areas that does not overlap with gap2, since gap2 probe does not show any binding with either NLP6 or NLP7 DB.

Interestingly, both NLP6 and NLP7 DB appear to bind to the same binding sites in the 3 prime region of the nia109-bp enhancer fragment. Figure 5b shows NLP6 DB binding to the 3 prime mutations m1 to m3 and m6, suggesting that these regions are not essential for binding. However, mutations m4 and m5 eliminated binding indicating that this 8 base-pair region between m3 and m6 contains critical recognition sequences for this transcription factor. Similar results were seen with NLP7 DB in Figure 5c. On the

other hand, mutant analysis of the middle region showed that NLP6 DB can only bind to MR mutant 1 (Figure 4b), while NLP7 DB can bind to both MR mutants 1 and 5 (Figure 4c). This suggests that the DNA region spanning m2 to m5 of MR is critical for NLP6 binding, while only DNA region spanning m2 to m4 of MR is critical for NLP7 binding. Although both NLP6 and NLP7 bind to the same sequence at the 3 prime end of the nia109-bp enhancer element, the binding of NLP6 to the middle region of this enhancer fragment requires a larger consensus binding site.

Transient Expression of NLP6 and NLP7 transcription factors in *Nicotiana benthamiana*

In vitro EMSA data strongly indicates that the transcription factors NLP6 and NLP7 bind to the nia109-bp enhancer fragment. This suggests that these factors might activation expression of a gene driven by the nia109 enhancer fragment *in vivo*. To investigate this question and to test for any nitrate-dependent activation, the *in vivo* transient expression system using *Nicotiana benthaminana* was employed.

The process of generating transgenic *Arabidopsis thaliana* plants via Agrobacteria transformation can take several months. However, the expression of proteins of interest without the use of stable transgenic lines can be achieved using agroinfiltration into *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 that then transfer the test DNA into plant cells for expression. The infiltrated sections may then be sampled and utilized for further assays or analyses (Goodin et al., 2008).

In this experiment, full length NLP6 and NLP7 cDNAs were cloned into pGreen vectors and transformed into Agrobacteria. Each NLP protein construct was subsequently co-infected into *N. benthamiana* leaves with a nitrate-responsive promoter GUS construct containing the Nia109-bp enhancer sequence, the Nir130bp enhancer sequence, and the 35S minimal promoter fused to the beta-glucoronidase (GUS) reporter gene (Figure 6a). *N. benthamiana* plants were treated with either 10mM of nitrate or 10 mM of KCl 30 minutes prior to leaf infection. Figure 6b shows that leaves from plants treated with 10mM nitrate 30 minutes prior to infection with empty vector, NLP6 insert, or NLP7 insert showed relative GUS activity that is 2.2x, 5.1x, and 9.3x higher than KCl-treated control plants, respectively. The 2.2 fold induction of GUS protein in the nitrate-induced, empty-vector plants is likely caused by endogenous *N. benthamiana* nitrate regulatory genes. Overall, the significant increase in GUS activity in response to transient overexpression of NLP6 and NLP7 suggests that these transcription factors can activate the nia109-bp enhancer element *in vivo*.

DISCUSSION

In this project, I have produced experimental data indicating that NLP6 and NLP7, members of the NIN-like protein family with the RWP-RK predicted DNA binding motif, can bind to a known enhancer fragment from the nitrate reductase NIA1 gene. Through mutant analysis, I have shown that NLP6 and NLP7 bind the enhancer fragment through interaction with an 8bp sequence 5'-CGCCACTT-3' found near the 3 prime end of nia109-bp enhancer fragment. In addition NLP6 and NLP7 were also shown to interact with a 16bp sequence 5'-GACCCGTCCCTTTGTT-3'and a 12bp sequence 5'-GACCCGTCCCTT-3' in the middle region of nia109-bp enhancer fragment. Finally, I used the transient expression system in *Nicotiana benthamiana* to demonstrate the nitrate dependence of gene activation *in vivo*. The significance of these results will be discussed below.

The NLP6 and NLP7 binding site 5'-CGCCACTT-3' corresponds to a potential regulatory motif

During the past decade, several groups have successfully identified N-responsive *cis*-elements; however, most groups failed to address the involvement of their sequence of interest in the activation of transcription by nitrate due to binding of specific transcription factors. For instance, Hwang et al. (1997) identified a 12bp sequence element that is essential for transcription of nitrate reductase gene in *Arabidopsis thaliana*. These elements are composed of several copies of a core Ag/cTCA sequence preceded by a 7bp AT rich sequence. Although the finding was significant, the group did not show that these elements could confer nitrate-regulation to heterologous promoters

and was unable to demonstrate regulation of the 12bp *cis*-acting sequence by a specific transcription factor, since they used the crude cell extracts to demonstrate interactions. In 2007, Girin et al. identified a 150bp *cis*-acting element of the Arabidopsis nitrate transporter NRT2.1 promoter; however, the group did not address trans-acting factors. In 2010, Kinoshi et al., identified a nitrate-responsive *cis*-element in the Arabidopsis NIR1 promoter; however, the group suggests that no known putative transcription factors of the nitrate signaling pathway, except for members of the Dof family of transcription factors and novel transcription factors, could bind to the 43bp *cis*-element.

Wang et al., in 2010 identified an element within the 109 bp enhancer fragment of Arabidopsis NIA1 that contains a potential regulatory motif for Alfin1, predicted by AthaMap analysis software. Deletions within the Alfin1 sequence reduce the activity of the enhancer fragment by 75% (Wang et al., 2010). The Alfin1 sequence 5'-CGCCACT-3' almost completely matches the binding site of NLP6 and NLP7 transcription factors examined in this project. Thus my data indicates that the putative Alfin1 regulatory motif is in fact and NLP-binding site. This is one of the few instances where a specific transcription factor was shown to bind to a N-responding *cis*-element at a crucial regulatory site.

Steps taken for the elucidation of the complete nitrate signaling pathway could, in the future, result in better utilization of nitrate based fertilizers. Currently, farmers use a large excess of nitrate to ensure high crop yield. After each growing season, over 80% of the nitrate still remains in the soil (Spiertz 2009). These often leech into ground water supplies or lakes, resulting in water pollution and eutrophication, which is damaging to both humans and marine life (Spiertz 2009). In the past, traditional breeding experiments

have produced plants that have modified root structures to efficiently take up nitrate (Schlüter et al., 2012). In the future, with a complete understanding of nitrate signaling, perhaps genetically engineered crop strains with some modified component of nitrate signaling pathway could be produce to utilize nitrate more effectively. This could decrease the heavy uses of nitrate fertilizers, which would lessen the negative environmental impacts compared to our current traditional method of agriculture.

Summary for Future Experiments

Although the use of *in-vitro* mutagenesis analysis for the identification of binding sites of NLP6 and NLP7 provided some clear data, additional experiments may be necessary to increase the credibility of the data set. A future experiment could be performed using mutated nia109-bp *cis*-element fused to the GUS reporter gene. Mutations should occur at either of the DNA binding sites obtained from this project or both. Co-injection of the new mutated constructs with either NLP6 or NLP7 would allow the determination of whether the binding sites obtained from mutagenesis analysis could inhibit binding of transcription factors in-vivo.

MATERIALS AND METHODS

NLP6 and NLP7 expression and purification

NLP6 spanning residues 511-591 was cloned into pGEX-2TK vector with a glutathione S-transferase (GST) tag followed by thrombin cleavage site. The pGEX-2TK vector was also used for cloning residues 578-657 of NLP7. Recombinant NLP76 and NLP7 were expressed as N-terminal fusion protein with GST in BL21 cells and induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2.5 h at 25 ^{0}C. The fusion proteins were affinity purified using columns packed with Sepharose Gluthathione beads in pH 7.4 PBS buffer of 137mM Nacl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4. Recombinant fusion proteins were eluted with 10mM reduced gluthathione, and was finally concentrated to 1mg/ml in a HiTrap 10000 desalting column (GE Healthcare).

EMSA

EMSA was performed with biotin labeled probes (Figure S1) and 700ng of either NLP6 or NLP7 recombinant purified protein in 20µl of EMSA reaction buffer (1ug of poly(dI-dC), 10mM Tris, 50mM KCl, 1mM DTT, 2.5mM MgCl2, and 4% glycerol). Samples were electrophoresed in 4% polyacrylamide gel using 0.5X TBE as running buffer (45mM Tris, 45mM boric acid, 1.M EDTA, pH 8.3) at 70V for 1.2 hours. Following a 30 minute transfer onto N+ Nylon membrane and crosslinking, membranes were developed for color as described on manufacturer's website (www.thermoscientific.com/pierce).

Transient expression *Agrobacteria* preparation

NLP6 and NLP7 were individually cloned into pGreen vectors. pGreen recombinant constructs and pSoup were transformed into *Agrobacterium tumefaciens* strains C58. Single colonies were picked and grown in 5-mL of Luria broth (5 μg/mL tetracycline, 25 μg/mL gentamycine) for approximately 20 hours at 28 ^{0}C. 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^{0}C. Bacteria were then spun down, pelleted, and resuspended in infiltration buffer (150 μM acetosyringone, 10 mM MES, 10 M MgCL2) to an OD600 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 KPO4, 2 mM EDTA, 5% glycerol, 2 mM DTT). GUS assays were performed in GUS assay buffers (50 mM NaPO4, 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.

FIGURES

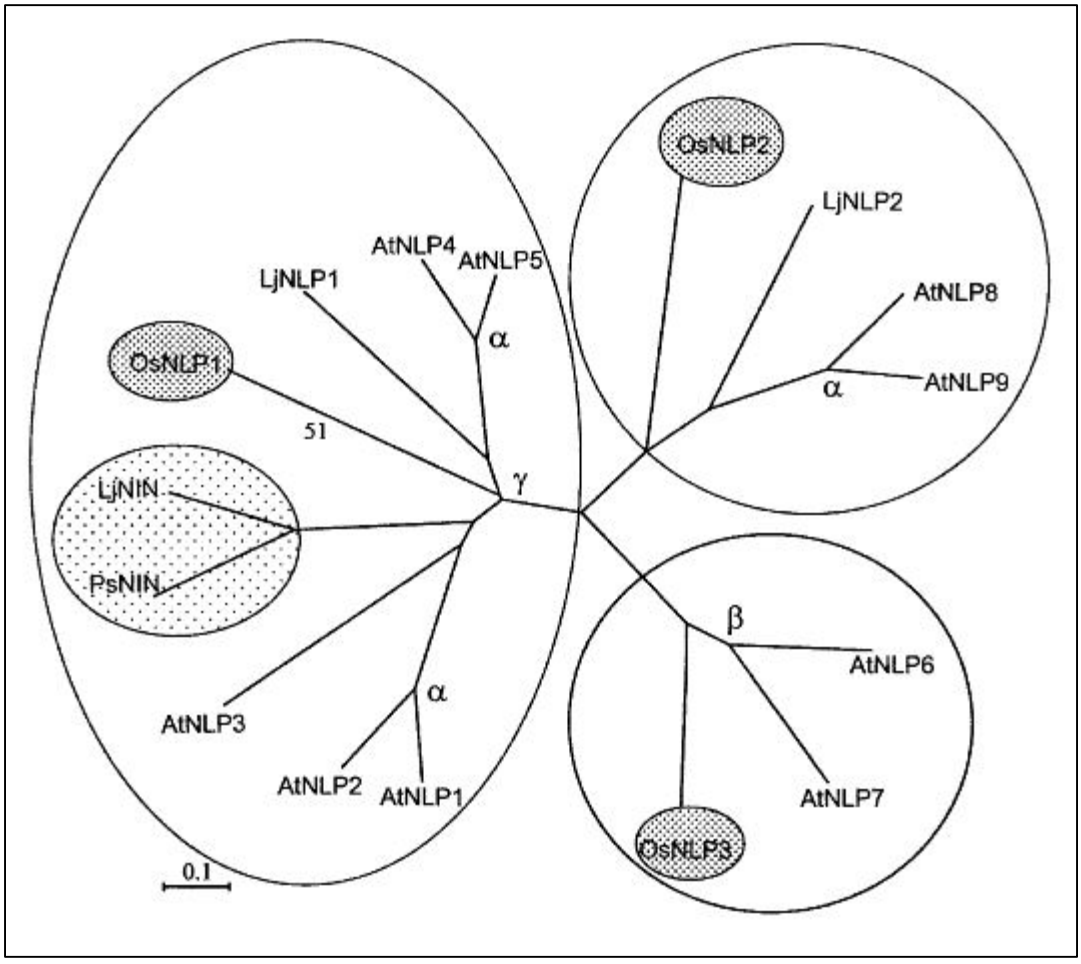
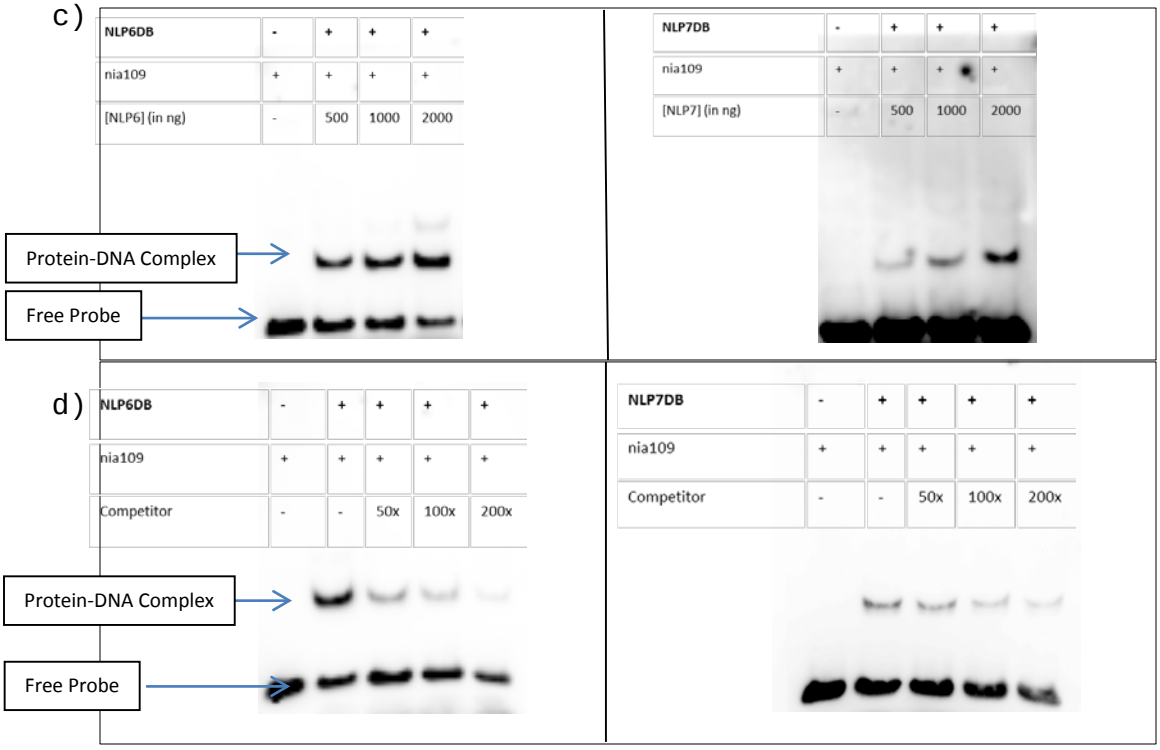


Figure 1. Phylogenetic analysis of the NIN-like protein family (Schauser et al., 2005). Sequence alignment of amino acids from the different NLPs was used to construct of this phylogenetic tree. Arabidopsis NLP6 and NLP7 shared a common ancestor until approximately 150 million years ago, where a duplication event occurred.

a) AtNLP6
VKKSERKRGKTEKTISLEVLQQYFAGSLKDAAKSLGVCPTTMKRICRQHGISRWPSRKINKVNRSLTRLKHVIDSVQGA
AtNLP7
KKKTEKKRGKTEKTISLDVLQQYFTGSLKDAAKSLGVCPTTMKRICRQHGISRWPSRKIKKVNRSITKLKRVIESVQGT

b) Nia109-bp probe:
TCGAAAATTCAAACAAGTGACAAAAATAATATTACGACCCGTCCCTTTGTTTGGTTGCCCCGTTC
GTGTATTTGCTTGGCCGTTGGTGTTCCCATGCGCCACTTAGCCT

c)



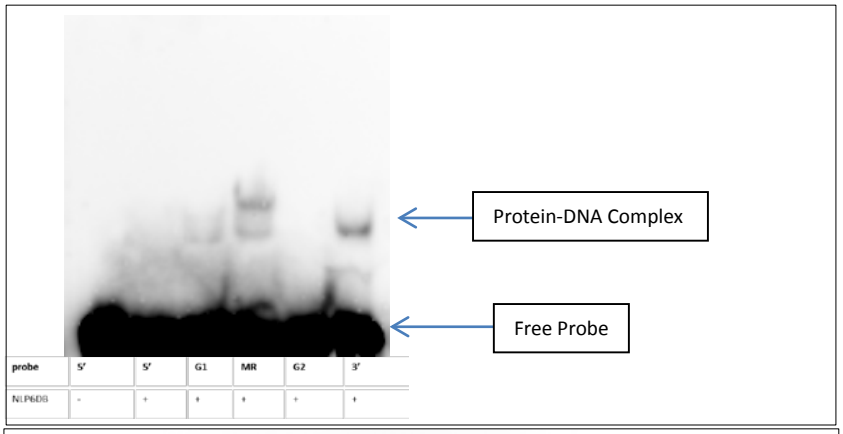
d)

Figure 2. DNA binding activities of NLP6 and NLP7 transcription factors to the biotin labeled 109-bp enhancer fragment.

a) Amino acid sequence comparison between the DNA binding domains of NLP6 and NLP7 showing roughly 87% sequence similarity. Sequence in gray indicates the conserved RWP-RK conserved motif.
b) Sequence of the N-responsive nia109-bp *cis*-element used in EMSA experiment.
c) EMSA images of NLP6 (left) and NLP7 (right) DB with biotin labeled nia109-bp probes. The first lanes in both images contained nia109-bp biotin-labeled free probes. The second, third, and fourth lanes in both images contained increasing concentrations of NLP6 (left image) and NLP7 protein (right image).
d) EMSA images of NLP6 (left) and NLP7 (right) DB domain with biotin labeled nia109-bp probes in the presence of 50, 100, and 200 fold molar excess of non-labeled nia109-bp competitor probes.

a)
```
5′: TCGAAAATTCAAACAAGTGACAAAAATAATATTA
G1:               CAAGTGACAAAAATAATATTACGACCCGTCCCTTTG
MR:                           TTACGACCCGTCCCTTTGTTTGGTTGCCCCGTTCGTG
G2:                                                   GTTGCCCCGTTCGTGTATTTGCTTGGCCGTTGGTG
3′:                                                               GTGTATTTGCTTGGCCGTTGGTGTTCCCATGCGCCACTTAGCCT
```

b)



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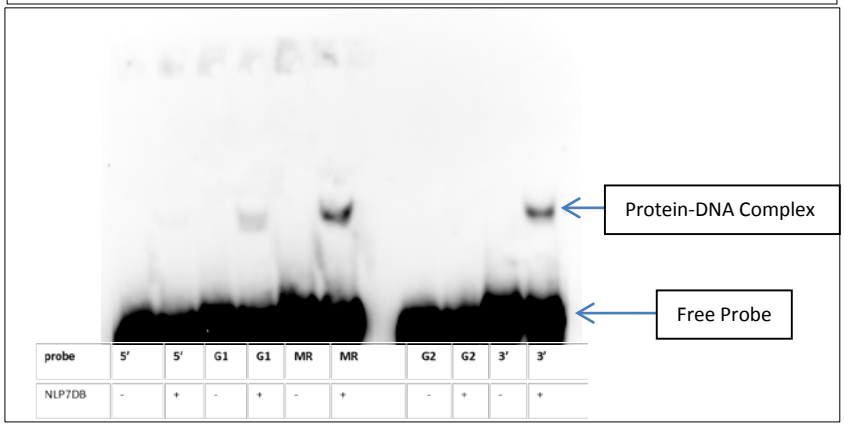
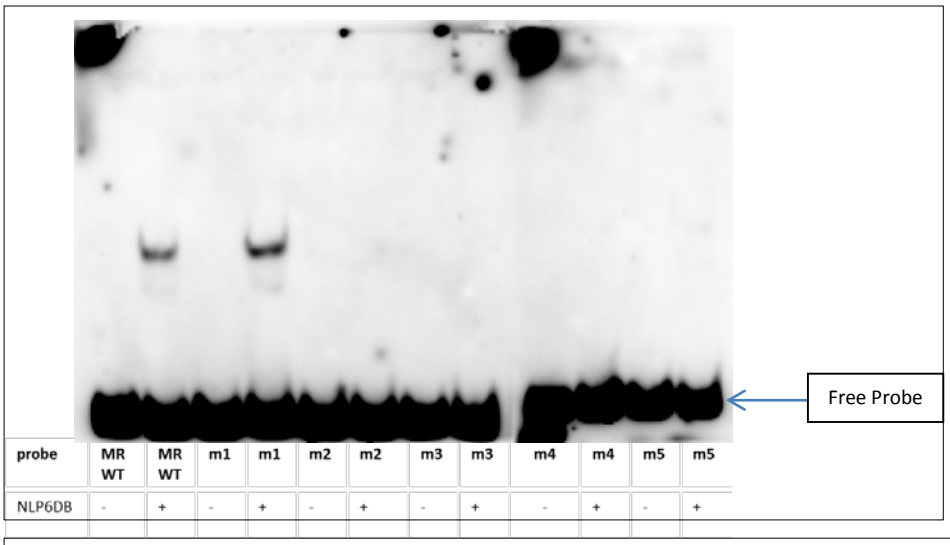


Figure 3. NLP6 and NLP7 DB bind strongly to the middle (MR) and 3 prime (3') region of nia109-bp enhancer.

a) List of the five biotin-labeled overlapping probes spanning the nia109-bp enhancer fragment
b) Gel shift assay of NLP6 DB with the five overlapping probes of nia109-bp enhancer fragment. The lengths of 5', G1, MR, G2, and 3' probes were 34, 36, 37, 35, and 44 bp, respectively. The first lane contained biotin-labeled 5' free probe. In lanes 2, 3, 4, 5, and 6, NLP6 DB protein was used along with biotin-labeled 5', G1, MR, G2, and 3' probes, respectively.
c) Gel shift assay of NLP7 DB with the five overlapping probes of nia109-bp enhancer fragment. The lengths of 5', G1, MR, G2, and 3' probes were 34, 36, 37, 35, and 44 bp, respectively. Odd numbered lanes 1,3,5,7 and 9 contained 5', G1, MR, G2, and 3' biotin-labeled free probes, respectively. NLP7 DB protein was used in the even-numbered lanes 2,4,6,8, and 10.

a) MR: TTACGACCCGTCCCTTTGTTTGGTTGCCCCGTTCGTG
MRm1 TCTGTACCCGTCCCTTTGTTTGGTTGCCCCGTTCGTG
MRm2: TTACGCGAAGTCCCTTTGTTTGGTTGCCCCGTTCGTG
MRm3: TTACGACCCACAACTTTGTTTGGTTGCCCCGTTCGTG
MRm4: TTACGACCCGTCCTCCCGTTTGGTTGCCCCGTTCGTG
MRm5: TTACGACCCGTCCCTTTACACGGTTGCCCCGTTCGTG

b)



c)

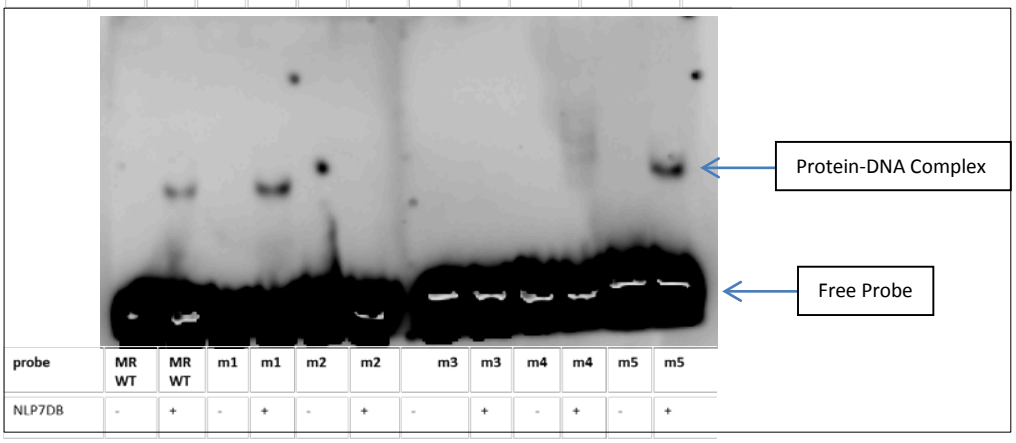
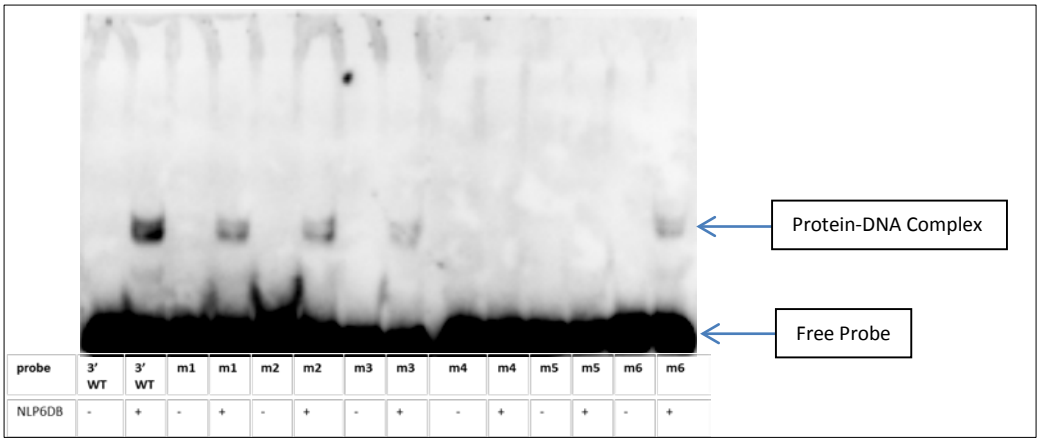


Figure 4. Mutant analysis of the 37bp middle region of nia109-bp enhancer fragment.

a) List of the five middle region probes, each containing a 4bp mutation. Underlined letters indicate mutated nucleotides compared to wild-type sequence.
b) EMSA image of NLP6 DB binding activity to the five mutated versions of MR. Lane 2, a positive control, shows binding of NLP6 DB to the Wild-type MR probe. NLP6 DB protein only binds to m1, but not m2 – m5 of the mutant probes.
c) EMSA image of NLP7 DB binding activity to the five mutated versions of MR. Lane 2, a positive control, shows binding of NLP7 DB to the Wild-type MR probe. NLP7 DB protein binds to mutant probes m1 and m5; no binding was detected for m2 – m4.

a) 3′WT: TGGCCGTTGGTGTTCCCATGCGCCACTTAGCCTTGCA
3′m1: TGGCCGTTATCATTCCCATGCGCCACTTAGCCTTGCA
3′m2: TGGCCGTTGGTGGCATCATGCGCCACTTAGCCTTGCA
3′m3: TGGCCGTTGGTGTTCCTGGACGCCACTTAGCCTTGCA
3′m4: TGGCCGTTGGTGTTCCCATGTAATACTTAGCCTTGCA
3′m5: TGGCCGTTGGTGTTCCCATGCGCCGACCAGCCTTGCA
3′m6: TGGCCGTTGGTGTTCCCATGCGCCGACTGAACTTGCA

b)



c)

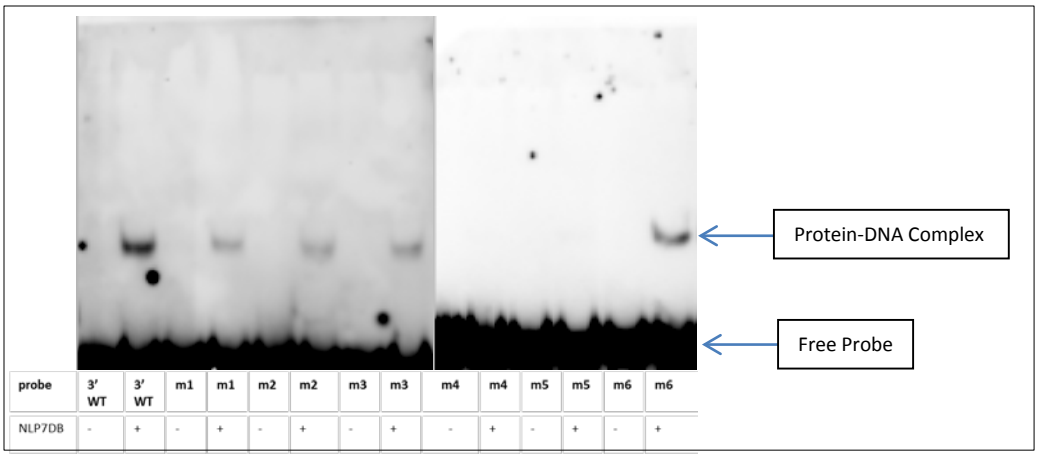
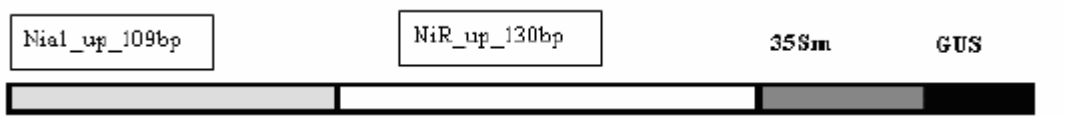


Figure 5. Mutant analysis of the 37bp 3 prime end of nia109-bp enhancer fragment.

a) List of the six nia109-bp 3 prime probes, each containing a 4bp mutation. Underlined letters indicate mutated nucleotides compared to wild-type sequence.
b) EMSA image of NLP6 DB binding activity to the six mutated versions of the 3' probe. Lane 2, a positive control, shows binding of NLP6 DB to the Wild-type 3' probe. Bands that are indicative of protein-DNA complex formation appear for m1, m2, m3, and m6; but not for m4 and m5.
c) EMSA image of NLP7 DB binding activity to the six mutated versions of the 3' probe. Lane 2, a positive control, showed binding of NLP7 DB to the Wild-type 3' probe. NLP7 binds to mutants m1 – m4 and m6, but not to mutants m4 and m5.

a)



b)

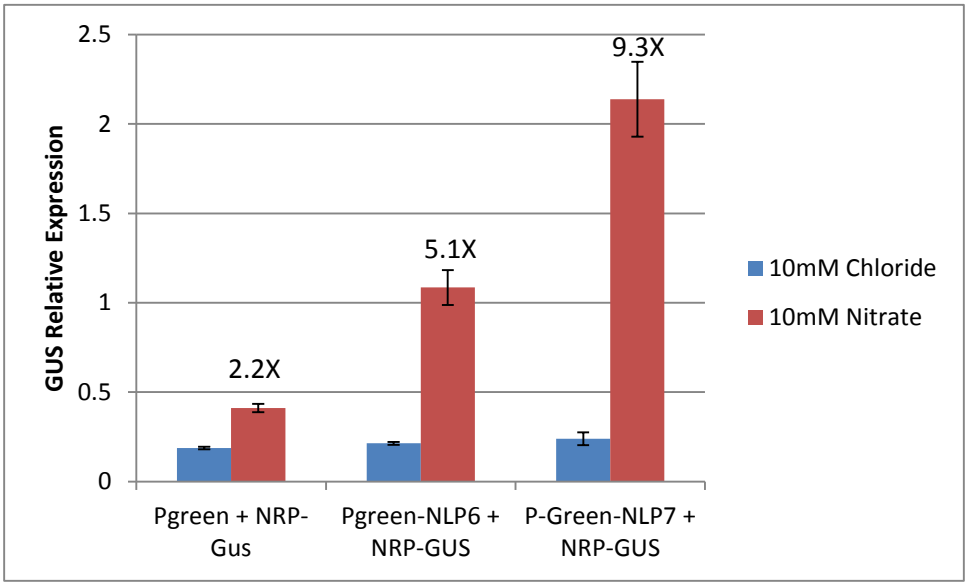


Figure 6. Nitrate induction of NRP-GUS reporter gene activity.

a) Schematics of the NRP tripartite construct. Nia1 is an enhancer fragment from the nitrate reductase NiA1 gene. NiR is an enhancer fragment from the nitrite reductase NiR gene. The 35S minimal promoter is unable to promote transcription of the reporter gene in the absence of enhancer activity.

b) *Nicotiana benthamiana* plants were grown for 3 weeks in perlite and irrigated with nitrate-free hydroponic solution. Prior to agroinfiltration, plants were watered with either 10mM of nutrient solution containing nitrate or 10mM nutrient solution containing KCl. Leaves of *Nicotiana benthamiana* were co-infected with agrobacteria containing NRP-GUS construct and either empty vector, NLP6 construct, or NLP7 construct. GUS assay was performed 3 days post-infection. Experiments were done with n=4 for each experimental condition, error bars showing the standard error (SE).

REFERENCES

Castaings L., Camargo A., Pocholle D., Gaudon V., Texier Y.. (2009). The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. *Plant Physiology 57,* 426-435.

Crawford N., Herbert A. (1993). The molecular genetics of nitrate assimilation in fungi and plants. *Annual Reviews Genetics 27,* 115-146.

Dechorgnat J., Nguyen C., Armenguad P., Jossier M., Diatloff E., Filleur S., Vedele F. (2011). From the soil to the seeds: the long journey of nitrate in plants. *Journal of Experimental Botany 62,* 1349-1359.

Goodin M., Zaitlin D., Naidu A., Lommel A. (2008). Nicotiana benthamiana: its history and future as a model for plant pathogen interactions. *Molecular Plant-Microbe Interactions 21,* 1015-1026.

Ho C., Tsay Y. (2012). Nitrate, Ammonium, and Potassium Sensing and Signaling. Current Opinion in Plant Biology 13, 604-610.

Hu H., Wang Y., Tsay Y. (2009). AtCIPK8, a CBL Interacting Protein Kinase, Regulates the low-affinity Phase of the Primary Nitrate Response. The Plant Journal *57,* 264-278.

Hwang C., Lin Y., D'Souza T., Cheng C. (1997). Sequences necessary for nitrate-dependent transcription of Arabidopsis nitrate reductase genes. *Plant Physiology 113,* 853-862.

Konishi M., Yanagisawa S. (2011). Roles of the transcriptional regulation mediated by the nitrate-responsive cis-element in higher plants. *Biochemical and Biophysical Research Communications 411,* 708-713.

Krouk G., Crawford M. N., Coruzzi M. G., Tsay Y. (2010). Nitrate signaling: adaptations to fluctuating environments. *Current Opinion in Plant Biology 13,* 266-273.

Llave, C., Kasschau, K. D., & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences of the United States of America 97,* 13401-13406.

Orsel M., Chopin F., Leleu O., Smith S., Krapp A., Vedele F., Miller A. (2006). Characterization of the two-component high-affinity nitrate uptake system in Arabidopsis Physiology and Protein-Protein Interaction. *Plant Physiology 142,* 1304-1317.

Schlüter U., Mascher M., Colmsee C., Scholz U., Bräutigam A. (2012). Maize source leaf adaptation to nitrogen deficiency effects not only N and C metabolism but also control of P homeostasis. Plant Physiology Preview (unpublished)

Schauser L., Andreas R., Jiri S., Jens S. (1999). A plant regulator controlling development of symbiotic root nodules. Nature *402,* 223-228.

Schauser L., Wieloch W., Stougaard J. (2005). Evolution of NIN-like proteins in Arabidopsis, rice and Lotus Japonicus. *Journal of Molecular Evolution 60*, 229-237.

Spiertz J.H.J. (2009). Nitrogen, sustainable agriculture and food Security. A review. *Agron. Sustain. Dev. 30*, 43-55.

Tsay Y., Chiu C., Tsai C., Ho C., Hsu P. (2007). Nitrate transporters and peptide transporters. *FEBS Letters 581*, 2290-2300.

Wang R., Mamoru O., Xiujuan X., and Nigel M. C. (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 Physiology 132*, 556-567.

Wang R., Karl G., Samuel T. L., and Nigel M. C. (2000). Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *The Plant Cell 12*, 1491-1509.

Wang R., Guan P., Chen M., Xing X., Zhang Y., Crawford N. (2010). Multiple regulatory elements in the Arabidopsis NIA1 promoter act synergistically to form a nitrate enhancer. *Plant Physiology 154*, 423-432.

Wang R., Xing X., Wang y. (2009). A Genetic screen for nitrate regulatory mutants captures the nitrate transporter gene NRT1.1. *Plant Physiology 151*, 472-478.

Wang Y., Hsu P., Tsay Y. (2012). Uptake, allocation and signaling of nitrate. *Trends in Plant Science 17*, 1360-1385.