DISSERTATION

CHARACTARIZATION OF A NITRATE RESPONSIVE MYB TRANSCRIPTION FACTOR IN *ARABIDOPSIS*

Submitted by

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ABSTRACT

CHARACTARIZATION OF A NITRATE RESPONSIVE MYB TRANSCRIPTION FACTOR IN *ARABIDOPSIS*

NRM1 (AT1g13300) is a gene that was initially uncovered in a microarray experiment where *Arabidopsis* was starved of nitrate and then re-fed varying concentrations of nitrate. NRM1 was one of a few genes that was up-regulated in all treatments. NRM1 has a single MYB like domain and a leucine-zipper like domain. We hypothesized that NRM1 is a transcription factor that plays a role in the plants response to nitrate availability.

NRM1-GFP fusions showed that NRM1 was localized to the nucleus of the cell. We did not see any evidence of differential localization of NRM1 when examining its location under changing levels of nitrate availability. The yeast two hybrid system was utilized to test if NRM1 interacts with itself to form a homodimer or with another protein to form a heterodimer that would contain the two MYB domains needed for specific DNA binding. No protein - protein interactions were found in our experiments nor are there reports of NRM1 interactions in publicly available databases of interactions.

Recombinant expression plasmids containing NRM1 have been constructed and the protein expression has been examined. Purification of recombinant protein was successful but purity of the product needs to be improved. Once the pure protein is obtained, antibodies will be made and chromatin immuno precipitation experiments can be performed.

Experiments performed with NRM1 promoter::GUS plants confirmed that NRM1 had reduced expression in response to nitrate and phosphate starvation. The experiments also showed NRM1 had reduced expression in response to calcium and sulphate starvation. Spatial expression patterns showed that NRM1 was mostly expressed in areas of lateral root formation and in the elongation zone of the root. In young seedlings (5-9 days) NRM1 showed expression in a large portion of the root but not in the root tip. In older plants (10-18 days) NRM1 was expressed in the lower portion of the root, but, again not in the root tip. This suggests that NRM1 may play a role in root elongation and lateral root emergence.

NRM1 is part of a family of genes that contains two closely related genes NRM2 and MRM3. NRM3 is most similar to NRM1 and has a similar expression profile. NMR2 is slightly less similar and is expressed throughout the plant. To determine what role NRM1 might play in root architecture, NRM1-NRM2 double mutants, NRM1 overexpressors and NRM1-NRM3 microRNA plants were made. NRM1-NRM2 double mutant plants showed shorter primary roots than wild type plants when grown on low nitrate media. These plants also produced fewer lateral roots when grown on complete, low nitrate and low phosphate media. Plants overexpressing NRM1 showed differences in root architecture but these changes were not consistent between lines. Further experiments will need to be performed to deduce exactly how these changes in root architecture are related to the function of NRM1. NRM1-NRM3 microRNA plants are being produced, but are not ready for analysis at this point in time.

Some evidence that NRM1 is a transcription factor and is involved in the root architecture response to nitrate has been uncovered and several tools to further explore its function have been developed.

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Chapter 1

Introduction

Introduction

Nitrogen is an essential macronutrient for plant growth. Nitrogen is incorporated into the fundamental building blocks of life: nucleotides and amino acids (Dechorgnat et al. 2011). Nitrate (NO_3^{2-}) is the primary form of nitrogen absorbed by plants from the soil. Nitrate is also the nutrient that most limits plant productivity (Vance 2001). *Arabidopsis thaliana* response to nitrate is very complex and includes changes in the expression of many genes, up to 10% of the detectable transcriptome (Krouk et al. 2010). Some of the best known and most important members of the nitrate response pathway are: NIN Like Protein 7 (NLP7), Ethylene Insensitive 2 (EIN2), CBL-Interacting Protein Kinase 8 (CIPK8), *Arabidopsis* Nitrate Refulated 1 (ANR1), Auxin signaling F-box 2 (AFB3) (Walch-Liu and Forde 2008; Gojon et al. 2011). *Arabidopsis* uses members of the Nitrate Transporter (NRT) family of transporters to acquire nitrate from the soil and some of these transporters also play a role in the nitrate signaling pathway (Little et al. 2005; Remans et al. 2006; Miller et al. 2007; Lin et al. 2008; Mounier et al. 2013). Nitrate can be converted to ammonia by nitrate reductase and nitrite reductase (Crawford 1995). The ammonia is then incorporated into organic molecules by glutamine synthase after which it can be eventually incorporated into the many nitrogen containing molecules in the plant (Figure 1.1). Because nitrogen is such an essential element it is very important for the plant to maximize acquisition from the soil while minimizing expenditure of resources in root production.

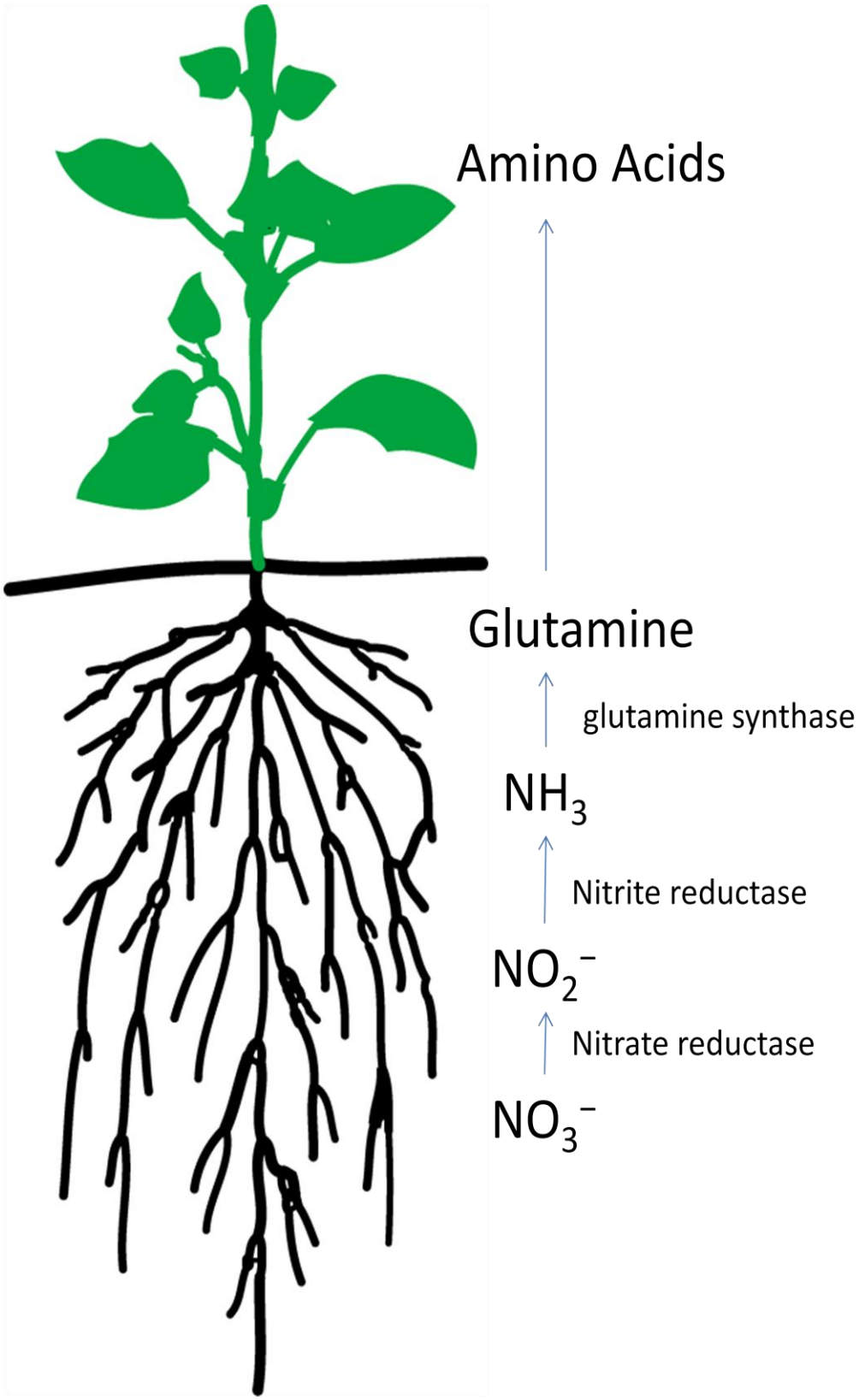


Figure 1.1 Nitrogen Uptake and Assimilation in *Arabidopsis*

Phosphate (PO_4^{3-}) is another essential macronutrient for plants. Phosphate is the second most limiting nutrient in plants after nitrogen (Todd et al. 2004). Phosphate is required in important molecules including nucleotides and cell membranes (Peret et al. 2011). Phosphate in the soil is much less mobile than nitrate. The most common source of phosphate fertilizers (rock phosphate) is a non-renewable resource and will be depleted within 60-90 years (Hammond et al. 2004). Thus, understanding the key steps in phosphate acquisition is important for increasing uptake efficiency. The major genes in the phosphate signaling pathway discovered to date are Phosphate 2 (PHO2), microRNA399 and Photolyase 1 (PHR1) (Bari et al. 2006). Phosphate is must also be acquired in as large quantities as possible with minimal investment of resources.

Root architecture changes in varied ways in response to nitrate and phosphate availability(Linkohr et al. 2002; Osmont et al. 2007; Desnos 2008; Smith and De Smet 2012) (Figure 1.2). Root architecture can be altered in three distinct ways: primary root elongation, lateral root proliferation (Malamy and Ryan 2001) and root hair formation (Lopez-Bucio et al. 2002; Lopez-Bucio et al. 2003). Each of these alter the plant's ability to mine the soil for these vital nutrients. Primary root elongation is induced by low nitrate supply. Lateral root development can be induced by local patches of high nitrate and inhibited by uniformLy high nitrate in media or a high sucrose to nitrate ratio (Casimiro et al. 2003). This response helps to access the greatest volume of soil.

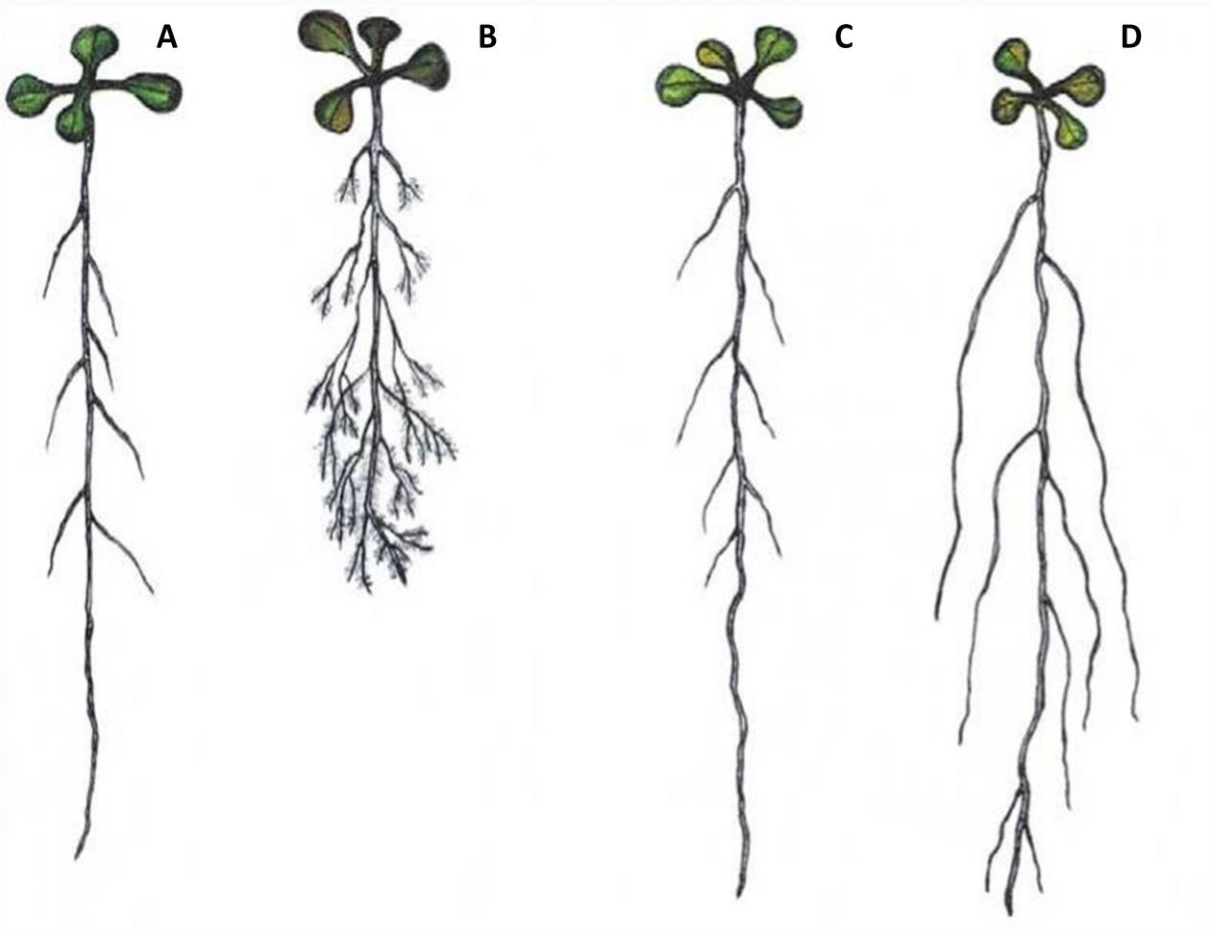


Figure 1.2 Root architecture changes due to nutrient starvation

A. Murashige and Skoog Media plus 1% Sucrose with standard Phosphate (1.25mM)

B. Murashige and Skoog Media plus 1% Sucrose with reduced Phosphate (0mM)

C. Murashige and Skoog Media plus 1% Sucrose with standard Nitrate (60mM)

D. Murashige and Skoog Media plus 1% Sucrose with reduced Nitrate (6mM)

Modified from figure obtained in (Lopez-Bucio et al. 2003)

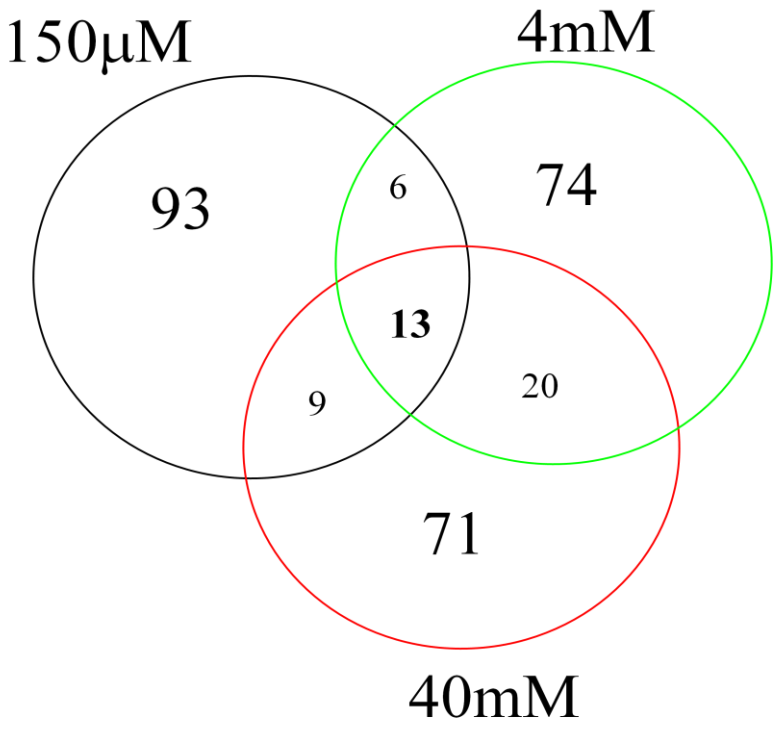
The root response to phosphate is quite different from nitrate. Primary roots are significantly shorter in response to low phosphate (Webb and Loneragan 1985; Linkohr et al. 2002). In low phosphate conditions lateral root number and elongation is increased (Williamson et al. 2001). The combination of more lateral roots and a shorter primary root increases lateral root density (Peret et al. 2009), which maximizes surface area in the upper portion of the soil, which is where phosphate is most concentrated (Lynch 2001). These differences in response to nitrate and phosphate are thought to occur because nitrate is much more mobile in the soil. Localized high concentrations of phosphate have also been shown to increase lateral root density and elongation (Linkohr et al. 2002). Plants respond to localized areas of nutrients in order to take advantage of different portions of heterogeneous soil. In the case of nitrate this is also thought to be a response to competition with other plants rather than being due to a necessity to absorb the nitrate (Hodge et al. 1999; Svistoonoff et al. 2007). In addition to external phosphate, the concentration inside the whole plant also affects root morphology. In split root experiments where half of the roots were placed on high phosphate and half on low phosphate the roots on low phosphate media did not show reduced primary root length or increased lateral root growth (Bonser et al. 1996). This is presumably due to plants maintaining phosphate supply via the roots on high phosphate. In contrast, *Arabidopsis* with a mutation in the nitrate reductase gene, which causes the plants to have low nitrate concentration, did not show an altered root response (Zhang and Forde 1998; Zhang et al. 2007) on plates with half high nitrate and half low nitrate, suggesting local nitrate concentration in the soil is responsible for changes in root architecture. These differences suggest quite different pathways for root architecture response to either nitrate or phosphate.

Background

The focus of my research is to learn more about plant responses to nitrate availability. To identify early players in the nitrate signaling pathway a microarray experiment was designed. In this experiment *Arabidopsis thaliana* Columbia (Col-0) plants were grown for 2 weeks on nitrogen replete Murashige and Skoog (MS) media, and then grown for 4 days on nitrogen free media. Previous work in the lab indicated that 4 days of nitrogen starvation is enough time for the plants to use all of its nitrogen reserves. The plants were then transferred to modified MS plates containing 150µM, 4mM or 40mM potassium nitrate or as a control potassium chloride. The roots were harvested 20 minutes after transfer, and RNA was purified for microarray gene expression analysis. We chose the 20 minute time point in order to identify early response genes. Thirteen genes were found to be significantly induced at all 3 concentrations of nitrate (Figure 1.3). This list included genes known to be induced by nitrate, such as nitrate reductase, which provides confidence in the data set. Within this set of genes there were 2 related putative MYB like transcription factors AT1g13300 and AT1g25550. Our lab named these 2 genes Nitrate Responsive MYB like transcription factor 1 and 2 or NRM1 and NRM2.

When NRM1 and NRM2 were searched against the *Arabidopsis* genome using BLAST five other very similar genes were discovered; these genes were named NRM3-7 (Figure 1.4). NRM1 was most similar to NRM3 (57% amino acid identity) and NRM2 was most similar to NRM 4 (59% amino acid identity)(Figure 1.5). RT-PCR showed that NRM1 was expressed

A



B

Gene ID	Name
At1g13300	Myb like transcription factor
At1g25550	Myb like transcription factor
At1g77760	nitrate reductase 1 (NR1)
At2g15620	ferredoxin--nitrite reductase
At1g24280	glucose-6-phosphate 1-dehydrogenase
At1g78040	phosphoglycerate mutase 1 like protein
At3g44870	AtPP -like protein
At4g18340	beta-1,3-glucanase-like protein
At5g13930	chalcone synthase
At5g41670	6-phosphogluconate dehydrogenase
At2g33550	Unknown
At3g49940	Unknown
At3g54900	Unknown

Figure 1.3 Genes Induced by Starvation and Readdition of different concentrations of Nitrate

Seedlings were starved of nitrate for 4 days then transferred to modified MS plates containing 150µM, 4mM or 40mM potassium nitrate. The roots were harvested 20 minutes after transfer and RNA was purified for microarray gene expression analysis.

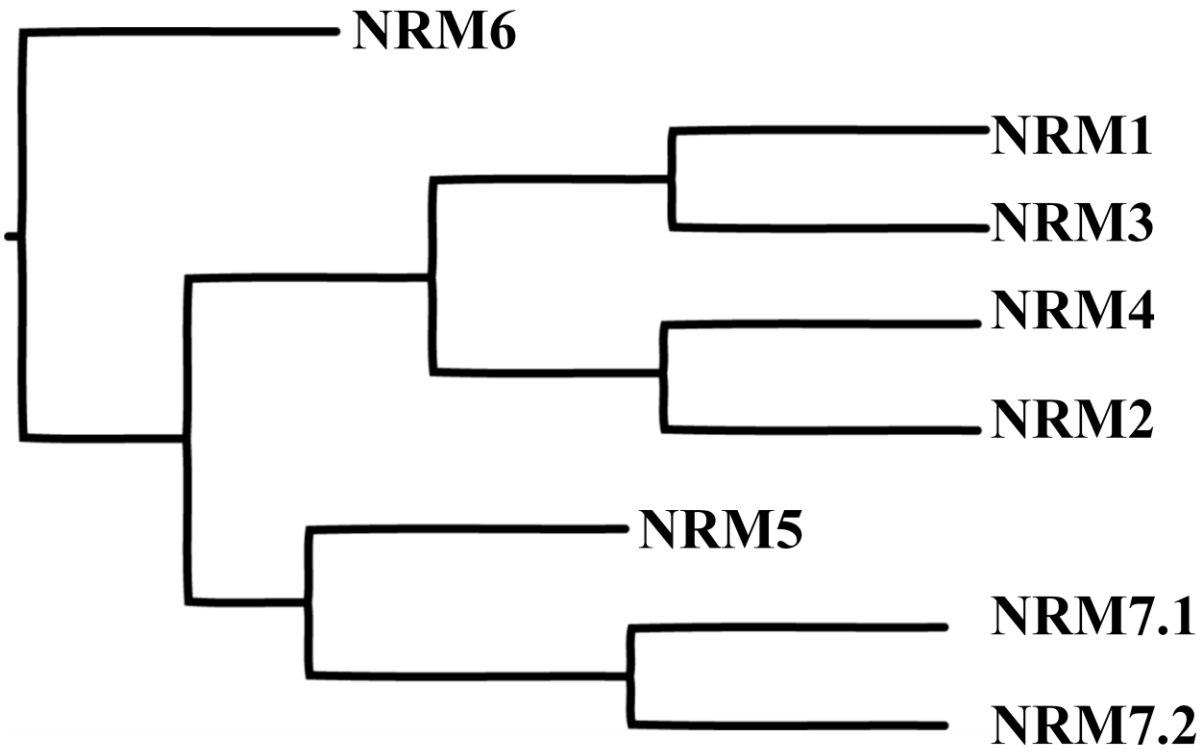
A. Diagram of number of genes induced in each treatment

B. List of genes induced in all three treatments

When NRM1 and NRM2 were searched against the *Arabidopsis* genome using BLAST five other very similar genes were discovered; these genes were named NRM3-7 (Figure 1.4). NRM1 was most similar to NRM3 (57% amino acid identity) and NRM2 was most similar to NRM 4 (59% amino acid identity)(Figure 1.5). RT-PCR showed that NRM1 was expressed predominantly in roots while NRM2 was expressed in most/all tissues tested (Figure 1.6). This data was confirmed by publicly available microarray data (Schmid et al. 2005). In addition to their amino acid similarities, NRM 1 and NRM3, and NRM2 and 4 shared similar expression profiles (Figure 1.7 and 1.8).

The NRM gene family is part of the larger Golden 2 like transcription factor family (Rossini et al. 2001)(Figure 1.9), which is notable in that all members have a single MYB DNA binding domain (as opposed to 2 that are known to be required for specific DNA binding (Ording et al. 1994) and a leucine zipper-like domain (Figure 1.10). We hypothesized that NRMs work as homo-or hetero-dimers, as has been demonstrated for other single MYB domain proteins (Spink et al. 2000; Zhai et al. 2010).

Recent data suggests that NRM1 function may not be limited to plant response to nitrate, but may have a broader role in nutrient signaling. For example when over-expressed, NRM1 was shown to cause hypersensitivity to inhibition of primary root growth in low phosphate conditions (Liu et al. 2009). This suggests that NRM1 might be involved in a central response that is sensitive to multiple major nutrients.



Gene ID number	
At1g13300	**NRM1**
At1g25550	**NRM2**
At3g25790	**NRM3**
At1g68670	**NRM4**
At2g03500	**NRM5**
At1g49560	**NRM6**
At4g37180	**NRM7**

Figure 1.4 NRM Family in *Arabidopsis*

	NRM1	NRM2	NRM3	NRM4	NRM5	NRM6	NRM7
NRM1	100%*	43%	57%	46%	34%	27%	26%
NRM2		100%	42%	59%	33%	23%	28%
NRM3			100%	43%	29%	24%	28%
NRM4				100%	34%	27%	28%
NRM5					100%	21%	31%
NRM6						100%	17%
NRM7							100%

Figure 1.5 NRM Family Percentage Amino Acid Identity

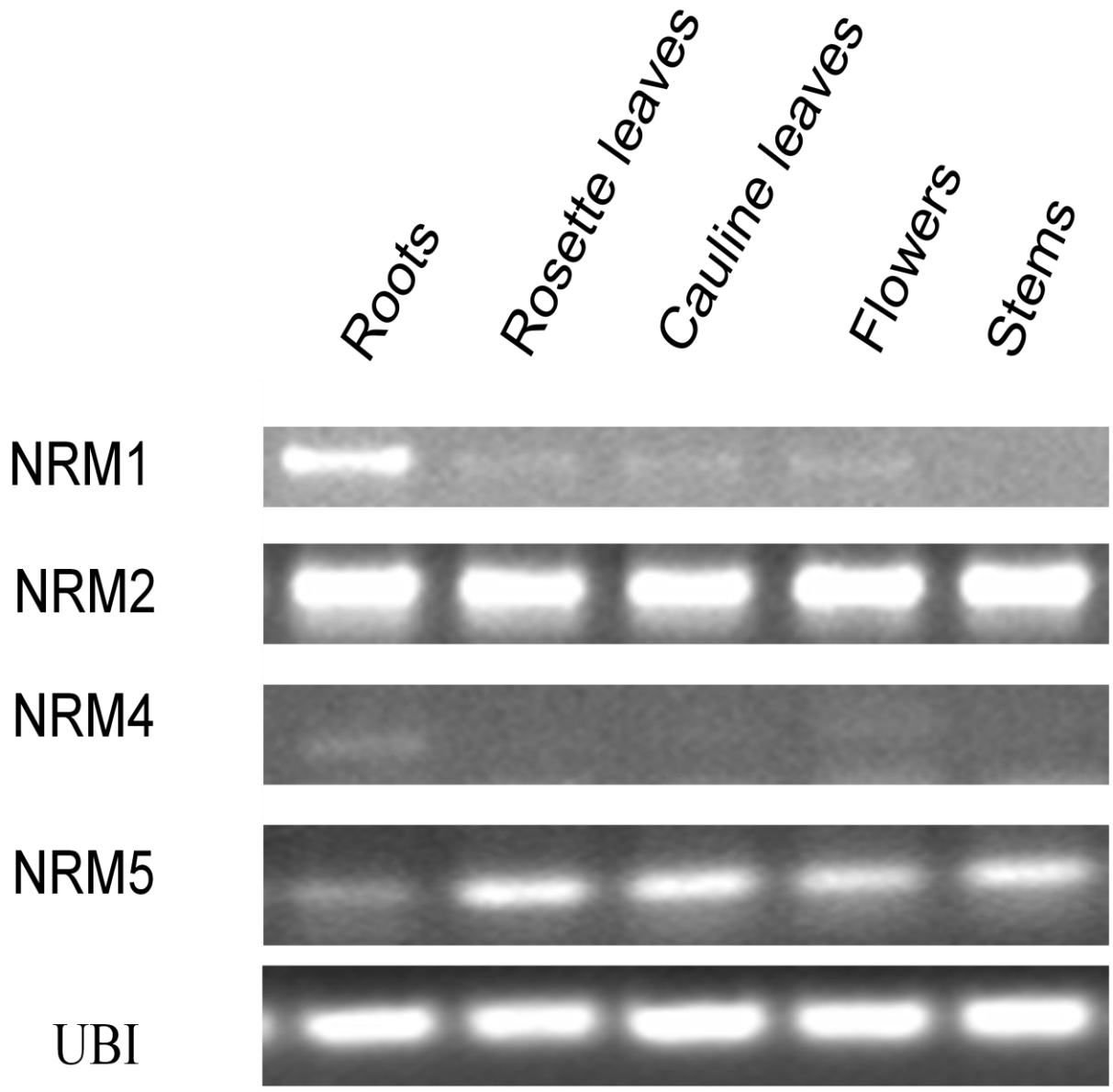


Figure 1.6 NRM Family Expression in Different Tissues

Expression of NRM1, 2, 4, 5 and Ubiquitin in various tissues in mature *Arabidopsis*. NRM3 expression was below detectable limits in this experiment.

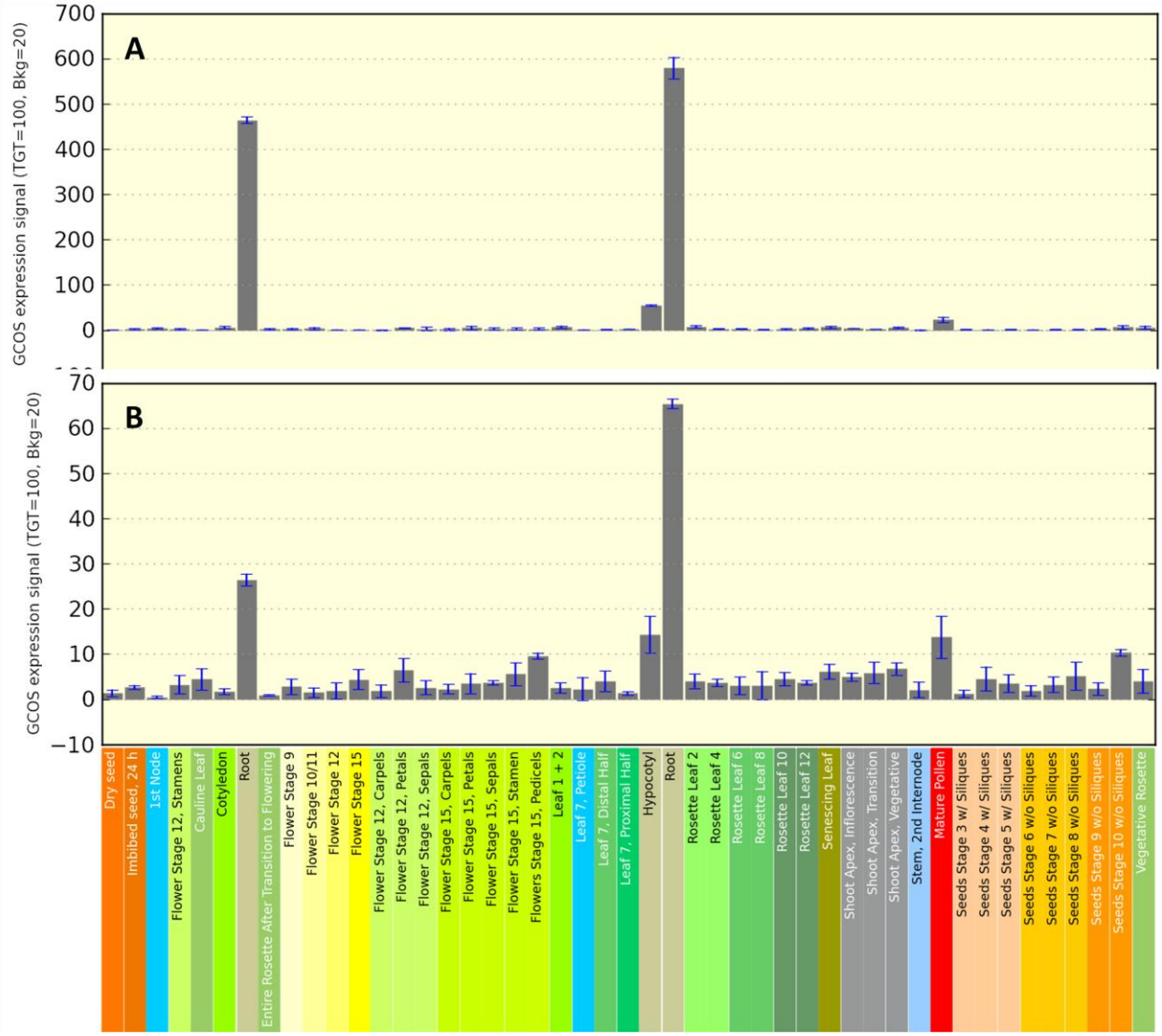


Figure 1.7 Expression Profile of NRM1 and NRM 3

A. NRM1

B. NRM3

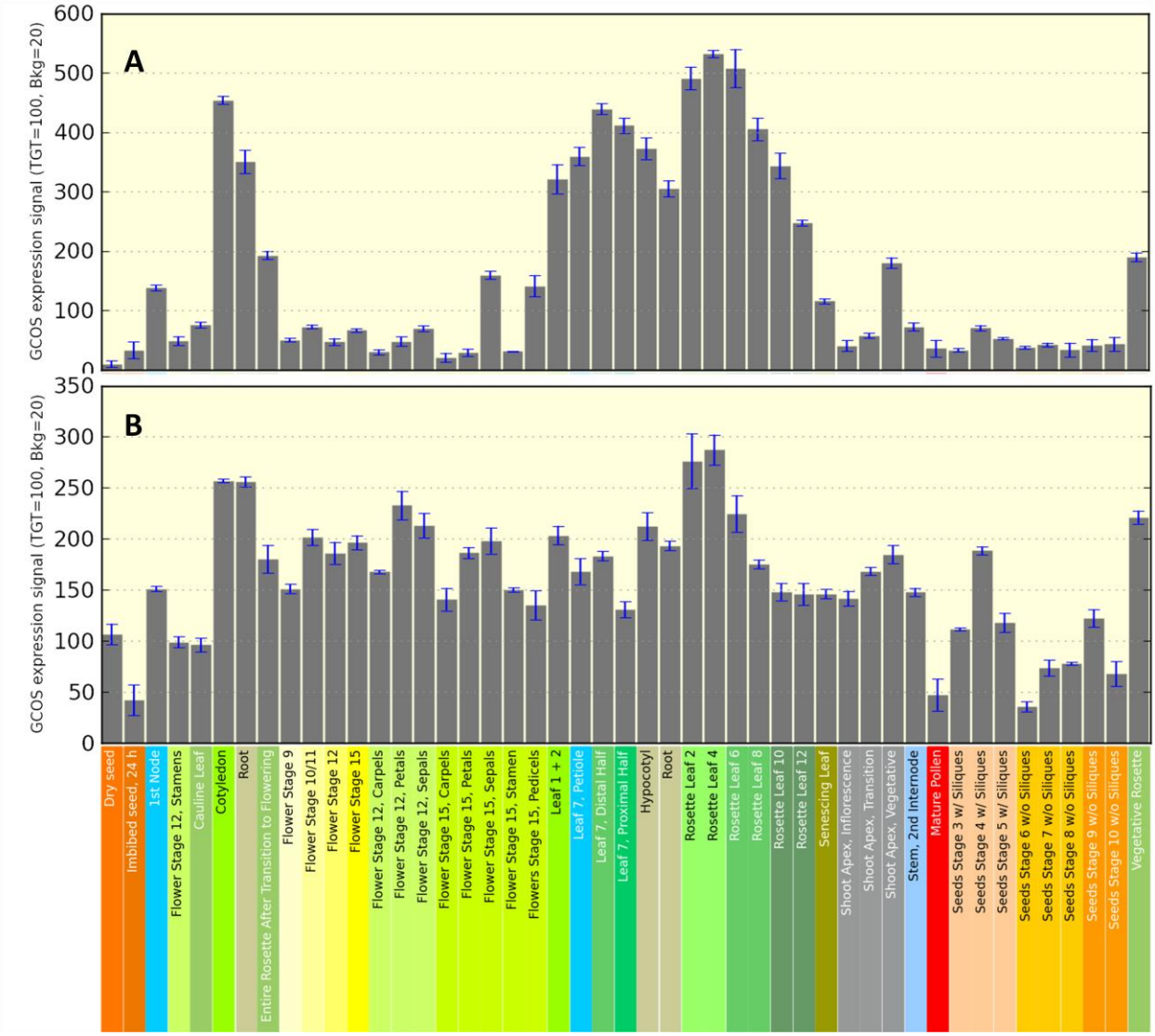


Figure 1.8 Expression Profile of NRM2 and NRM 4

A. NRM2

B. NRM4

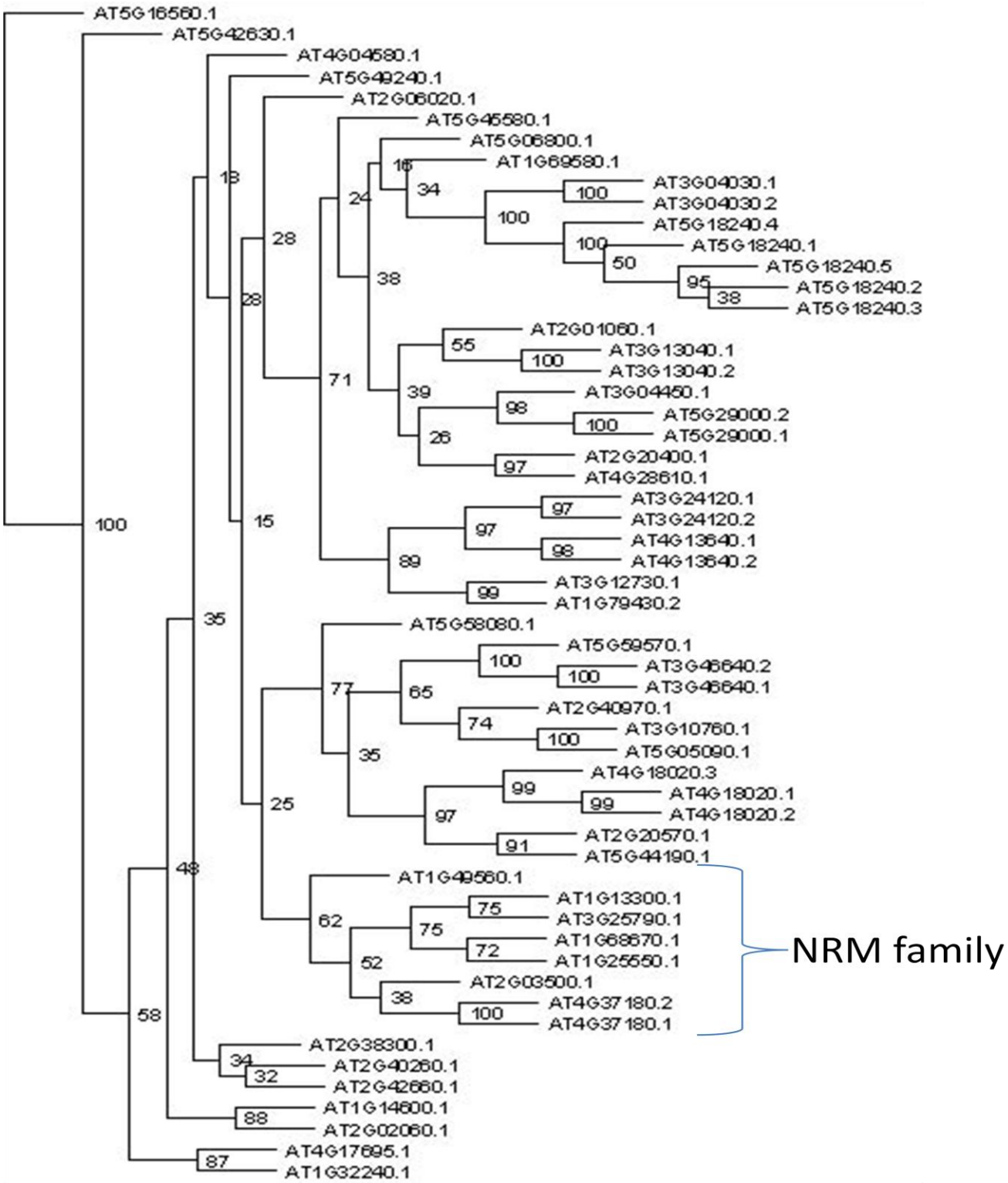


Figure 1.9 Golden 2 like Family in *Arabidopsis*

Gene family tree obtained from Database of *Arabidopsis* Transcription Factors

http://datf.cbi.pku.edu.cn/index.php

NRM1

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETMT

RDESMRKDPMVNGGEGR**KREAEKDGGGGRKQRR**CWSSQLHRRFLNALQH

LGGPHVATPKQIREFMKVDGLTNDEVKSHLQKYRLHT**RRPR**QTVPNNGNS

QTQHFVVVGGLWVPQSDYSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQW

PSHSNYRPSIIVDEGSGSHSEGVVVRCSSPAMSSSTRNHYVKNN

Figure 1.10 NRM1 Domain Organization

Amino Acid sequence of NRM1. The Leucine zipper-like domain is highlighted in blue and the MYB like DNA binding domain is highlighted in red. Nuclear localization sequences are bold and underlined.

Recent data suggests that NRM1 function may not be limited to plant response to nitrate, but may have a broader role in nutrient signaling. For example when over-expressed, NRM1 was shown to cause hypersensitivity to inhibition of primary root growth in low phosphate conditions (Liu et al. 2009). This suggests that NRM1 might be involved in a central response that is sensitive to multiple major nutrients.

To evaluate the possibility that NRM1 is a transcription factor several experiments were performed and are described in the following chapters. To determine cellular localization of NRM1, fusion proteins were produced and analyzed by fluorescent microscopy. Because NRM1 only has one MYB domain, experiments were performed to determine if NRM1 interacts with other proteins using the yeast two hybrid system. In order to set the groundwork for future work to determine which DNA sequences NRM1 interacts with, recombinant NRM1 protein was produced in *E. coli.*

To determine if NRM1 has a role in the root architecture response to nutrient availability in *Arabidopsis*, two types of experiments were performed. GUS staining experiments were used to determine tissue localization and altered expression in response to nutrient starvation. The expression of NRM1 was altered through ectopic expression, micro RNA or double gene knockouts and plants with varying NRM1 expression levels were analyzed on a variety of media.

Chapter 2

Tissue Localization of NRM1

Introduction

In order to determine tissue specific patterns of expression and the temporal expression patterns, promoter GUS constructs were made and tested in a variety of conditions. To determine the tissue localization of NRM1, the beta-glucuronidase (GUS) (Battraw and Hall 1990) gene was expressed in transgenic *Arabidopsis* plants under the control of the sequence upstream of the NRM1 gene.

The GUS gene is used as a reporter system to determine expression pattern of genes in a variety of organisms. The GUS gene catalyzes the hydrolysis of certain complex carbohydrates that can be easily visualized or quantified. This gene is usually expressed under the control of a promoter sequence of a gene that is being studied. The GUS gene is very useful in determining the cell and tissue specific localization of target genes because the protein is not very mobile and the product of the reaction is also only slightly mobile. This makes localization in tissues and even specific cell types precise and easy to determine. To see where the GUS gene is expressed or quantify expression, the substrates need to be in direct contact with the enzyme. For this reason the substrates have to be either infiltrated into the plant tissues or mixed with protein extract. This makes following the expression of GUS impossible in living plants. To follow changes in expression over time a large number of plants are needed so that many plants can be tested at multiple time points.

To determine which nutrients might change the expression of NRM1, all transgenic plants contained the same GUS construct. These transgenic plants were then grown under a variety of nutrient deficient conditions and examined at various time points. Thus, the NRM1

promoter::GUS plants are useful to show changes in expression levels using a quantitative assay with extracts in response to nutrients but are also useful to examine changes in spatial expression patterns in intact tissues.

Materials and Methods

NRM1 Promoter: Gus Construct

The NRM1 promoter, defined as 1709 base pairs upstream of the start codon, was inserted in plasmid pBI101.3 upstream of GUS. The resulting construct was sequenced to confirm that no mutations were present and then transferred into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* (accession Col-0) was transformed using the floral dip as described (Clough and Bent 1998). Several independent insertion events were tested and were found to show the same GUS expression pattern. 35S::GUS seeds were also obtained to serve as a positive control for GUS experiments.

Media composition

For all assays modified Murashige and Skoog (MS) media was used (Murashige and Skoog 1962) . Macronutrient composition for complete and drop-out media is given in (Figure 2.1). Initially, macronutrients and micronutrients (the latter purchased as a 10X stock from Sigma (M0529)) were supplied as individual stocks, but more recently macronutrients and micronutrients were added as modified MS salt mixtures from Phytotech. The Phytotech media used was Murashige & Skoog modified basal salt mixture without nitrogen (M531) or Murashige & Skoog modified basal salt mixture without nitrogen, phosphorus and potassium

(M407). Potassium nitrate, potassium phosphate and potassium chloride were added from individual stocks at the concentrations listed in Table 1. Sucrose was added to 1% and the pH adjusted to 5.7. Agar was added at 8 grams per liter before autoclaving. After autoclaving the media was then poured into 110mm square gridded Petri plates and allowed to cool. For reference, a working protocol for media composition is given in Table 1.

Seed sterilization and growth conditions

The seeds were first sterilized to prevent contamination of the agar plates. To sterilize seeds, two different protocols were used. Initially, seeds were sterilized using ethanol and bleach as follows. Seeds (in 1.5 mL tubes) were incubated for 10 minutes in 1 mL of 95% ethanol. After removal of the ethanol the seeds were resuspended in 1 mL of 20% Clorox bleach with 0.1% Tween 20 for five minutes before the solution was removed by aspiration. The seeds were then rinsed 3 times with 1 mL of sterile water for at least 30 seconds. The seeds were then suspended in a sterile 0.1% agargel solution and either pipetted individually onto a plate or pipetted in a pool of agargel (2 mL) in the middle of a plate and swirled to distribute evenly. The plates were allowed to dry for 20 to 30 minutes before sealing with miratape. Seeds were stratified for at least 2 days in the dark at 4^{o}C before being transferred to the appropriate growth chamber.

More recently, a chlorine vapor phase sterilization method was used. This method is much less labor intensive, results in less contamination and makes placing seeds easier. This

Table 2.1 Macronutrient Composition of Dropout Media

Media	Abreviation	Macro Nutrient Concentrations
Complete Media	HN, HS or HP	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM
Low Nitrate	LN	Potassium Nitrate 6 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 10 mM
No Nitrate	0N	Potassium Nitrate 0 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 10 mM
No Phosphate	0P	Potassium Nitrate 60 mM Potassium Phosphate 0 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM Magnesium Chloride 1.5mM
No Sulfate	0S	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 0 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM
No Calcium	0Ca	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 0 mM Potassium Chloride 1 mM

A

Stock	Recipe	MW	Stock concentration
Calcium Chloride, dihydrate	44.0 g/L	147.01	299 mM
Magnesium Sulfate, 7-hydrate	37.0 g/L	246.47	150 mM
Potassium Phosphate, monobasic	17.0 g/L	136.09	125 mM
Potassium Chloride	7.495 g/L	74.55	100 mM
Potassium Nitrate	60.66 g/L	101.1	600 mM
10X MICRONUTRIENT STOCK	Sigma M0529		

B

Component	Amount per liter	Final concentration
ddH_2O	500 mL	
Sigma Micronutrient stock	100 mL	
Potassium Phosphate	10 mL	1.25 mM
Magnesium Sulfate	10 mL	1.5 mM
Calcium Chloride	10 mL	3.0 mM
Potassium Chloride	10 mL	1 mM
Potassium Nitrate	100 mL	60 mM
Sucrose	10 grams	1%
Agar	8 grams	0.8%

Figure 2.1 Component Media for Vertical Plates

A. Stock Solutions

B. Recipe for Complete Media

method was performed in the fume hood. Seeds were placed in 1.7mL tubes that were labeled using a super permanent industrial strength 'Sharpie' pen that is resistant to chlorine gas. The tubes of seeds were opened and placed in a tube rack inside a Tupperware cake saver container. In a 250mL beaker containing 100mL of Clorox bleach, 3mL of concentrated HCl was added and the cake saver was immediately closed. The sealed container was then allowed to sit for 5 hours before the tubes were closed and removed. The seeds were then sprinkled dry onto plates or individually placed with a moist pipette tip. The plates were then wrapped in miratape to seal. All seeds were stratified for at least 2 days in the dark at 4^{o}C before being transferred to the appropriate growth chamber.

For all GUS experiments plants were grown on 110mm gridded square plates. The seeds (up to 12) were placed in a line near the top of the plate as outlined in Figure 2.2. The plants were grown at 21^{0}C with 16 hours of light and 8 hours of darkness for most of the experiments and also tested at 8 hours of light and 16 hours of darkness. The plates were placed on racks in the incubator at about a 75% angle.

For starvation and re-addition experiments plants were transferred with ethanol sterilized forceps to fresh plates. Plants were grown for 8, 10 or 12 days on complete or deficient media then transferred. Plants grown on low nitrate plates were transferred to complete media plates and samples were taken daily. Plants grown on complete media were transferred to no nitrate for 4 days and then transferred back to complete media for 4 days and samples were taken daily. Plants grown on complete media were transferred to no phosphate media and daily samples were taken.

β-glucuronidase (GUS) Staining

GUS staining solution (made fresh daily) was composed of 100 mM sodium phosphate buffer pH 7, 10 mM EDTA, 0.1% Triton X-100, 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$ and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl ß-D glucuronide. A whole seedling or group of seedlings was placed directly into the GUS staining solution. Depending on the number of treatments and size of plants containers ranging from 1.7mL microcentrifuge tubes to 50 mL conical tubes were used. The containers with stain and plants were placed in a vacuum chamber to facilitate stain infiltrattion of the tissue. The vacuum was applied briefly and released two times. The vacuum was then applied and held for 30 minutes. The containers were then transfered to a 37°C incubator for 24 hours. Staining solution was removed with a pipette. To clear the chlorophyll 70% ethanol was added and changed every 24 hours until the tissue was cleared.

Quantitative GUS assay

Seedlings were collected at varying time points and placed in preweighed 2mL centrifuge tubes containing a stainless steel bead. Samples were weighed and then flash frozen in liquid nitrogen. Samples were ground to a fine powder using a Qiagen tissuelyser II (frequency= 30 s^{-1}, for one minute). Three volumes of extraction buffer (50 mM $NaHPO_4$, 10 mM beta-mercaptoethanol, 10 mM Na_2EDTA, 0.1% SDS and 0.1% Triton X-100) was added to the disrupted tissue. The tube was then vortexed for 10 seconds and the suspension was

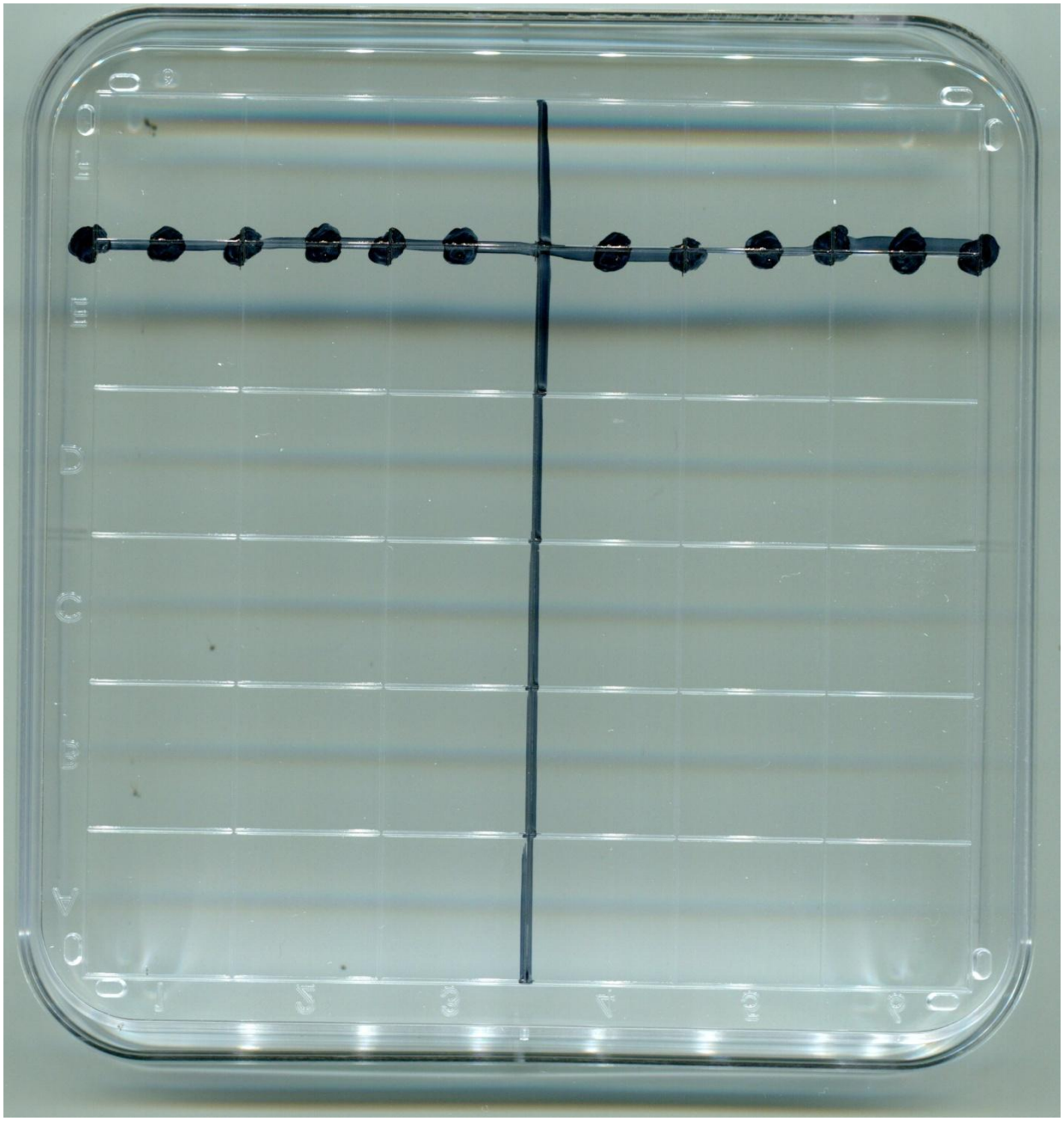


Figure 2.2 Seed distribution on vertical plates

Seeds were placed along the top of the plate (represented by black dots).

transferred to a new 1.7 mL tube. The suspension was then centrifuged for 10 minutes and the supernatant was transferred to a new 1.7 mL tube. For each reaction 25 µL of sample was added to 25 µL extraction buffer supplemented with 0.88 mg/mL 4-methylumbelliferyl-beta-D-glucuronide (MUG) and incubated at 37^0C for thirty minutes. After incubation 250µL stop buffer (0.2M Na_2CO_3) was added. To prepare a standard curve 4-methylumbelliferone (4-MU) standards of 0, 0.25, 0.5, 1.0, 2.0 and 3.0 µM were prepared in stop buffer. To determine protein concentration 10µL of a 5-fold dilution of the sample was added to 200µL Bradford reagent, mixed thoroughly and incubated for 10 minutes (Bradford 1976). Standards of 0, 0.1, 0.2, 0.4, 0.6 and 0.8 mg/mL BSA were also assayed to prepare a standard curve for protein concentration. The Bradford reactions were read at 595 nm on a spectrophotometer. The GUS reactions were then read with a DyNA Quant 200 Fluorometer (Hoefer, Inc., San Francisco, CA). The fluorometer was zeroed with 1.9 mL of stop buffer, then 100 µL of the 4-MU standard solution to the stop buffer was added and calibrated to 500 units. The values obtained were plotted against the standard curves and the data for each sample was calculated as nanomoles 4-MU/minute/mg protein.

Results

Expression profile data for NRM1 (Figure 1.6 and 1.7) shows strong expression in *Arabidopsis* roots, with limited expression in other tissues. To confirm root specificity NRM1 promoter:GUS plants were grown on complete media on vertical plates and GUS expression was determined in 4-18 day old seedlings. No staining was observed in the shoots of any of the NRM1 promoter::GUS plants under any conditions, but roots consistently showed GUS staining. Upon closer examination, no GUS staining was observed in the root tips (Figure 2.3a). NRM1

promoter::GUS plants grown on complete media for 18 days showed staining fron the lower part of the root (except the root tip) just above the area where lateral roots were emerging. There was also staining in the upper portion of the older lateral roots except in their root tips (Figure 2.4). No staining was observed in wild-type plants. 35S:GUS plants had strong, uniform staining throughout the entire seedling.

Because NRM1 is induced in response to nitrate, we next asked if GUS expression was altered in response to nitrate as well as other nutrients in the media. In 8 day old plants on low nitrate media GUS staining was greatly reduced overall compared to complete media but strongest in the upper portion of the primary root (Figure 2.3b). As expected, these plants did have more lateral roots when grown on low nitrate media. In 8 day old plants on low phosphate media GUS staining was reduced overall relative to complete media and strongest in the lower portion of the primary root with no expression in the root tip area (Figure 2.3c).

These plants showed shorter primary roots and more lateral roots, consistent with expected root architecture when grown on no phosphate media. The 35S::GUS plants showed root architecture consistent with nutrient composition of the plates and had intense, consistent GUS staining throughout the plants (Figure 2.3d-f).

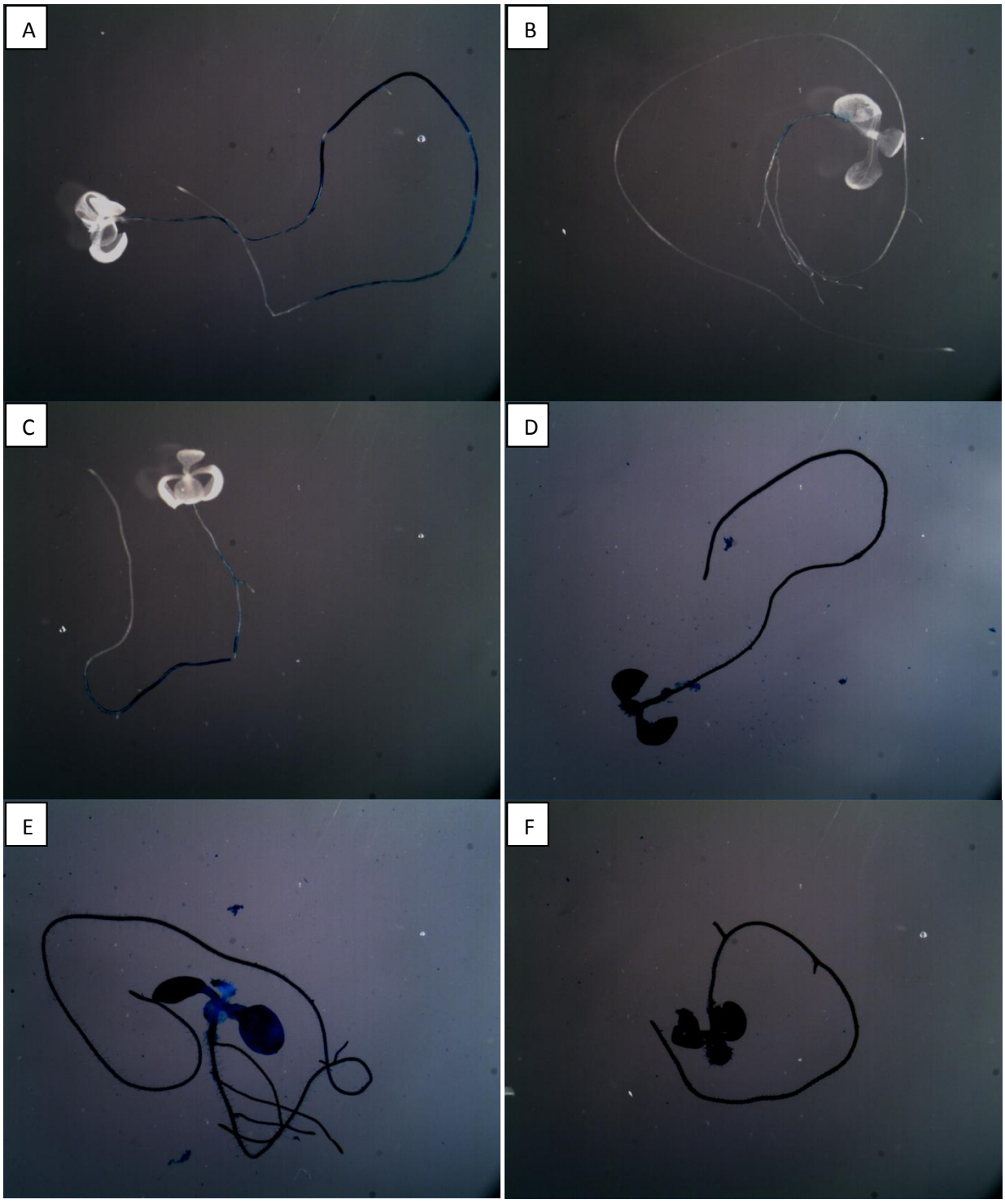


Figure 2.3 NRM1p::GUS and 35S::GUS Plants on Nutrient Deficient Media for 8 Days

A. NRM1p::GUS Complete Media
B. NRM1p::GUS Low Nitrate
C NRM1p::GUS No Phosphate
D. 35S::GUS Complete Media
E. 35S::GUS Low Nitrate
F. 35S::GUS No Phosphate

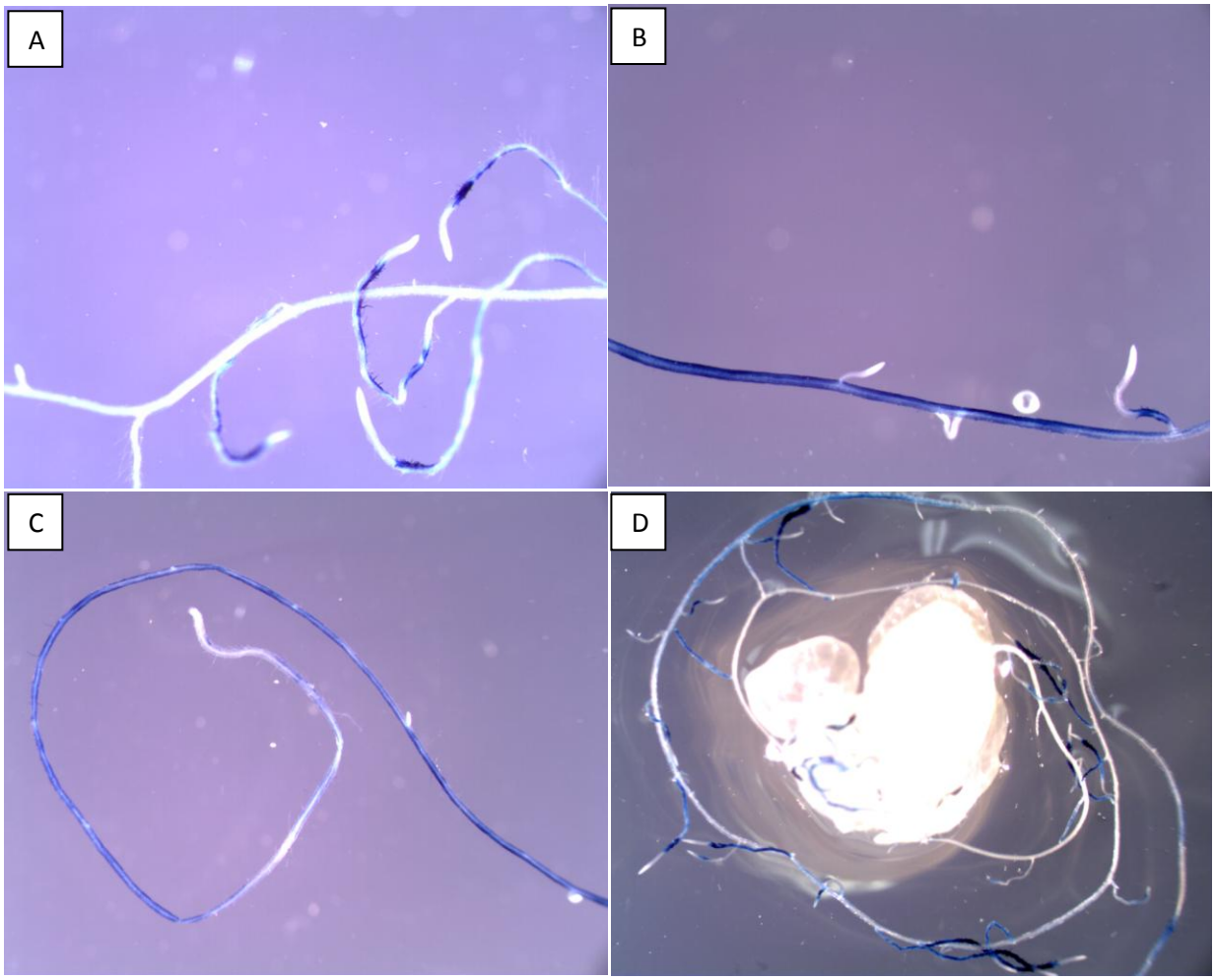


Figure 2.4 NRM1p::GUS Plants on Complete Media for 18 Days

A. Upper root with mature lateral roots

B. Middle root with emerging lateral roots

C. Elongation and root tip area

D. Complete plant

To explore the effect of nutrients on NRM1 expression in more detail, NRM1 promoter::GUS plants were grown for 10 days on media lacking nitrate, sulphate, phosphate, potassium or calcium. In 10 day old plants on low nitrate media GUS staining is greatly reduced and strongest in the upper portion of the primary root (Figure 2.5b). In 10 day old plants on no phosphate media GUS staining is almost completely absent (Figure 2.5c). In 10 day old plants grown on no sulphate media GUS staining is reduced and mostly present in the upper portion of the primary root (Figure 2.5d). In 10 day old plants on no calcium media GUS staining is almost completely absent (Figure 2.5e). In 10 day old plants on no potassium media GUS staining is similar to the complete media (figure 2.5f). Plants grown on media and transferred to nutrient deficient media showed very inconsistent staining. Data for these plants is not included because of the high level of variability.

Quantitative GUS analysis was performed using plant extract at a subset of time points to quantify the reduction in GUS staining in response to nitrate or phosphate starvation. Data from this experiment is shown in (Figure 2.6). Wild type Columbia plants and 35S::GUS plants were used as negative and positive controls, respectively. Wild-type plants showed very low GUS activity under all treatments as expected. 35S::GUS plants showed a very high level of GUS activity regardless of treatment. NRM1 promoter::GUS showed high levels of activity on complete media. When grown on low nitrate or no phosphate media GUS activity was reduce between 4 and 5 fold. This confirmed the reduction of staining seen in whole GUS stained plants.

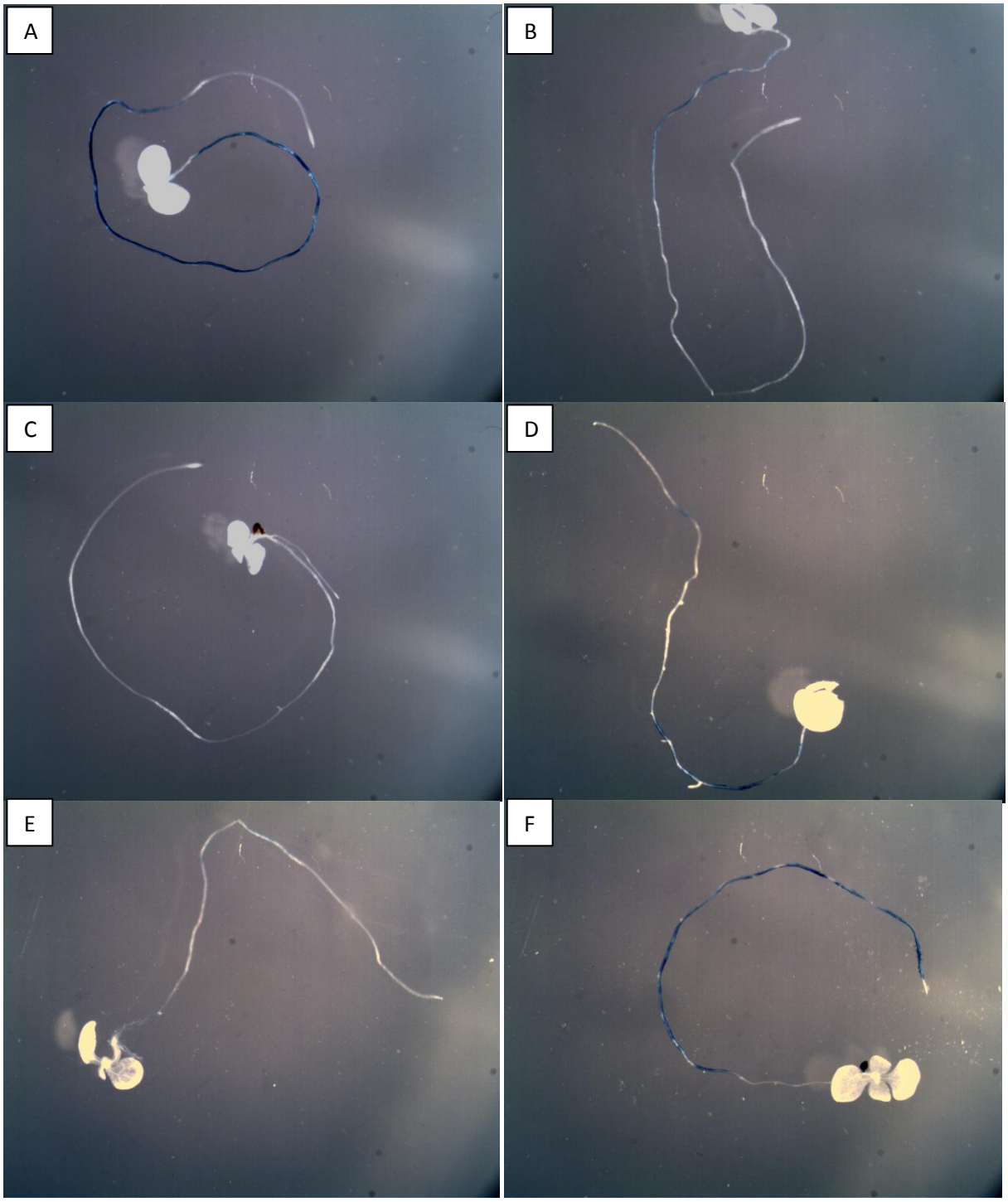


Figure 2.5 NRM1p::GUS Plants on Nutrient Deficient Media for 10 Days

A. Complete Media

B. Low Nitrate

C. No Phosphate

D. No Sulphate

E. No Calcium

F. No Potassium

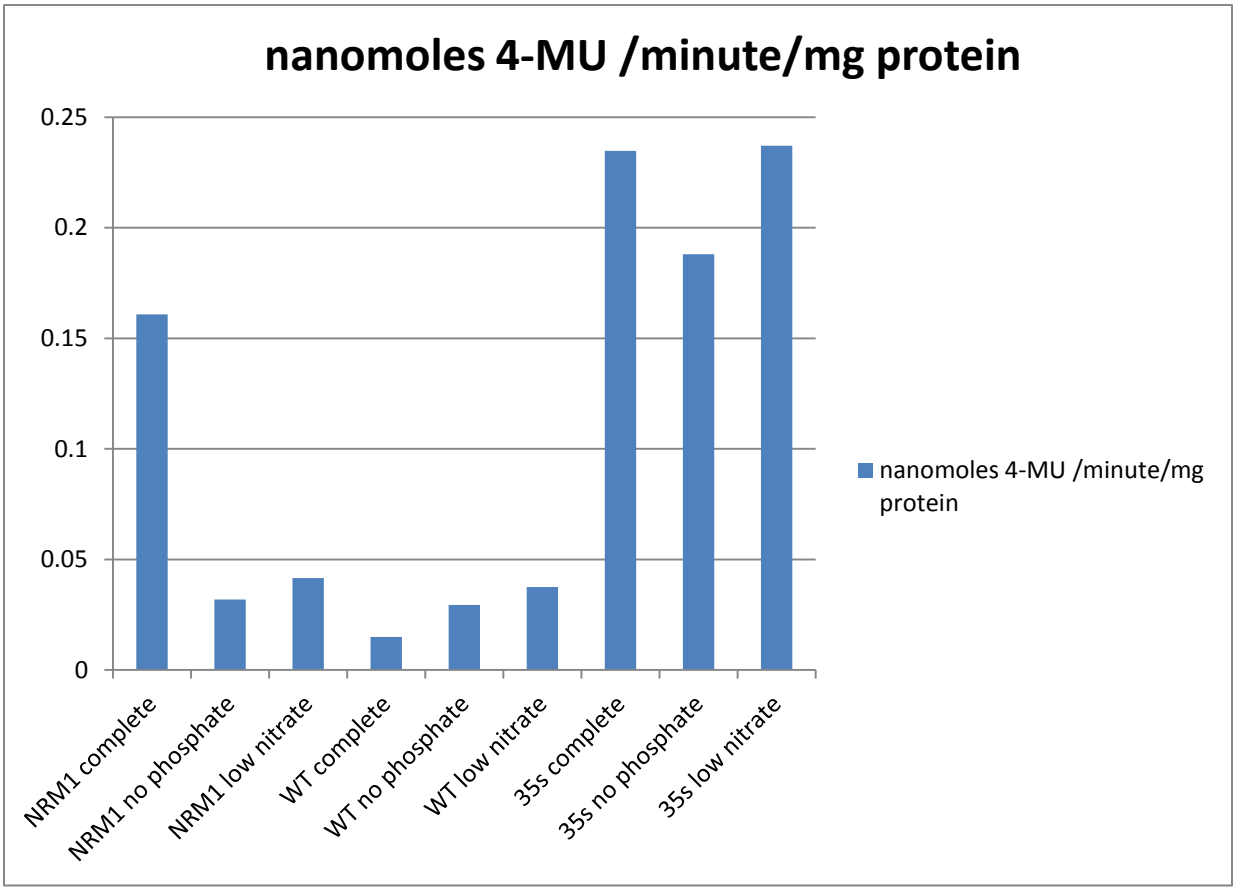


Figure 2.6 Quantitative GUS Results

Arabidopsis seedlings were grown for 8 days on complete media, low nitrate or no phosphate. Crude protein extract made from seedlings was analyzed by the quantitative GUS protocol.

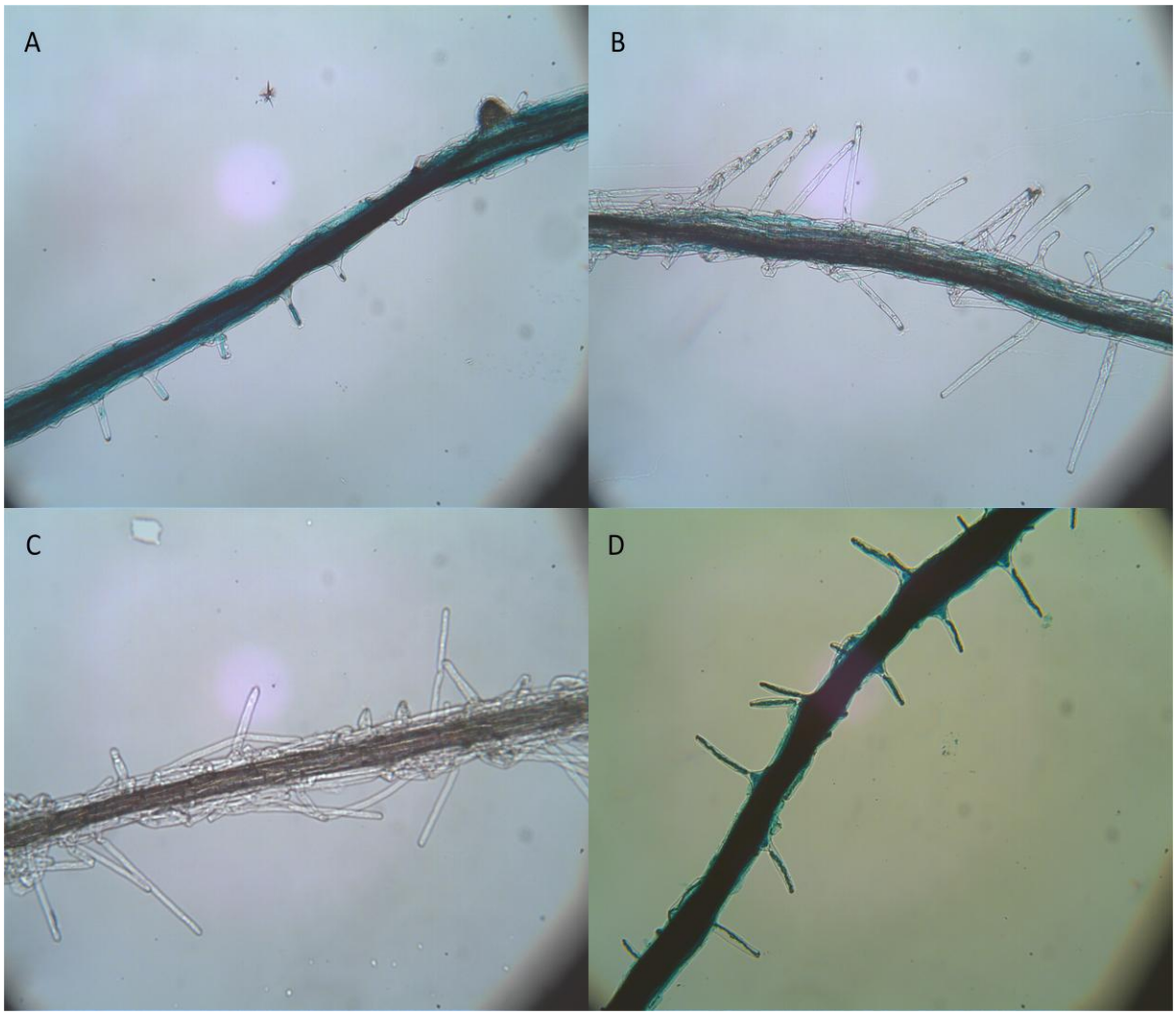


Figure 2.7 NRM1p::GUS Root Hair staining

A. Complete Media

B. Complete Media

C. Low Nitrate

D. 35S::GUS grown on Complete Media

All images captured at 100x total magnification.

Discussion

GUS expression experiments confirmed previous experiments that showed reduced NRM1 expression in response to low nitrate and low phosphate conditions, and provided insight into the spacial expression of NRM1 expression in response to these nutrients. NRM1 expression is also altered in response to sulphate and calcium. This suggests that NRM1 responds to a variety of nutrient deficiencies in *Arabidopsis*. This suggests that NRM1 may be involved in a general pathway of root response to limiting nutrients as opposed to specific nitrate responses as originally postulated. NRM1 could be involved in a pathway involved in root architecture changes in response to a wide range of nutrient availability.

In experiments where plants were transferred from one type of media to another produced were highly variable results. This could be due to a variety of reasons. Media variation caused by inconsistent agar quality were noticed in this experiment. Agar variation and type of agar used can play a large role in success of starvation experiments (Wiren et. Al. 2013). Small variations in media can be less influential when plants are grown on the same media for their entire life cycle. Transferring plants is also a very stressful process for seedlings. When transferred, the plants can have broken lateral roots and may not be in contact with the new media for the entire length of the root. The plants were transferred as carefully as possible but there is always a chance of damage. Contamination is also an issue when transferring plants. When plants are grown on the same media for their entire lifespan, the plate remains sealed. Every time plants are transferred, the plate has to be opened and there is

a risk of contamination. Any time contamination was noticed the plate was discarded, however there could have been some contamination that was not noticeable.

The spatial expression of NRM1 differs according to nutrient availability. NRM1 is not expressed in shoots in any experiments to date. This lends further support to the idea that NRM1 is involved in root response to nutrients. The expression of NRM1 is localized to areas of elongation and lateral root formation under nutrient replete conditions. No expression was seen in the primary root tip or lateral root tips under any treatment conditions. This suggests that NRM1 is involved in pathways related to lateral root formation and root elongation but not elongation growth at the root tips. Under low nitrate or no sulphate conditions expression is lower and islocalized near the upper portion of the root. This change in expression could lead to downstream responses such as increased lateral root formation and changes in root elongation.

Quantitative GUS data confirmed lower expression under low nitrate and no phosphate conditions. This data is useful to determine more precise levels of expression and further experiments could shed light on subtle changes in expression over shorter periods of time. Quantitative data would be very useful in hydroponic experiments where complete media could be replaced with nutrient deficient media and changes in expression could be monitored over time.

The GUS data shows a correlation between the availability of certain nutrients and NRM1 levels in the plant. This data does not, however, show a link between NRM1 expression and any changes in the plants in response to these nutrients.

Future experiments could be performed to find sequence elements in the NRM1 promoter that are responsible for nutrient responses. Portions of the promoter could be deleted and the truncated promoters could be used to drive the expression of the GUS gene. Truncated promoters could then be analyzed under nutrient deficient conditions to look for changes in expression. It would be interesting to see if there was one promoter element responsible for the changes in expression or if there were multiple promoter elements that respond to different nutrients.

Chapter 3

Subcellular Localization of NRM1

Introduction

NRM1 is hypothesized to be a transcription factor because it has a MYB like DNA binding domain and is expressed very early in response to nitrate. If this is the case it should be localized to the nucleus where it can interact with the promoters of the genes that it regulates. NRM1 also contains 2 putative nuclear localization sequences that should direct the protein to the nucleus (Figure 3.1). To determine the localization of NRM1, a fusion protein was made with Green Florescent Protein (GFP). GFP is a small (239 amino acids) protein originally isolated from *Aequorea victoria,* a species of jellyfish (Chalfie et al. 1994). It is commonly used in protein fusions to tag a protein of interest and is useful in determining the localization of proteins within living organisms and cells (Chiu et al. 1996; Davis and Vierstra 1998). The chimeric proteins usually have the same localization and function as the protein without GFP (Chopin et al. 2007). It can also be used to monitor changes in localization of proteins in response to different treatments. GFP fusions can be visualized easily by exciting with 488 nm light, with no substrate needed for fluorescence. Generally, this means that it is easy to visualize protein in cells of living organisms with little disruption to the organism. Correct folding of GFP which can be effected by factors such as temperature and the site of fusion to the protein of interest, is needed for fluorescence.

NRM1 Full Length

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETMT

RDESMRKDPMVNGGEGR**KREAEKDGGGGRKQRR**CWSSQLHRRFLNALQH

LGGPHVATPKQIREFMKVDGLTNDEVKSHLQKYRLHT**RRPR**QTVPNNGNS

QTQHFVVVGGLWVPQSDYSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQW

PSHSNYRPSIIVDEGSGSHSEGVVVRCSSPAMSSSTRNHYVKNN

Figure 3.1 Nuclear Localization Sequences in NRM1

Amino Acid sequence of NRM1. The Leucine zipper-like domain is highlighted in blue and the MYB like DNA binding domain is highlighted in red. Putative nuclear localization sequences are bold and underlined.

Stably transformed plants are the ideal platform for examining the localization of NRM1-GFP chimeric protein. Once transgenic plants are obtained, it is easy to routinely grow and analyze them. The plants can be germinated on a wide variety of media and can be imaged directly without manipulation. However, it is quite time consuming to make the transgenic plants. The constructs have to be cloned and verified while plants are grown to the flowering stage. The constructs must be transformed into *Agrobacterium* and the plants infected with these transformed bacteria. The seeds from these plants have to be selected on media containing the selective agent and these selected plants have to be self-pollinated. The seeds from this generation then have to be grown on selective media and selfed again and seeds are harvested from individual plants. The resulting seed are selected again and the lines with all resistant plants (from homozygous parents) are saved. This takes several months to complete even in *Arabidopsis* with a 4 month generation time.

Alternatively, onion epidermal cells are easily transformed and transgenic cells can be obtained in a few days. Onion epidermal cells are very useful for quick visualization of multiple gene constructs. These cells are a different species, however, which can cause problems when expressing *Arabidopsis* genes. Moreover, only a small percentage of cells are transformed in a typical experiment. These cells cannot be examined on different media and at different developmental stages. An additional disadvantage of this system is that the cells must be transformed each time an experiment is performed. The cells also do not contain chloroplasts, which can be very helpful with florescent imaging by eliminating auto-fluorescence.

Arabidopsis protoplasts are plant cells that are stripped of their cell wall and are floating freely in solution. One advantage of protoplasts is that they are from the same species as the NRM1 gene. Protoplasts are also easily transformed and transgenic cells can be obtained in a few days. Again, a small percentage of cells are transformed in each experiment. The plants from which protoplasts are obtained can be treated with different media as can the protoplasts themselves. The process of making protoplasts is very stressful to the plant cells, however, and this can complicate any physiological experiments. With commonly used protocols, only leaf cells are obtained so only expression in this type of tissue is examined. These cells contain chloroplasts, which can complicate fluorescent imaging. While protoplasts are not as useful as stably transformed plants they can be obtained quickly and constructs can be tested in protoplasts to make sure they function properly, before stable transformation of plants is attempted.

In this chapter, I describe experiments analyzing NRM1 localization in all three kinnds of transformed plant cells: onion epidermal cells, stably transformed *Arabidopsis* and *Arabidopsis* protoplasts

Materials and Methods

Construction of pUC118_NRM1-smGFP

To construct a vector for transient expression, plasmid pUC118smGFP was used. This plasmid contains a CaMV35S promoter, the coding sequence for GFP, and a multiple cloning site to allow C-terminal fusion of a gene of interest with GFP. The GFP protein used in this plasmid is smGFP that is codon-optimized for plants and has enhanced solubility. The full

length NRM1 minus the stop codon was ligated into the vector upstream of the GFP coding region in the same reading frame to for a fusion protein. This construct was verified by sequencing and used in transient expression experiments.

Onion Transient Expression

Tungsten particles were sterilized by adding 60 mg of tungsten particles to 1 mL of 70% ethanol, vortexing and incubating at room temperature for 15 minutes. The mixture was then centrifuged for 5 minutes at 13000g and the supernatant was discarded. The particles were washed with 1 mL of sterile water then vortexed, centrifuged at 13,000 g for 5 minutes and the supernatant was discarded. The particles were then suspended in 1 mL of 50% glycerol and stored at -20C.

Plasmid DNA was precipitated onto the tungsten particles. 5 µg of plasmid DNA was added to 50µL of sterilized tungsten plus 50 µL 2.5M $CaCl_2$ and vortexed. Twenty µL 0.1M spermidine was added and the mixture was vortexed for 10 minutes. The mixture was then centrifuged at full speed for 5 seconds then the supernatant was removed. The pellet was washed with 140 µL 70% ethanol, vortexed, then centrifuged at max speed for 5 seconds and the supernatant was discarded. The mixture was washed a second time with 140µL of 100% ethanol then centrifuged at maximum speed for 5 seconds and the supernatant was discarded. The pellet was then resuspended in 48µL of 100% ethanol and vortexed.

For particle bombardment a Biorad PDS1000/He Biolistic Gene Gun was used(Hagio 1994). This gene gun uses pressurized helium behind a plastic rupture disc. When the pressure

reaches a critical point the rupture disc ruptures and creates a shockwave which propels the flying disc and tungsten particles at high velocity into the tissue below.

The cells for transient expression were onion epidermal cells. A fresh healthy white or yellow onion was obtained from a supermarket. The outer layers of the onion were removed and discarded. The onion was then segmented and the inner epidermal layer was then peeled from many of these segments and placed onto a MS + 1% sucrose plates. Enough onion epidermal segments were placed on the plate to cover most of the center of the plate.

The rupture discs and flying discs were sterilized in 70% ethanol for fifteen minutes then placed on a Kimwipe to dry. The machine was turned on along with the vacuum pump and helium valve. Six micro liters of the particle DNA solution was spotted onto the rupture disc and the ethanol was allowed to evaporate. A rupture disc was placed in the top fitting and hand tightened. After the ethanol had evaporated from the flying disc it was placed facing downward into the holder facing down and the securing cap was tightened. The MS plate with the onion epidermal layer was then placed at the bottom of the vacuum chamber. The vacuum switch on the machine was placed in the open position until the vacuum reached 25 inches of mercury. The vacuum was then moved into the hold position and the fire switch was held until the rupture disc failed and an audible pop was heard. The vacuum switch was then moved into the vent position until the chamber reached equilibrium with the atmosphere. This procedure was then repeated for several replicate plates of the control and 35S-NRM1-GFP constructs.

After bombardment, the plates were wrapped in aluminum foil and placed in a growth chamber at 21^0C for 18 hours. The cells were then imaged using a Nikon 76516 inverted

compound microscope and Image Pro Plus software located in the Mykles lab. The epidermal layers were stained with DAPI (4',6-diamidino-2-phenylindole) nucleic acid stain to confirm the location of the nucleus. This was done by adding 80 µL of 1μg per mL DAPI on the slide and placing the epidermal layers on top followed by another 80 µL of 1μg per mL DAPI. A standard sized slide was used in place of a cover slip to better flatten the epidermal layer. The epidermal layer was manually scanned at 40x magnification using a 41020 HQGFPNB filter to observe GFP fluorescence and minimize visualization of autofluorescence. The cells that expressed GFP well were then imaged with the same filter at 400X magnification. During the time it took to image the cells with the GFP filters DAPI was absorbed into the cells. The filter was then switched to a 31000 DAPI filter at the same location and magnification to image the nucleus stained with DAPI.

Construction of pCAMBIA 2300_NRM1-GFP

A vector was constructed to stably express a chimeric fusion of the NRM1 and GFP. The binary vector pCAMBIA 2300 with GFP inserted into Nde 1 and Xho 1 sites was used as a starting point. Expression of the chimeric protein was driven by the constitutive promoter 35S. The 35S promoter was amplified using the 35Sleft and 35Sright primers and ligated into pCAMBIA 2300_GFP using the restriction enzymes Sph I and Hind III. The coding sequence for NRM1 (minus the stop codon) was amplified from cDNA using NRMLeft and NRMnostop primers. The stop codon was deleted using these primers so the protein would continue to include the GFP segment as well. The construct was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The coding sequence was then ligated into the

pCAMBIA 2300 35S_GFP using the restriction enzymes Sal I and BamH I. The completed vector was then sequenced to verify correct assembly with the primers FOR_1, FOR_2, FOR_3, FOR_4, FOR_5, REV_1, REV_2, REV_3, REV_4 and REV_5.

Construction of GFP-GA_5-NRM1

The vector pGFP2-$(GA)_5$ II contains the 35S constitutive promoter and GFP followed by linker consisting of 5 repeats of glycine-alanine. For insertion into pGFP2-$(GA)_5$ II, the coding sequence for NRM1 was amplified from cDNA using gfp2 for 1 and gfp2 re 1 primers. The construct was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The coding sequence was then cloned into the pGFP2 (GA)5 II plasmid using the restriction enzymes Sal I and Sma I. The completed vector was then sequenced with the primers ga5 gfp seq L and ga5 gfp seq R.

Protoplast Transient Expression Methods

Protoplasts were isolated from healthy leaves of 3-4 week old *Arabidopsis* Col-0 plants grown on soil with 8 hours of light and 16 hours of dark at 21^0C. Five millimeters of the leaf tip of each leaf was removed with a razor blade and ~25 leaves were cut into 0.5- 1 mm strips. The blade was changed after cutting about 5 leaves. The leaves were transferred quickly into 10 mL prepared enzyme solution (solutions composition in Figure 3.2) in a 50mm Petri plate using flat forceps. It was important to fully submerge the leaves in the enzyme solution. The leaf strips were then vacuum infiltrated for 30 min in the dark, using a vacuum chamber. The plate was then removed from the vacuum chamber and the digestion was continued for 3.5 hours in the dark without shaking. The protoplasts were checked using a microscope to make sure the

protoplasts were released and not lysed . The enzyme/protoplast solution was diluted with an equal volume(10mL) of W5 solution in the Petri dish. The enzyme/protoplast solution was then filtered gently with a 70 µM nylon mesh strainer (BD Falcon 352360) using a 25 mL pipette for the transfer. The flow-through was centrifuged at 1000 rpm in round bottom centrifuge tubes for 2 min. The supernatant was removed and the pellet was resuspended in 4 mL W5 pellet by gentle swirling.

Cells were counted under the microscope using a hemocytometer. During that time, the protoplasts were kept on ice. With coverslip in place, a Pasteur pipette was used to transfer a small amount of cell suspension to both chambers of the hemocytometer. All the intact cells in the 1 cm center square and four 1 mm corner squares were counted. The number of cells per mL was calculated by averaging the count per square and multiplying by 10^4. Alternatively the cells were counted with a BIO-RAD TC20 automated cell counter. 10µL cell suspension was transferred into the cell counter slides and the machine was programmed to count cells between 15nm and 40nm. Cell counts using the hemocytometer vs the cell counter produced very similar results. The protoplasts were diluted to a concentration of 2×10^4 cells/mL in W5 solution. The protoplast solution was centrifuged at 1000 rpm in round bottom centrifuge tubes for 2 min. The protoplast pellet was resuspended in the same total volume of MMG solution and kept at room temperature.

W1		[final]	[stock]	dilution	To add
	MES pH 5.7	4 mM	100 mM	25	2 mL
	Mannitol	0.5 M	0.8 M	1.6	31.25 mL
	KCl	20 mM	1 M	50	1 mL
	Water				15.75 mL
					50 mL
W5		**[final]**	**[stock]**	**dilution**	**To add**
	MES pH 5.7	2 mM	100 mM	50	1 mL
	NaCl	154 mM	4 M	26	1.925 mL
	$CaCl_2$	125 mM	1 M	8	6.25 mL
	KCl	5 mM	1 M	200	0.250 mL
	Water				40.575 mL
					50 mL
MMg		**[final]**	**[stock]**	**dilution**	**To add**
	MES pH 5.7	4 mM	100 mM	25	2 mL
	Mannitol	0.4 M	0.8 M	2	25 mL
	$MgCl_2$	15 mM	1 M	66.7	0.75 mL
	Water				22.25 mL
					50 mL
PEG-Ca		**[final]**	**[stock]**	**dilution**	**To add**
	PEG 4000	40% (w/v)			20
	Mannitol	0.2 M	0.8 M	4	12.5 mL
	$CaCl_2$	100 mM	1 M	10	5 mL
	Water				to 50 mL

Enzyme "Pre"-solution		[final]	[stock]	dilution	To add
	MES pH 5.7	20 mM	100 mM	5	10 mL
	Mannitol	0.4 M	0.8 M	2	25 mL
	KCl	20 mM	1 M	50	1 mL
	Water				14 mL
					50 mL

Enzyme final solution	[final]	[stock]	dilution	To add
Enzyme "Pre"-solution				10 mL
Cellulase *(RPI C32200-10.0)*	1.5% (w/v)			150 mg
Maceroenzyme *(RPI M22010-1.0)*	0.4% (w/v)			40 mg
$CaCl_2$	10 mM	1 M	100	100 ul
BSA *(Calbiochem 126579)*	0.1% (w/v)			10 mg

Figure 3.2 Protoplast Isolation Solutions

To transform the protoplasts, 10- 20 µg/L DNA (10-20 µg of plasmid DNA of 5-10 kb in size) and 100 µl of protoplasts (2 x 10^4) were pipetted into a round bottom 2 mL microfuge tube and mixed gently by inversion. 110 µl of PEG solution was added and the tube was gently taped to mix the solutions but not burst the protoplasts. The mixture was incubated at room temperature for 10 minutes. To stop the transformation 440 µl W5 solution at RT was added and mixed by gently inverting the tube. The transformation was centrifuged 200 rcf for two minutes at room temperature and the supernatant was removed. The pellet was resuspended gently in 1 mL W1 and incubated overnight under continuous light before imaging.

Transgenic Seedling Growth for Confocal Microscope Imaging

To view stably transformed seedlings more effectively on an inverted confocal microscope they were grown directly on a large cover slip. This allows the seedlings to be imaged without the stress of being removed from their growth media and placed onto a cover slip. Several 24 x 48mm cover slips were autoclaved along with 250 mL of half strength MS media plus 1% sucrose and 1% phytagel. The cover slips were placed in sterile 100mm petri plates in the laminar flow hood and the media was pipette onto the cover slip before it could cool. To cover most of the cover slip one mL of media was used. The media stayed on the cover slip because of surface tension as long as it was pipetted slowly. The media was allowed to cool and solidify in closed petri plates to avoid contamination. Sterilized seeds were placed individually on the cover slip with sterile forceps. The seeds were pushed into the gel so the root system would not develop on the surface of the gel. The seedlings were then grown for 6 days at a 45^0 angle at 22^0C with 16 hours of light. The advantage of this growth technique is

that the roots grew through the media directly on the surface of the cover slip. When the plants were imaged the roots were in the perfect position to be viewed by the inverted microscope without any disruption in growth.

Hydroponic *Arabidopsis* Growth

In order to test if nitrate starvation had an effect on protein localization in *Arabidopsis* protoplasts a hydroponic system was designed (Figure 3.3). 4 liter sterilite containers were painted black to block light and prevent algal growth. $5/16^{th}$ inch holes were drilled in a grid pattern in the lid of the container. These holes were just big enough to insert a 0.65mL microcentrifuge tube. The 0.65 mL microcentrifuge tubes were prepared by removing the lid and drilling a $1/16^{th}$ inch hole in the bottom of each tube. The tubes were then placed in a chilled block. A solution of 0.6 % agargel was heated on a stir plate until boiling. The solution was pipette into the tube and the chilled block solidified the agar quickly so it did not leak out the bottom of the tube. The agargel was allowed to solidify and then a single seed was placed on the surface of the agargel in the center of the tube with forceps. The tubes were then stratified at $4^{o}C$ for 2 days wrapped in Saran Wrap to prevent drying. The tubes were then placed into the holes drilled into the 4 liter container lid. The containers were filled with 4 liters of modified Hoagland's media with quarter strength macronutrients, full strength micronutrients and 5mM KNO_3. After germination the seedlings grew normally, developing a root system through the hole in the bottom of the microcentrifuge tube and into the media. There was very little contamination of the non-sterile agargel in the tubes. After 3 weeks half of the plants were transferred to similar media lacking KNO_3 and $CaNO_3$. After 4 days on either

media, tissue was collected and protoplasts were made from the plus or minus nitrate plants. Protoplasts were transformed as described above except 1mM KNO_3 was added to all the solutions for the plus nitrate protoplasts to prevent nitrate starvation.

Protoplast and Stable Construct Imaging

All imaging of protoplasts and *Arabidopsis* seedlings was performed on Zeiss LSM510 META inverted confocal microscopes. Transgenic seedlings were imaged directly on media cover slips and protoplasts were imaged in petri plates with cover slips built into the bottom of the dish (*MayTek corp P356-0-10-C).*

GFP images were acquired using a plan-apochromat 63x objective lens with a 96µm pinhole. The 488nm laser was used to excite the GFP at 10% power. A broad pass filter that transmitted wavelengths from 505-530 nm was used on the emitted light.

To confirm the position of the nucleus, the nucleic acid stain DAPI was used. Ten minutes before imaging 10 µL of 1*µ*g per mL DAPI was added to the protoplasts. DAPI images were acquired using a plan-apochromat 63x objective lens with an 82µm pinhole. The 405nm laser was used to excite the DAPI at 6.1% power. A broad pass filter that transmitted wavelengths from 420-480 nm was used to filter the emitted light.

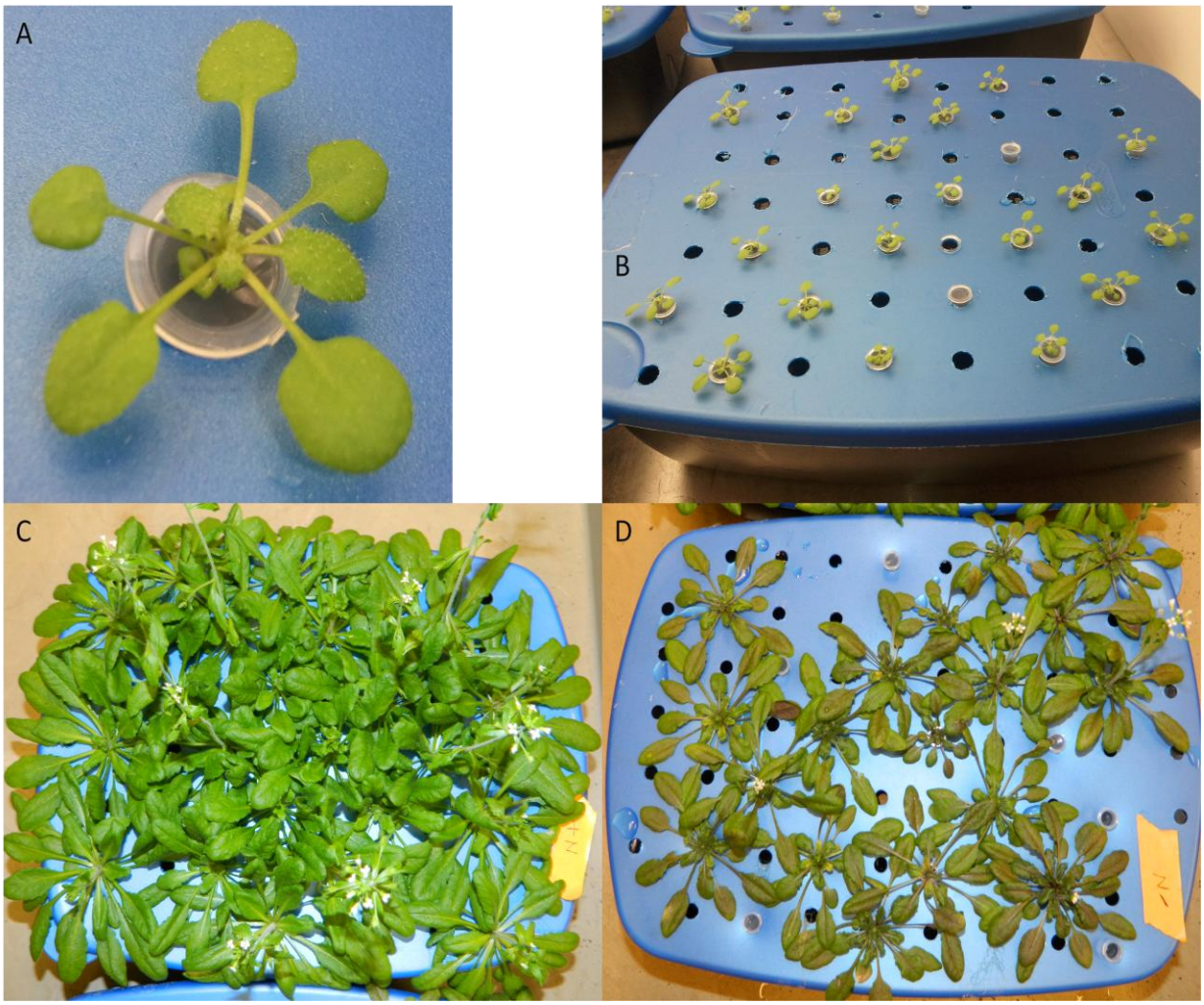


Figure 3.3 Hydroponic Growth of *Arabidopsis thaliana*

A. One week old seedling in modified microcentrifuge tube with agar

B. One week old seedlings with complete media

C. Four week old plants with complete media

D. Four week old plants with media lacking nitrate

Results

Transient expression of NRM1-GFP in Onion Epidermal Cells

To determine the subcellular localization of NRM1, I first tested expression of an NRM1-GFP fusion construct in the pUC118smGFP plasmid vector in onion epidermal cells. This plasmid contains the 35S promoter, NRM1-GFP fusion and the Nos terminator cassette, and is therefore useful for transient expression assays. Because this plasmid is not a binary vector it is not suitable for stable transformation using Agrobacterium. As a control, the empty pUC118smGFP vector was used, which should express GFP under the control of the 35S promoter. As expected, the 35S::GFP control construct showed GFP fluorescence throughout transformed cells, with the exception of the vacuole (Figure 3.4a). The GFP protein by itself is quite small and can diffuse freely into the nucleus through the nuclear pores. In onion epidermal cells, the vacuole occupies a very large percentage of the cell and pushes the cytoplasm against the cell walls. This is why the cells appear to have more protein localized near the cell wall. DAPI staining was used to confirm the position of the nucleus within the cells. There was some staining of the cell wall with DAPI, but because the nucleus was easily detectable this was not seen as a major problem.

In 35S::NRM1-GFP bombarded cells, the overall fluorescence was weaker than that seen in the controls, but was localized exclusively to the nucleus (Figure 3.4b). Nuclear staining was confirmed with DAPI (Figure 3.4d).

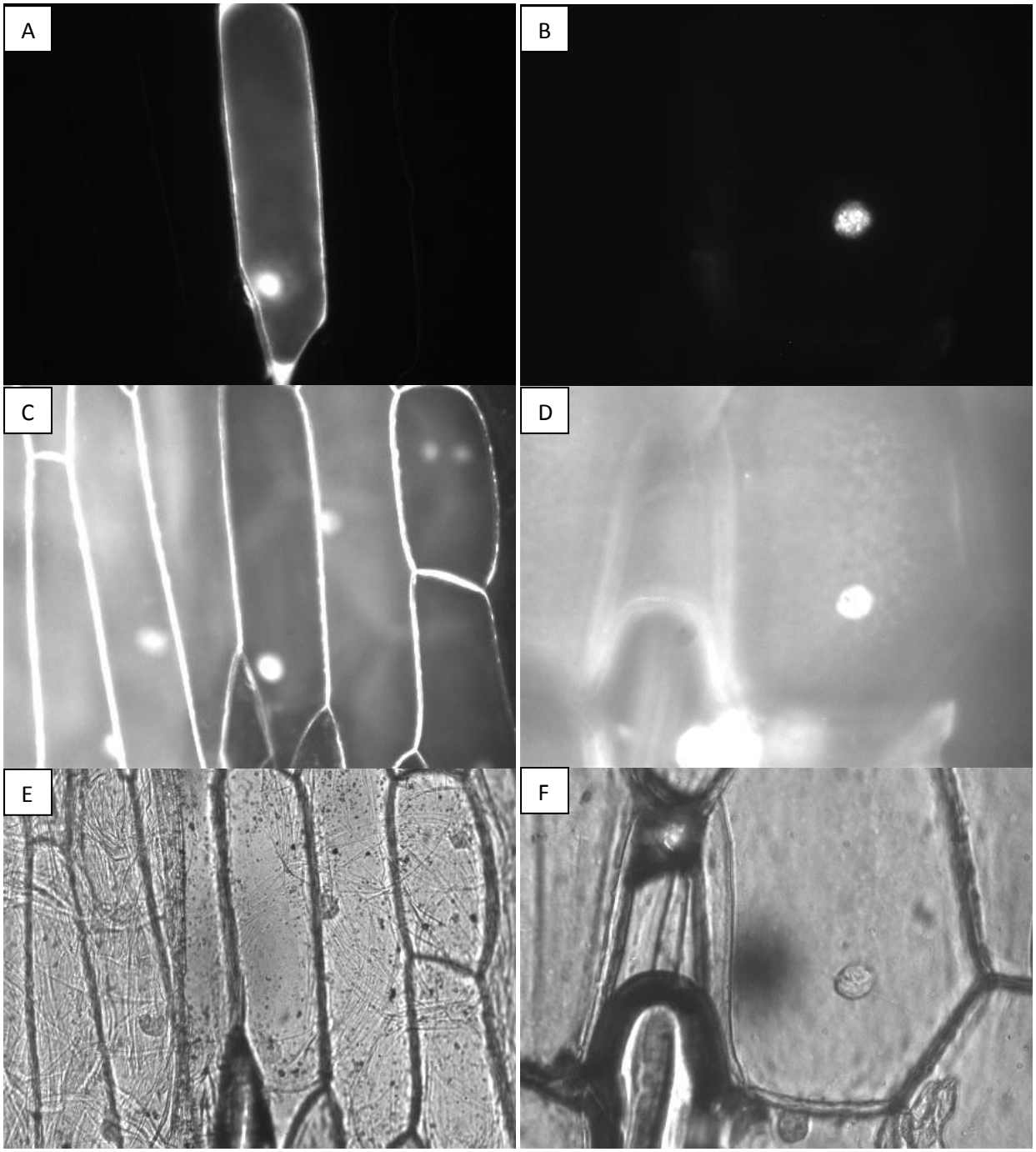


Figure 3.4 Onion Transient Expression of NRM1-GFP fusion Protein In Onion Epidermal Cells

A. 35S::GFP GFP florescence B. 35S::NRM1-GFP GFP florescence
C. 35S::GFP DAPI florescence D. 35S::NRM1-GFP DAPI florescence
E. 35S::GFP Bright field F. 35S::NRM1-GFP Bright field
All images are at 40x objective magnification.

Stable Expression of NRM1-GFP in *Arabidopsis*

In order to confirm expression of NRM1-GFP in stably transformed *Arabidopsis*, the NRM1-GFP expression cassette was moved into the pCAMBIA 1300 binary vector and used to transform *Arabidopsis* using Agrobacterium transformation. Stable transgenic plants expressing NRM1-GFP would be useful to look at expression in plants as well as in experiments to test whether NRM1-GFP localization was altered in response to different nutrient conditions. When NRM1-GFP seedlings, selected by growth media containing kanamycin were imaged, there was no detectable GFP fluorescence in the transgenic seedlings. Using the same experimental protocol, we were able to detect GFP fluorescence in seedlings expressing GFP under the control of the 35S promoter. Expression of GFP transcript in NRM1-GFP plants was confirmed using RT-PCR (Figure 3.5).

Transient expression of NRM1 GFP constructs in *Arabidopsis* protoplasts

Because the NRM1-GFP fusion was expressed (Figure 3.5), but did not fluoresce in *Arabidopsis*, we suspected that the fusion protein was not folded properly. As an alternative, we cloned NRM1 into the pGFP2_GA5II vector. In this vector NRM1 is expressed as a C-terminal fusion to GFP and is separated from GFP by a 10 amino acid linker (5- Gly-Ala repeats). The linker may help promote proper folding of each fusion partner by separating them from one another. This new construct (GFP-GA_5-NRM1) as well as GFP alone and NRM1-GFP were tested for expression using protoplasts of *Arabidopsis*. Protoplasts were used to perform this experiment quickly to make sure the construct would function properly.

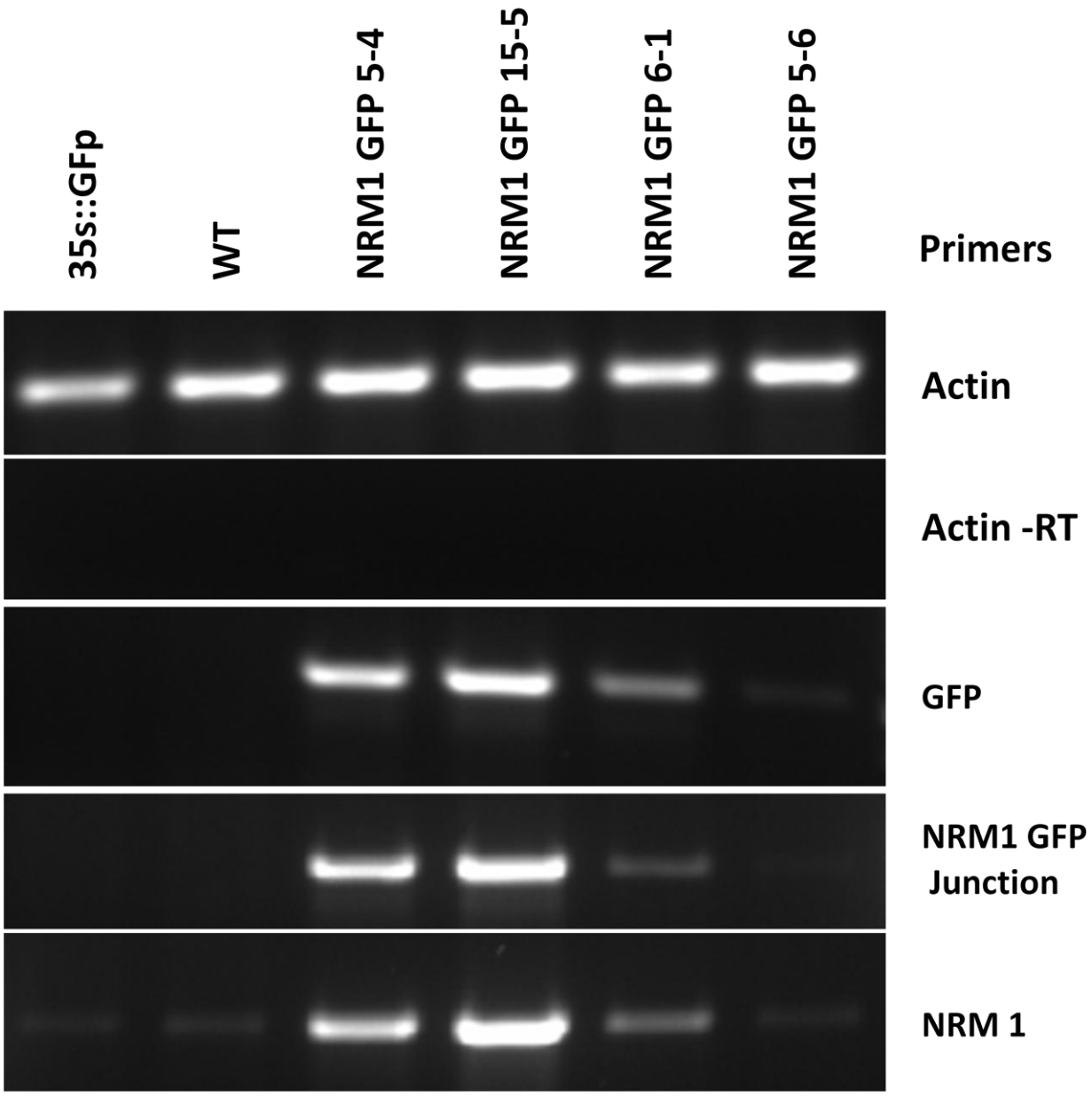


Figure 3.5 RT PCR to Confirm Expression of NRM1-GFP

RNA extracted from whole 8 day old transgenic seedlings.

-RT denotes lack of reverse transcriptase enzyme to check for genomic DNA contamination.

The 35S::GFP construct showed florescence throughout the cell with the exception of chloroplasts and the vacuole (Figure 3.6a). The DAPI staining was variable in the protoplasts, which might have been correlated with the quality of the protoplasts. No GFP fluorescence was observed in the 35S::NRM1-GFP construct with no linker between the proteins (data not shown). The 35S:GFP-GA_5-NRM1 construct showed florescence only in the nucleus, which was confirmed by DAPI staining (figure 3.6b).

To determine whether the subcellular localization of GFP-GA5-NRM1 was influenced by nitrate levels, protoplasts were isolated from nitrogen starved and nitrogen replete *Arabidopsis* plants grown hydroponically. No difference in fluorescence was observed for either 35S:GFP or 35S:GFP-GA5-NRM1 constructs. Because there is no nitrate present in any of the solutions used during protoplast isolation and transformation, it was possible that protoplasts isolated from nitrate-replete plants became nitrate starved during the experiment. To exclude this possibility, all solutions used to isolate protoplasts from nitrate-replete plants were supplemented with nitrate. Again, no difference in GFP localization was observed for either 35S:GFP or 35S:GFP-GA5-NRM1 under nitrate-replete and starved conditions; GFP-GA5- NRM1 protein was localized to the nucleus under both conditions. (Figure 3.7)

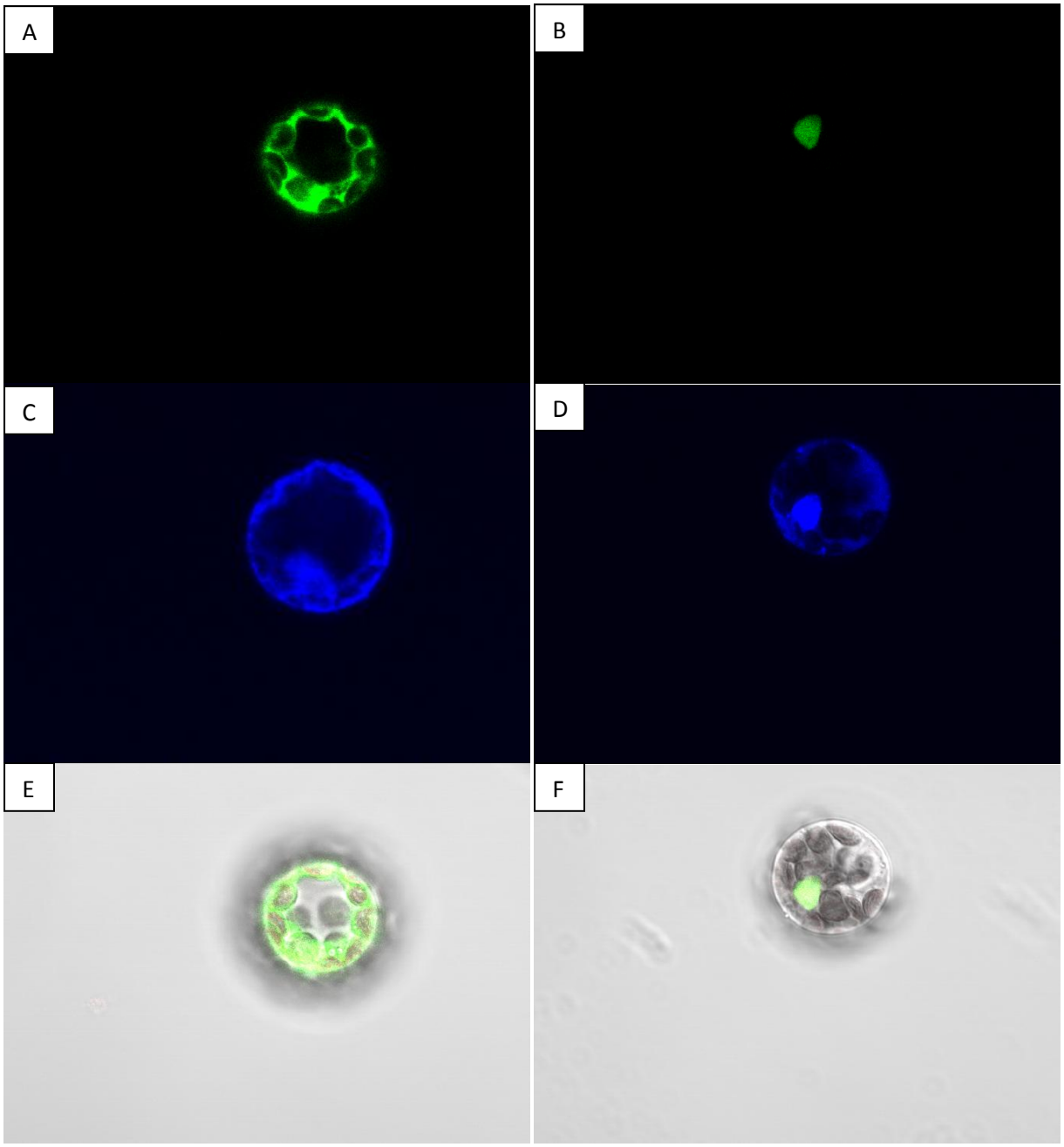


Figure 3.6 ***Arabidopsis thaliana*** **Protoplast Transient Expression of NRM1-GFP fusion Protein**

A. 35S::GFP GFP florescence

B. 35S:: GFP-GA5-NRM1 GFP florescence

C. 35S::GFP DAPI florescence

D. 35S:: GFP-GA5-NRM1 DAPI florescence

E. 35S::GFP Bright field

F. 35S:: GFP-GA5-NRM1 Bright field

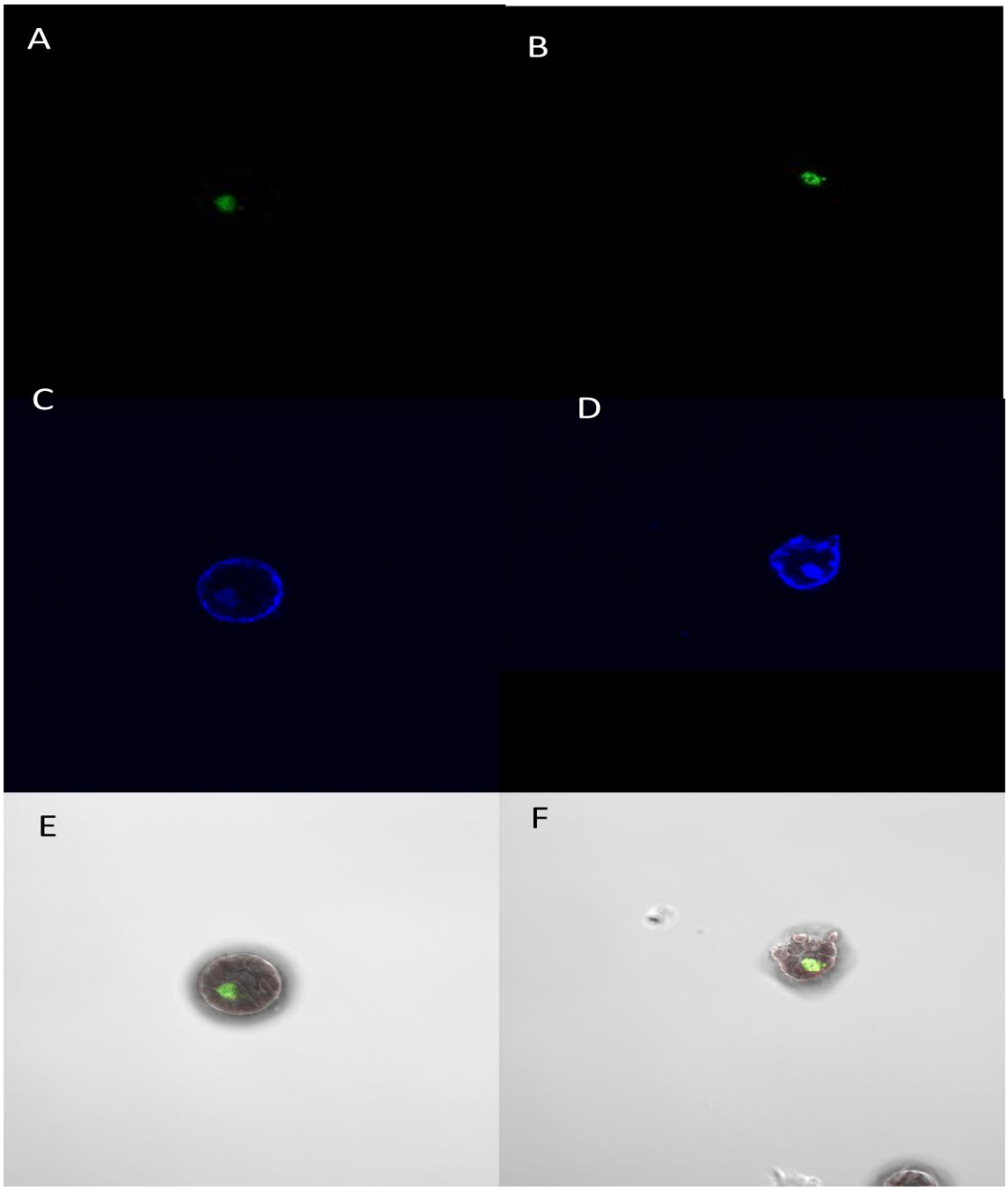


Figure 3.7 *Arabidopsis thaliana* Protoplast Transient Expression of NRM1-GFP fusion Protein Under High and Low Nitrate Conditions

A. 35S:: GFP-GA5-NRM1 GFP florescence low nitrate

B. 35S:: GFP-GA5-NRM1 GFP florescence high nitrate

C. 35S:: GFP-GA5-NRM1 DAPI florescence low nitrate

D. 35S:: GFP-GA5-NRM1 DAPI florescence high nitrate

E. 35S:: GFP-GA5-NRM1 bright field low nitrate

F. 35S:: GFP-GA5-NRM1 bright field high nitrate

Discussion

Localization of NRM1-GFP fusion constructs in onion epidermal cells and *Arabidopsis* mesophyll protoplasts indicated that NRM1 is localized to the nucleus. Transcription factors have been reported to be localized to different subcellular compartments under certain conditions. We tested whether NRM1 changed localization in response to nitrate levels in plants by isolating protoplasts from nitrate replete and nitrate starved plants. GFP-GA5-NRM1 florescence was not altered by nitrate levels, which does not support the hypothesis of differential localization. It is possible however that the fusion with GFP affected the protein in a way that could change its differential localization such as by obscuring a targeting domain.

Two different NRM1-GFP constructs pUC118_NRM1-smGFP and pCAMBIA 2300_NRM1-GFP were tested in onion epidermal cells and *Arabidopsis* plants respectively. The construct made for the onion cells was successful demonstrating nuclear localization while the one for plants was not since GFP fluorescence could not be detected. This difference could be for many reasons including that the protein fusions are being expressed in entirely different tissues and organisms. The onion cells are a very specific cell type from a plant that is not very closely related to *Arabidopsis*. This could result in differences in protein production and/or folding.

The NRM1-GFP construct did not produce fluorescent protein in stably transformed plants or protoplasts but the GFP-GA5-NRM1 performed as expected in protoplasts. This is most likely because the GFP-GA5-NRM1 construct was engineered to contain a GA5 linker peptide. The linker peptide is a flexible peptide that makes it more likely that the chimeric protein will fold properly by increasing the distance between the two proteins. In addition, the GFP-GA5-NRM1

construct has the GFP protein on the N-terminus of NRM1 while NRM1-GFP has the GFP peptide on the C-terminus of NRM1. This could also play a role in the successful expression, folding and targeting of the chimeric protein.

The GFP-GA5-NRM1 construct fluoresced in protoplasts, so it is likely that the GFP-GA5-NRM1 construct would produce properly functioning protein when inserted into a binary vector and stably transformed into *Arabidopsis*. It would also be useful to make a construct with a NRM1 promoter driving the NRM1-GFP chimeric protein. This construct could be used to further study the response of NRM1 to nitrate and other nutrients. An NRM1 promoter NRM1-GFP construct was being made with the same NRM1-GFP sequence without a linker peptide as the stable transformation construct but it was abandoned when the 35S::NRM1-GFP construct did not produce a fluorescent protein in protoplasts.

All of the data obtained from the GFP fusion experiments, combined with the fact that NRM1 has a recognizable bipartite nuclear localization sequence lead to the conclusion that NRM1 protein is localized primarily to the nucleus of the cell. This is consistent with the hypothesis that NRM1 is a transcription factor because in order to interact with gene promoters, NRM1 would need to be localized to the nucleus.

Chapter 4

Protein Interactions of NRM1

Introduction

The NRM family of genes contains a MYB-type repeat domain that is common to MYB transcription factors. Two MYB domains are normally required for specific DNA binding (Yanhui et al. 2006). The NRM family of genes contains only one MYB repeat (Figure 4.1), so it would make sense if these proteins interacted with other MYB-containing proteins to form a complex with at least 2 MYB domains. This complex could then bind specifically to a DNA region and influence transcription of other genes. In addition to its MYB-domain, NRM1 contains a putative protein interaction domain that further suggests it might interact with other proteins. In order to determine which other proteins NRM1 might interact with, I used the Yeast 2 hybrid system (Chien et al. 1991; Amberg et al. 2005).

The Yeast 2 Hybrid system utilizes a transcription activating protein, GAL4, that has been divided into two halves. The portion of the GAL4 protein with the DNA binding domain is fused to the bait protein. The bait protein is the gene that will be tested for interaction against a cDNA library or other pre-selected proteins. The plasmid that contains the bait fusion also contains a gene for tryptophan synthesis to select for transformed yeast. The portion of the GAL4 protein that contains the transcription activation domain is fused to the prey protein. The genes encoding the prey proteins are usually provided in a cDNA library but genes encoding a protein that is hypothesized to interact with the protein of interest can also be used. The plasmid that contains the prey fusion also contains a gene for leucine synthesis to select for transformed yeast.

```
NRM1        -MIKKFSNMDY-NQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIEACKRELPE 58
NRM3        -MIKNLSNMKNDNQKREKCCEYIEALEEERRKINVFQRELPLCVELVTQAIEAYKREISG 59
NRM2        MMMFKSGDMDY-TQKMKRCHEYVEALEEEQKKIQVFQRELPLCLELVTQAIESCRKELSE 59
NRM4        -MMV---EMDY-AKKMQKCHEYVEALEEEQKKIQVFQRELPLCLELVTQAIEACRKELSG 55
            *:   :*.  :* ::* :*:******:.**:*********::*******: ::*:.
                                                  ____________________________________________
                                                  Leucine Zipper like domain

NRM1        MTTENMYGQPECSEQTTGECG-PVLEQFLTIKDSSTSNEEEDEEFDD---EHGNHDPDND 114
NRM3        TSTDNLYGQSECSEQTTGECG-RILDLFIPIKHSSTSIEEEVDDKDDDDEEHQSHETDID 118
NRM2        S-SEHVGGQSECSERTTSECGGAVFEEFMPIKWSSASSDETDKDEEA---EKTEMMTNEN 115
NRM4        T-TTTTS--EQCSEQTTSVCGGPVFEEFIPIKKISSLCEEVQEEEEE---DGEHESSPEL 109
             :     :***:**. **  ::: *:.** *: :* .:: :    :    .

NRM1        SEDKNTKSDWLKSVQLWNQP-------------DHPLLP-KEERLQQETMTRDESMRK-D 159
NRM3        FDDKNMKSEWLKSVQLWNQS-------------DAVVSNNRQDRSQEKTETLVELIKIND 165
NRM2        NDGDKKKSDWLRSVQLWNQSPDPQPNN----KKPMVIEVKRSAGAFQPFQKEKPKAADSQ 171
NRM4        VN--NKKSDWLRSVQLWNHSPDLNPKEERVAKKAKVVEVKPKSGAFQPFQK-RVLETDLQ 166
            : : **:**:******:.            :   .   :  .      :

NRM1        PMVNGGEGRKR-----EAEKDGG-----------------GGR----KQRRCWSSQLHR 192
NRM3        EAAKKNNNIKSPVTTSDGGSGGG-----------------GGRRGQRKNRRCWSQELHR 207
NRM2        PLIKAITPTSTTTTSSTAETVGG-------GKE---FEEQK-QSHSNRKQRRCWSPELHR 220
NRM4        PAVKVASSMPATTTSSTTETCGGKSDLIKAGDEERRIEQQQSQSHTHRKQRRCWSPELHR 226
             :          . **                 *:***** :***
                                                                          ____________

NRM1        RFLNALQHLGGPHVATPKQIREFMKVDGLTNDEVKSHLQKYRLHTRRP---RQTVPNNGN 249
NRM3        RFLNALKQLGGPHVATPKQIRDIMKVDGLTNDEVKSHLQKYRLHARRP---SQTTPNNRN 264
NRM2        RFLHALQQLGGSHVATPKQIRDLMKVDGLTNDEVKSHLQKYRLHTRRP-ATPVVRTGGEN 279
NRM4        RFLNALQQLGGSHVATPKQIRDHMKVDGLTNDEVKSHLQKYRLHTRRPAATSVAAQSTGN 286
            ***:**::***.*********: **********************:***    . .  *
                                  ____________________________________________
                                  MYB Like DNA Binding Domain

NRM1        SQTQHFVVVGGLWVPQSD-----YSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQWPSHS 304
NRM3        SQTQHFVVVGGIWVPQTN-----HSTANAVNAVASG---ETTGIYGPMVSSLPSEWPRHS 316
NRM2        PQQRQFMVMEGIWVPS-------HDTTNN-------------RVYAPVATQPPQ------ 313
NRM4        QQQPQFVVVGGIWVPSSQDFPPPSDVANKG------------GVYAPVAVAQS------- 327
            * :*:*: *:***.      .. :           :*.::.  .

NRM1        NYRPSIIVDEGSGSHSEGVVVRCSSPAMSSSTRNHYVKNN--- 344
NRM3        NFGRKISEDRSRCSNNG--FFRCSSPAMSCSTRTKTKDAKIIS 357
NRM2        ---SSTSGERSN--------RGCKSPATSSTTTHTPHLLPLS- 344
NRM4        ---PKRSLERS----------CNSPAASSS-TNTNTSTPVS- 354
             .  :..        *.*** *.:
```

Figure 4.1 Protein Alignment of NRM1, 2, 3 and 4

Alignment of the most similar NRM family members. The Leucine zipper like domain is highlighted in blue and the MYB like DNA binding domain is highlighted in red.

If two proteins interact, they will bring the DNA binding domain and the transcriptional activation domain that they are fused to into close proximity with each other. The two domains will then be able to activate transcription of reporter genes for beta galactosidase and histidine synthesis.

Two bait constructs were made to test for interactions. One was a full length NRM1 protein and the other was the portion of the gene without the DNA binding domain to minimize the possibility of false positives if this DNA binding region could interact with the yeast reporter gene promoters. These two constructs were cloned into the pACT plasmid at the C-terminal end of the GAL4 activation domain. These two bait constructs were tested against two different *Arabidopsis* cDNA libraries obtained from the *Arabidopsis* Biological Resource Center. The first cDNA library is the Walker Two-hybrid cDNA library. This library was made from size selected mRNA isolated from mature leaves and roots and cloned into the lambdaACT prey vector. The second cDNA library is the Kim & Theologis LAMBDA-ACT Two-hybrid cDNA library. This library was made using from mRNA isolated from 3 day old etiolated seedlings cloned into ƛACT prey vector**.** There were several positive colonies indicating apparent interactions detected during the screen. When the potential interactors were sequenced they were found to be several different tRNA synthetase genes that are known to be common false positives in Yeast 2 Hybrid experiments. To date there have been no genuine interactions found in the library screens. In addition to the library screens, a full length and truncated NRM1 gene without the MYB domain were cloned into the pMC86 plasmid which contains the GAL4 binding domain upstream of the NRM1 constructs. These were made to test for the possibility that NRM1 forms homo-dimers through the protein interaction domains. There were no

interactions detected when these plasmids were transformed into yeast lines containing the NRM1 constructs in pACT vector. Each of these experiments was performed twice with similar results each time.

Materials and Methods

Yeast Lines and Media

The primary yeast line used in these experiments was the Y190 yeast strain. This strain is grown on YPD complete yeast media that supplies all the amino acids that this yeast strain cannot synthesize including tryptophan, leucine and histidine. Once the yeast was transformed with the bait plasmid pMC86 it was grown on Synthetic Dropout or SD media lacking tryptophan to select for colonies transformed with this plasmid which contains a Tryptophan synthesis gene. When the yeast have been transformed with both bait and prey plasmids it is grown on SD media lacking Tryptophan, Histidine and Leucine. The media was also supplemented with 3amino triazol or 3AT to competitively inhibit basal levels of histidine synthesis activity. The lack of Leucine is to select for the pACT2 plasmid that contains a Leucine synthesis gene. The media did not contain histidine and did contain 3AT to select for yeast colonies in which interacting proteins formed a functional GAL4 transcription activator complex and allow transcription of a reporter. If there were interacting proteins, they form a gene required for Histidine synthesis along with a β galactosidase gene. The *E. coli* strain BNN132 was used to host the phage and excise the cDNA libraries. The bacterial cells were grown on LB media with 50 μg/mL carbenicillin.

cDNA Library

Two cDNA libraries were used in this experiment. The libraries were ordered from the *Arabidopsis* Biological Resource Center at The Ohio State University. These libraries were chosen from the many available libraries for two main reasons. First, the libraries represented two stages in the life cycle of the plants. Second both libraries were made from tissues that included roots where NRM1 is expressed.

The first library is the Walker two-Hybrid cDNA library. This library was constructed using leaf and root tissue from mature *Arabidopsis thaliana*. The cDNA was made using random primers and size selected for inserts greater than 300 base pairs. The fragments were ligated to adapters then into the ƛACT plasmid and 1.9 x 10^6 primary transformants were obtained.

The second library that was used was the Kim and Theologis LAMBDA-ACT two-hybrid cDNA library (Kim et al. 1997). This library was made using oligo dtt primers to make cDNA from mRNA obtained from 3 day old etiolated *Arabidopsis thaliana* seedlings. This produced 36 million independent recombinants.

The libraries arrived in phage and were subsequently converted into a plasmid library following the guidelines included with the library. Approximately 10^8 phage particles were added to 2mL logarithmically grown BNN132 E. coli cells, and $MgCl_2$ was added to 10mM concentration. This solution was incubated at 30^0C for 30 minutes without shaking. The cells were incubated for 1 hour at 37^oC after the addition of 2 mL LB media. 200 µL cells were spread on each of 10 plates of LB plus 50µg/mL carbenicillin and 0.2% glucose. 10 mL of liquid LB media was added to each plate and the cells were re-suspended using plastic cell spreaders.

The liquid from each plate was pooled and used to inoculate 3 L of terrific broth media plus 50μg/mL carbenicillin (CSHP, 2006). The cells were incubated overnight after which plasmid DNA was extracted using a Qiagen maxi-prep kit and following the included protocol. This method was repeated for each of the two libraries.

Bait Constructs

Two bait constructs were made to test for interactions between NRM1 and itself or any gene included in the cDNA libraries. The first construct was a full length NRM1 gene (Figure 4.2) amplified from cDNA with the primers VSNMY1 and VSNMY2. The second construct was the first portion of the NRM1 gene (Figure 4.2). This portion contains the leucine zipper like domain but not the MYB domain. This construct was designed to test the protein interaction domain without the putative DNA binding domain which could possibly bind to the reporter gen promoter without interacting with a prey protein. This construct was amplified from cDNA using the primers VSNMY1 and VSNM01. The two constructs were ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. Constructs with the correct sequence were ligated into the pMC86 bait plasmid using SalI and BglII restriction enzymes.

NRM1 Prey constructs

The full length gene and the portion that contains the leucine zipper like domain were used to test as prey as well as bait. The full length construct was amplified from cDNA using the primers VSPACTFL-L and VSPACTFL-R. The truncated construct was amplified from cDNA using the primers VSPACTFL-L and VSPACT0-r. The two constructs were ligated into pDRIVE and the

sequence was verified using M13 forward and reverse primers. Constructs with the correct sequence were ligated into the pACT2 bait plasmid using NcoI and BglII restriction enzymes.

Yeast Transformation

All transformations were performed using protocols adapted from the Clonetech Yeast Protocols Handbook (Clontech Laboratories 2009). Untransformed yeast was first grown in 50 mL cultures overnight at 30^0C shaking at 250 rpms. The culture was then diluted to bring the OD_{600} to 0.2-0.3 and incubate until the OD_{600} was between 0.4 and 0.6. The culture was then pelleted at 1000g then re-suspended and combined in sterile TE buffer and pelleted again. The washed pellet was re-suspended in 1.5 mL 10mM Tris-HCL 1mM EDTA and 100mM lithium acetate. Bait Plasmid DNA was then placed in a fresh tube along with 0.1 mg of Herring testes carrier DNA. The full length NRM1 bait construct was added at 102 ng/µL concentration and the truncated NRM1 bait construct was added at 112 ng/µL concentration. 100µL of the yeast cells and 600µL PEG/LiAc solution were added and mixed by vortexing. This mixture was then incubated at 30^0C for 30 minutes followed by the addition of 70µL DMSO. The mixture was then heat shocked for 15 minutes at 42^0C and quenched on ice for 2 minutes and pelleted. The cells were re-suspended in 500µL TE buffer and split between 5 plates and spread evenly. For the bait plasmid transformation the cells were grown on SD minus tryptophan plates. The colonies that grew successfully were re-suspended in 1mL YPD + 25% glycerol and stored at -80^0C to later be transformed with prey construct plasmids.

A.

NRM1 Full Length

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETM

TRDESMRKDPMVNGGEGRKREAEKDGGGGRKQRRCWSSQLHRRFLNALQH

LGGPHVATPKQIREFMKVDGLTNDEVKSHLQKYRLHTRRPRQTVPNNGNS

QTQHFVVVGGLWVPQSDYSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQW

PSHSNYRPSIIVDEGSGSHSEGVVVRCSSPAMSSSTRNHYVKNN

B.

NRM1 Leucine Zipper

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETM

TRDESMRKDPMVNGGEGRKREAEKDGGGGRK

Figure 4.2 Amino Acid Sequence of NRM1 Constructs

The Leucine zipper like domain is highlighted in blue and the MYB like DNA binding domain is highlighted in red.

A. Full length amino acid sequence

B. Amino acid sequence of the portion of NRM1 with the leucine zipper domain and without the MYB like DNA binding domain.

This procedure was repeated to transform the NRM1 prey construct plasmids with the minor changes that the untransformed cells were grown in SD minus tryptophan media and the transformed cells were selected on SD plus 3 Amino Triazol minus tryptophan, Leucine and Histidine.

The cDNA libraries were transformed into cells containing either NRM1 bait construct plasmid. Both cDNA libraries were used at separate times and each pairing was repeated two times. The transformation was the same as the prey construct transformation except it was scaled to ten times volume and the transformants were plated on 25 large 150mm SD plus 3 Amino Triazol minus tryptophan, Leucine and Histidine plates per transformation.

Screening Possible Positive Colonies

Colonies from the prey construct transformations that grew on selective plates were transferred to new plates. The colonies that survived the second screening were then transferred to Whatman number five filter paper by placing the filter paper on the plates then rubbing with forceps until the filter is evenly moist. The filter was then removed and placed in liquid nitrogen for 10 seconds. The filter was then placed on top of another filter pre soaked in X-gal solution. The filter was then incubated at 30^0C for 6 hours and checked for appearance of blue coloration that would indicate β-galactosidase activity.

To obtain plasmid DNA, yeast cells were treated using the Qiagen user submitted Yeast Miniprep Protocol. The plasmid DNA obtained was transformed into DH5α *E. coli* selected for transformed colonies on LB plus 50mg/mL carbenicillin. The plasmids were then extracted from yeast cultures using the Qiagen miniprep protocol and tested to determine if the plasmid was

the bait or prey plasmid using Pact F and Pact R sequencing primers. Plasmids that were confirmed to be pACT were then sent for sequencing using the same pair of primers.

Results

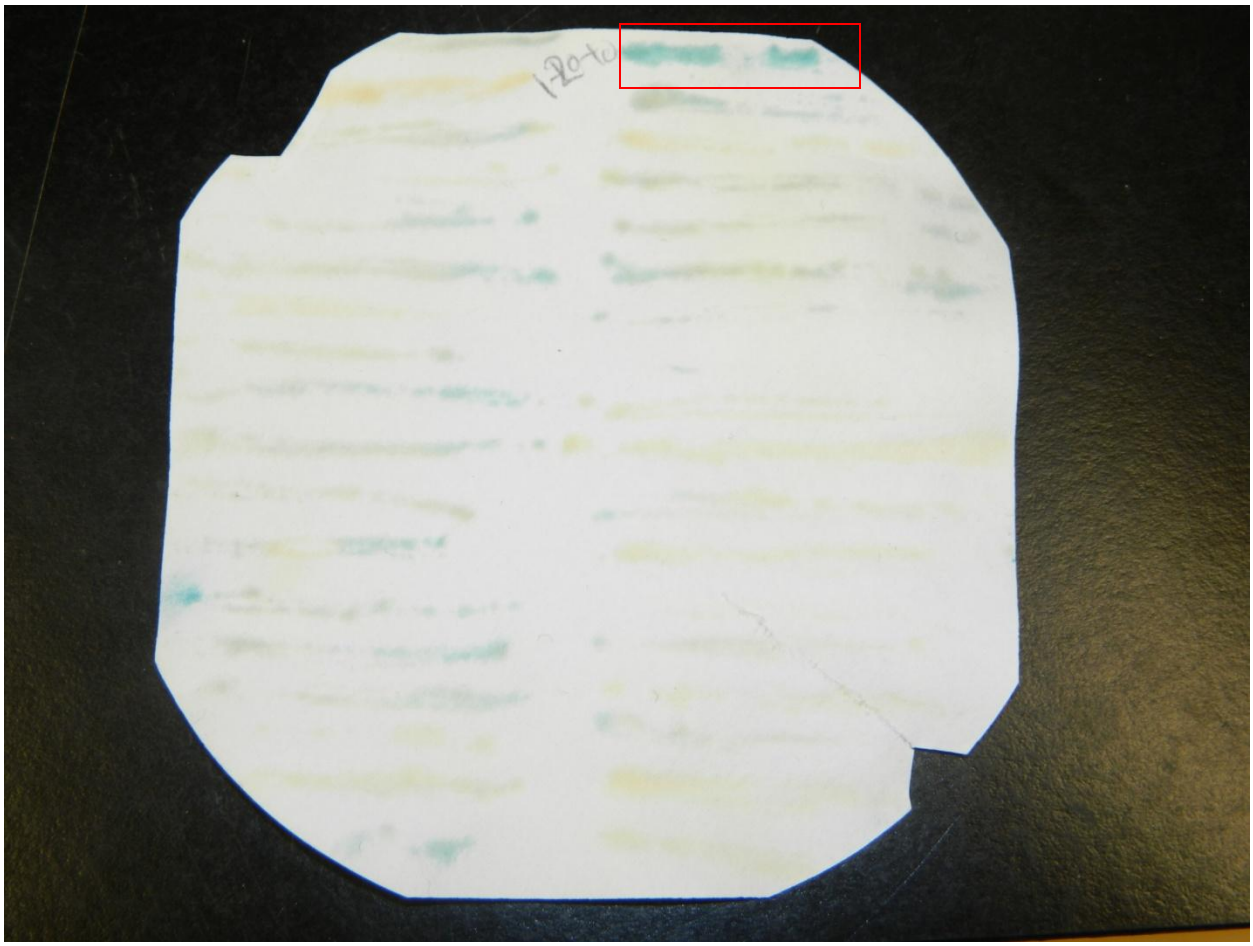
Library Prey Plasmids

A positive control line obtained from Sungbong Shin (Colorado State University) grew on selective plates and showed dark blue coloration when treated with x-gal (Figure 4.3). Several colonies that grew on selective media and showed significant β-galactosidase activity after repeated screening were sequenced to determine the specific cDNA in the prey plasmid. Sequence obtained from these plasmids was compared to the *Arabidopsis* genome using the NCBI BLAST web search. The results showed several ribosome associated genes that are known to interact with a large number of proteins, and are a common false positive result in Yeast 2 Hybrid experiments. Two of these genes At5g56670 and At5g26707 were detected many times. The complete results are shown in Figure 4.4

NRM1 Prey Plasmids

The NRM1 bait and NRM1 prey plasmids were tested in pairs using small scale transformations. Each pairing was repeated at least 3 times with repeated transformations. There were no positive, (His+) colonies in any of the pairings. To verify that both bait and prey plasmids were successfully transformed the cells were grown on SD minus tryptophan and leucine plates (Figure 4.5). All 4 pairing showed normal growth on these plates indicating both bait and prey were present even though no interaction was detectable.

A.



B.

Figure 4.3 Positive Control

A. Positive control in red box was much darker blue than the false positive colonies.
B. Positive control grew robustly on SD media minus tryptophan, histidine and leucine.

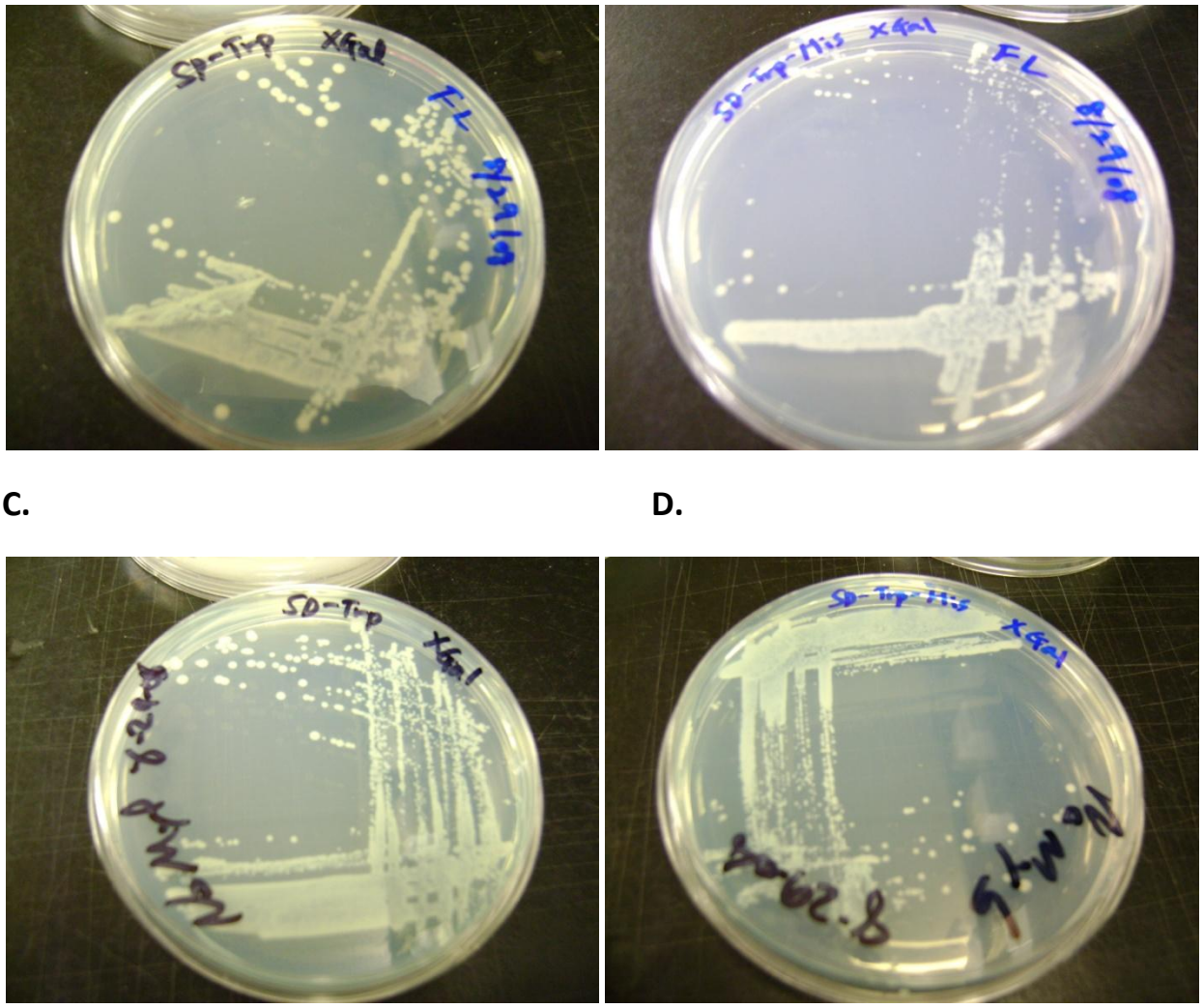
(Note, this strain of yeast has red spots when growing very densely)

Clone	Description	Gene ID Number
y13	40S ribosomal protein S30 mRNA	At5g56670
y31	40S ribosomal protein S30 mRNA	At5g56670
y40	glutamyl tRNA synthase	At5g26707
y45	26s Ribosomal rna	At3g41950
y46	glutamyl tRNA synthase	At5g26707
y55	40S ribosomal protein S30 mRNA	At5g56670
y56	glutamyl tRNA synthase	At5g26707
y57	40S ribosomal protein S30 mRNA	At5g56670
y59	glutamyl tRNA synthase	At5g26707
y63	glutamyl tRNA synthase	At5g26707
y66	40S ribosomal protein S30 mRNA	At5g56670
y84	40S ribosomal protein S30 mRNA	At5g56670
y86	glutamyl tRNA synthase	At5g26707

Figure 4.4 Possible Protein Interactions

Colonies that showed growth on selective media and blue staining when treated with X-gal were cultured. Plasmid DNA was purified and sent for sequencing. The sequence results were BLASTED against the *Arabidopsis* thaliana database.

A. B.



C. D.

Figure 4.5 Growth of NRM1 bait and Prey Constructs on Dropout Media

A. NRM1 full length bait construct on SD media lacking Tryptophan

B. NRM1 full length bait and prey constructs on SD media lacking Tryptophan and Leucine

C. NRM1 leucine zipper bait construct on SD media lacking Tryptophan

D. NRM1 leucine zipper bait and prey constructs on SD media lacking Tryptophan and Leucine

Discussion

No legitimate protein-protein interactions with NRM1 were detected using the yeast two hybrid technique. These results do not shed any light on what proteins NRM1 may or may not interact with. There are a few possible reasons that could explain why no interactions were detected. The first and most obvious is that NRM1 does not interact with any other proteins. While we cannot rule this possibility out we cannot support it either.

A second possibility is that NRM1 interacts with other proteins in *Arabidopsis thaliana* but the interaction is not detectable in the yeast two hybrid system. There could be problems with protein folding in yeast that would interfere with the interactions by obscuring or mis-forming the interacting domain of NRM1. There could also be some post translational modifications to NRM1 that are needed before any interaction could occur, even though normally the yeast is a very good system for properly expressing proteins from *Arabidopsis thaliana* because it is also a eukaryotic organism.

A third possibility is that NRM1 will only interact with a protein complex containing more than one other protein. It is possible that a protein with which NRM1 interacts would undergo a conformational change when interacting with another protein that would allow it to interact with NRM1. This would be impossible to detect using the yeast two hybrid technique since it only tests the interaction of two proteins. NRM1 could also interact with portions of multiple proteins that would only be in close enough proximity when they were part of a larger complex. NRM1 is a putative transcription factor and it is possible that it would interact with the RNA polymerase complex in some way. If this were the case it would be very hard to detect

this interaction because of the size of the complex and the large number of interacting proteins in the functional complex.

After the yeast two hybrid experiments were completed, another group performed a very ambitious set of experiments testing around 10,000 *Arabidopsis thaliana* proteins in pairs (Mukhtar et al. 2011). This experiment tested individual pairs of proteins using a modified yeast two hybrid technique adapted for very high throughput. The proteins were tested as bait and prey against all the other proteins in the experiment. The results of this experiment are available in an online searchable database at http://interactome.dfci.harvard.edu/A_thaliana/index.php?page=search . NRM1 was one of the proteins tested in this experiment. There were no interactions discovered in this experiment as well. While this does not shed any more light on NRM1, it did make me more confident about the negative results from my own experiments. It is more likely that there are no interactions that can be detected using this approach and not that there was just experimental error. An interesting side note is that there were several Interactions detected with the NRM2 protein. The interactions detected were with AT3G54190 (Teosinte Branched1), AT1G69690 (Transducin/WD40 repeat-like superfamily protein) and AT1G35670 (Calcium-Dependent Protein Kinase 2).

Chapter 5

Altered Expression of NRM1

Introduction

Reverse genetics is a very useful and commonly used tool to determine the function of a gene. Reverse genetics is the process of examining alterations to a known gene to look for a phenotype as opposed to forward or classical genetics which tries to find the gene or genes responsible for a phenotypic alterations. Plants with T-DNA insertions that abolish NRM1 expression show no obvious phenotype. This is not at all uncommon for genes that are members of multigene families in plants. NRM1 is part of a gene family with seven similar members. It is commonly asserted that similar genes can compensate for knockouts of other gene family members. To explore this in more detail I made plants that are lacking multiple members of the NRM family. These plants were analyzed in a variety of growth conditions to determine if they have a phenotype different from wild type *Arabidopsis*.

Salk knockout lines are a large collection of randomLy inserted T-DNA lines. To generate the collections a T-DNA cassette was transformed into *Arabidopsis* and these plants were genotyped to determine the location of the insertion event. Thousands of lines with different insertion locations were cataloged and propagated. These lines are available online in an easily searchable database organized by insertion location http://www.arabidopsis.org/. These lines are very easy to obtain and inexpensive. The lines have to be selected for homozygosity and tested for lack of expression of the intended knockout.

Single Salk knockout lines for NRM1 or NRM2 were selected for homozygosity and analyzed by RTPCR to confirm lack of expression did not have a detectable phenotype. NRM1 and 3 are very similar and have similar expression patterns, mostly in the root tissue, so the

could act redundantly and double mutant plants without NRM1 or NRM3 could display a phenotype. However there are no available NRM3 T-DNA insertion lines available. There are two common explanations for lack of insertional mutants in specific genes. The first is that the lack of the protein, in this case NRM3 protein, is lethal to the plants and therefore no knockouts are recovered. Although the large number of insertion lines should provide a high probability of insertion events in every gene in the genome, the other possibility is that there simply was no T-DNA inserted into this specific region of genome. NRM2 is also similar to NRM1 (43% amino acid identity) and is expressed in every tissue of the whole plant so there is concurrent expression in the roots. NRM2 also has the highest level of expression throughout the plant and was detected along with NRM1 in the original microarray experiment. For these reasons, double mutants of NRM1 and NRM2 were made.

MicroRNAs targeting a gene of interest is another method for knocking down gene expression. This method was also used to obtain plants with decreased NMR gene expression. Transgenic plants expressing miRNA constructs can be produced using constructs designed at Web MicroRNA Designer at wmd3.weigleworld.org (Ossowski et al. 2008). The first step in producing these constructs is to design primers that will modify the stem loop portion of MiR319a from the RS300 plasmid obtained from the website. The primers are designed using the website and they modify the existing miRNA to have a 21 base pair sequence on the stem that will silence the genes you choose. This region has several requirements for efficient silencing, including not having more than 5 mismatches or 2 mismatches in a row with the target sequence and hybridization energy 80-95% of perfect match with absolute hybridization energy between -35 and -38 kcal /mol(Schwab et al. 2006). Because the design of these miRNA

constructs is not 100% effective, multiple sites are targeted in each set of genes. If no T-DNA insertion mutants are available because knocking out this gene is lethal then the DEX inducible promoter will allow us to generate plants that will grow normally until the miRNA is induced then to down regulate NRM3 expression.

Another common tool of reverse genetics to gain insight into gene function is expressing a gene at high levels, or overexpression. Plants expressing genes at high levels can shed light on a pathway by overloading the system and negating any change in expression level under different conditions. These overexpressors are a very good compliment to knockout lines to determine the function of a gene.

The standard promoter for over-expressing plants is the 35S promoter. It drives expression at very high levels throughout the plant. The 35S promoter will cause the gene of interest to be expressed at very high levels in all tissues, including those in which it would not normally be expressed. Therefore, an alternative approach is to use a tissue specific promoter that matches the expression pattern of the gene of interest. The pea metallothionein-like gene promoter has been shown to drive high levels of GUS expression in the roots of *Arabidopsis (Liu et al. 2009)*. By using this promoter, NRM1 is overexpressed only in tissues where it is normally expressed. By expressing NRM1 at high levels in only root cells there is less chance of detecting phenotypes resulting from mis-expression of NRM1 in other tissues.

In this section I will present the results of experiments using plants with altered expression of NRM1. Root architecture of NRM1-NRM2 grown on complete media and media

lacking nitrate and phosphate were examined. Root architecture of NRM1 overexpressors grown on complete media and media lacking nitrate was also be examined.

Materials and Methods

Salk Double Mutants

Experiments performed by previous lab members had shown that NRM1 knockout lines had no detectable phenotype. To address possible gene redundancy, multiple knockouts of the NRM family were obtained or made. The closest family member NRM 3 had no T-DNA insertion in any of the readily available collections. NRM2 also has high sequence similarity to NRM1 and is highly expressed in all tissues and has T-DNA insertion lines available. For these reasons NRM1/NRM2 double mutant lines were produced. Salk T-DNA insertion lines were ordered from the *Arabidopsis* Biological Resource Center at The Ohio State University. The T-DNA insertion lines SALK_067195 and SALK_014754 were chosen for NRM1 knockouts and the T-DNA insertion lines SALK_083547, SALK_144656C and SALK_083545 were chosen for NRM2 knockout lines. SALK_144656C was tested first among the NRM2 lines because it was a confirmed line that had already been confirmed to be an insertion in NRM2. These lines were grown on MS + 50µg/mL kanamycin to select for plants with T-DNA inserts. Lines that showed evidence of antibiotic gene silencing were set aside and only those that showed resistance were used. Several resistant plants from each line were transferred to soil and allowed to self-fertilize and produce seeds. The seeds from these individual plants were collected and plated on MS + 50µg/mL kanamycin. The lines that showed 100% resistance were selected as homozygous and representative plants were tested using the SALK test primers for that line

listed in the primer appendix. When confirmed homozygous plants were obtained, RT-PCR was performed to confirm lack of expression of either NRM1 or NRM2 in the corresponding lines.

The NRM2 line SALK_144656C was crossed with both NRM1 knockout lines SALK_067195 and SALK_014754. For ease SALK_144656C and SALK_067195 crosses are referred to as double mutant 1 (DM1) and SALK_144656C and SALK_014754 crosses are referred to as double mutant 2 (DM2). For each pair of genes reciprocal crosses were performed to eliminate the possibility of any segregation distortions complications. Healthy flowering plants from each line were chosen for the crosses. Flowers from those plants that had not yet opened were chosen as recipient stigmas because they had not had a chance to be self-pollinated. The closed flowers were observed under a dissecting microscope at low power and the stems were immobilized several millimeters below the flower with tape. The sepals and petals were carefully removed to reveal the anthers and stigma. The anthers were then carefully removed so the pollen does not contact the stigma. A newly opened flower from the donor plant was then carefully rubbed against the stigma to transfer pollen. The stigma were then labeled with a small piece of tape to identify the flowers that had been crossed. The siliques from successful crosses were harvested individually when mature and the seeds were plated on MS + 50µg/mL kanamycin. Resistant plants heterozygous for both genes from each cross were transferred to soil, allowed to self-fertilize and produce seeds. These seeds were plated on MS + 50µg/mL kanamycin and many resistant plants were transferred to soil. DNA was obtained from these plants and separate PCR reactions were performed with primers for both genes involved in the cross. A small portion (one in sixteen in theory) of these plants were homozygous for insertions in both genes. These double homozygous plants were allowed to

self fertilize and produce seeds**.** These seeds were plated on MS + 50µg/mL kanamycin and the seedlings were tested for expression of both genes by RT-PCR. The confirmed plants were then grown on a variety of media to test for phenotypic variation from wild type plants.

Artificial Micro RNA Constructs

A set of vectors was designed to stably express a artificial micro RNA (miRNA) designed against either NRM3 or NRM1 and NRM3 in *Arabidopsis* thaliana under the control of an inducible promoter. Two constructs were made for NRM1 and NRM3 and two more were made for just NRM3. These constructs were designated NRM1-3b, NRM1-3d, NRM3a, NRM3b. Primers for each construct are listed in the primer table. The vector pTA7002 that contains a DEX inducible promoter was used as a starting point. Expression of the artificial micro RNA was driven by the DEX promoter, only in the presence of dexamethasone.

The artificial micro RNA sequences were designed using Web Micro RNA Designer at wmd3.weigleworld.org (Ossowski et al. 2008). This website uses the complete annotated sequence of *Arabidopsis thaliana* to design artificial micro RNAs that can target specific genes and not others in the same family or some, but not all, members of multigene families. The artificial microRNA designer WMD produced 4 oligonucleotide sequences (I, II, III and IV), which were used to make an artificial micro RNA from the miR319a precursor by site-directed mutagenesis. The plasmid pRS300 which contained the miR319a sequence was used as a template for the site directed mutagenesis PCR reactions. The primers A and B that flank the miR319 a region were used in every set of reactions and the primers I, II, III and IV were

changed depending on the artificial micro RNA being produced. The first round of 3 separate PCR reactions contains pairs of primers that use the miR319a template to produce changes in the sequence in the stem loop region that confer specificity to the desired gene or genes. The pairs of primers used were A/IV, III/II and I/B. It was important to use a proofreading TAQ polymerase such as Pfu Ultra polymerase that did not add an adenine to the end of the molecule so that these sequences from different PCR reactions would complement each other at the ends. The products from the first round of reactions were gel purified using the Qiagen gel purification kit. The next step was a PCR reaction that combined the three products from the first round of reactions into one reaction with the primers A and B. This reaction combines all 3 fragments into a contiguous sequence that is identical to the miR319a precursor except in the stem loop region. For this PCR reaction Easy A polymerase was used because it has proofreading capability and adds an adenine at the end of the molecule which facilitates TA cloning into the pDRIVE shuttle vector.

The constructs were ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The artificial micro RNA backbone that was unchanged by the site directed mutagenesis contained unique multiple cloning sites at each end inside the A and B primers. Fortunately there were a pair of restriction enzymes contained in these two multiple cloning sites that worked to ligate the sequence into the vector in the correct orientation. The artificial micro RNA sequences were ligated into the pTA7002 plasmid using the restriction enzymes xho I and spe I. The completed vector was then sequenced with the primers CA013 and CA014.

After all constructs were verified they were transformed into *Arabidopsis* using the floral

dip method. The NRM1-3b and NRM1-3d constructs that were designed against both genes were transformed into wild type columbia plants. The NRM3a and NRM3b constructs were each transformed into two NRM1 Salk knockout lines (SALK_067195 and SALK_014754).

Over Expressing Constructs

A vector was constructed to stably express NRM1 at very high levels in *Arabidopssis*. The binary vector pCAMBIA 2300 was used as a starting point. First, the pea metallothionein-like gene promoter(Fordham-Skelton et al. 1997) was amplified using the RPleft and RPright primers and ligated into pCAMBIA 2300 using the restriction enzymes Sph I and Hind III. The coding sequence for NRM1 was amplified from cDNA using NRMLeft and NRMstop primers. The construct was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The coding sequence was then ligated into the pCAMBIA 2300 using the restriction enzymes Sal I and BamH I. The completed vector was then sequenced with the primers FOR_1, FOR_2, FOR_3, FOR_4, FOR_5, REV_1, REV_2, REV_3, REV_4 and REV_5.

***Arabidopsis* Transformation**

Arabidopsis plants were grown in soil with 16 hours of light per day until the first inflorescence was initiated. The first bolts were cut off with scissors to encourage proliferation of many secondary bolts. The plants were allowed to grow for one week after the bolts were clipped. *Agrobacterium tumefaciens* with the desired binary plasmid was inoculated into 3 mL of Luria Broth with an appropriate antibiotic along with gentamycin so select against other microbes. The culture was grown for 8 hours at 30^{o}C with shaking. The 3mL culture was then added to a one liter flask containing 250mL of Luria Broth and appropriate antibiotics. This

large culture was grown at 30^{o}C overnight with shaking. The OD600 of the culture was measured and recorded. The culture was centrifuged at 4050 RCF for 20 minutes to pellet the bacteria. The pellet was resuspended in 5% sucrose and 0.02% Silwet L-77 to an OD600 of 0.8. The solution was transferred to a 500 mL beaker and plants were inverted and the inflorescence was dipped in the bacterial solution and swirled for about 5 seconds. The plants were covered with a clear plastic dome to maintain high humidity and left in the lab for 24 hours to recover. The plants were then placed back in growth chambers with 16 hours of light per day. After 1 week the dipping was repeated to infect new flowers. The plants were then allowed to mature and the seeds were collected from individual plants. The seeds were grown on MS plates with appropriate antibiotics and resistant plants from each individual dipped plant were selected.

Plant Growth Conditions

For all altered expression experiments modified Murashige and Skoog (Murashige and Skoog 1962) or MS media was used. For the first several years of my research the media was prepared from liquid stock solutions. Recently the media was changed to take advantage of Phyto Technologies Laboratories low cost dropout MS media. The chemical makeup of the media was no different but the Phytotech media comes in stable powder form and is much easier to use.

The component media was made from the stocks outlined in Figure 5.1. The media was modified to include or exclude nutrients as outlined in Figure 5.2. The Phyototech media used was either Murashige & Skoog modified basal salt mixture without nitrogen M531 at 0.78

grams per liter or Murashige & Skoog modified basal salt mixture without nitrogen, phosphorus and potassium M407 at 0.61 grams per liter. Potassium nitrate, potassium phosphate and potassium chloride were added to the media at the concentrations listed in Figure 5.2. The pH of the solution was then adjusted to 5.7 and sucrose was added at 10 grams per liter and agar was added at 8 grams per liter before autoclaving. After autoclaving the media was then poured into 110mm square gridded petri plates and allowed to cool.

The first step in ethanol/bleach seed sterilization was to aliquot seeds in 1.5 mL tubes. One mL of 95% ethanol was added to the tube, inverted to mix and then incubated for ten minutes. The ethanol was removed with a vacuum aspirator. The seeds were then washed in 1 mL of 20% Clorox bleach and 0.1% tween 20 for five minutes before the solution was removed by aspiration. The seeds were then rinsed 3 times with 1 mL of sterile water for at least 30 seconds. The seeds were then suspended in a sterile 0.1% agargel solution. The seeds were then either pipetted individually onto a plate or a selected volume was placed in the middle of a plate with an addition 2 mL of 0.1% agargel solution and the plate was swirled to distribute seeds evenly. The plates were allowed to dry for 20 to 30 minutes to solidify the agargel before the plates were sealed with miratape. All seeds were vernalized for at least 2 days in the dark at 4^{o}C before being transferred to the appropriate growth chamber.

A

Stock	Recipe	MW	Stock concentration
Calcium Chloride, dihydrate	44.0 g/L	147.01	299 mM
Magnesium Sulfate, 7-hydrate	37.0 g/L	246.47	150 mM
Potassium Phosphate, monobasic	17.0 g/L	136.09	125 mM
Potassium Chloride	7.495 g/L	74.55	100 mM
Potassium Nitrate	60.66 g/L	101.1	600 mM
10X MICRONUTRIENT STOCK	Sigma M0529		

B

Component	Amount per liter	Final concentration
ddH_2O	500 mL	
Sigma Micronutrient stock	100 mL	
Potassium Phosphate	10 mL	1.25 mM
Magnesium Sulfate	10 mL	1.5 mM
Calcium Chloride	10 mL	3.0 mM
Potassium Chloride	10 mL	1 mM
Potassium Nitrate	100 mL	60 mM
Sucrose	10 grams	1%
Agar	8 grams	0.8%

Figure5.1 Component Media for Vertical Plates

A. Stock Solutions

B. Recipe for Complete Media

Media	Abreviation	Macro Nutrient Concentrations
Complete Media	HN, HS or HP	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM
Low Nitrate	LN	Potassium Nitrate 6 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 10 mM
No Nitrate	0N	Potassium Nitrate 0 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 10 mM
No Phosphate	0P	Potassium Nitrate 60 mM Potassium Phosphate 0 mM Magnesium Sulfate 1.5 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM Magnesium Chloride 1.5mM
No Sulfate	0S	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 0 mM Calcium Chloride 3.0 mM Potassium Chloride 1 mM
No Calcium	0Ca	Potassium Nitrate 60 mM Potassium Phosphate 1.25 mM Magnesium Sulfate 1.5 mM Calcium Chloride 0 mM Potassium Chloride 1 mM

Figure 5.2 Dropout Media Composition

The vapor phase sterilization method was performed in the fume hood inside of a Tupperware cake container adapted as a vapor chamber. The seeds were placed in 1.7 mL tubes and it is very important to label the tubes with super permanent industrial sharpie markers that are resistant to chlorine gas so the labels are not oxidized thus making seed identification impossible. The tubes of seeds were opened and placed on a tube rack inside the container. A 250 mL beaker was also placed inside the container. To the beaker 100mL of Clorox bleach was added. 3 mL of HCl was added to the bleach and the cake container was immediately closed. The sealed container was then allowed to sit for 5 hours before the tubes were closed and removed. It is important to leave the container and the beaker of bleach in the fume hood overnight to prevent noxious fumes from being released in the lab. The seeds were then sprinkled onto plates or individually placed. The plates were then wrapped in miratape to seal. All seeds were vernalized for at least 2 days in the dark at 4^{o}C before being transferred to the appropriate growth chamber.

Plants were grown on 110mm gridded square plates. The seeds were placed in a line near the top of the plate evenly spaced with 12 seeds per plate as outlined in Figure 5.3. The plants were grown 21^{0}C with 16 hours of light and 8 hours of darkness for most of the experiments and also tested at 8 hours of light and 16 hours of darkness. The plates were placed on racks in the incubator at approximately a 75% angle. Plants were grown up to 10 days and then imaged with a document scanner. Scanned pictures (Figure 5.4) were analyzed with both EZ-Rhizo (Armengaud 2009; Armengaud et al. 2009) and Image J software (Henriques et al. 2010) to compare the programs. Image J was used for the final analysis because of its ease of use. Primary root length and lateral root number (if lateral roots were present) were

recorded. Wild type seedlings and transgenic seedlings were grown side by side on the same plate to eliminate plate to plate variation. Seedlings of one line and one media were averaged together and compared with wild type seedlings from the same set of plates. The student t-test was used to determine if there was a significant difference between the wild type and transgenic plants.

Results

Overexpressors

Plants overexpressing NRM1 were grown on complete media and low nitrate media split plates with half overexpressor and half wild type plants. Five lines representing plants propagated from five individual dipped plants were analyzed. All lines showed increased expression of NRM1 when compared to wild type plants (Figure 5.5). Several of the lines showed no significant differences from wild type in either treatment. The other lines showed variable amounts of root growth (Figure 5.6). Thus, no conclusions regarding the impact of NRM1 overexpression on root growth can be made.

Double Mutants

Double mutants of NRM1 and NRM2 grown on high nitrate had primary roots that were statistically the same length as wild type plants grown on the same plates (Figure 5.7), but the number of lateral roots on high nitrate was lower than wild type. On low nitrate media the double mutants had significantly shorter primary roots than wild type plants and also had fewer lateral roots. When grown on media lacking phosphate, the primary roots of the wild type and double mutant plants were not significantly different, the number of lateral roots were similar

in one of the two line and double mutants had fewer lateral roots than wild type in the other line.

MicroRNA

MicroRNA constructs were successfully made and transformed into *Arabidopsis*. The seeds from the transformed plants are currently being selected and grown for seed. The seeds from these plants will need to be selected and analyzed for a 3 to 1 ratio of resistant to susceptible plants. The lines with single insertions will be saved and plants will be transferred to soil. The seeds from these plants will be selected and homozygous plants with all resistant offspring will be propagated. Once homozygous lines are obtained these lines can be analyzed for phenotypes on nutrient deficient media.

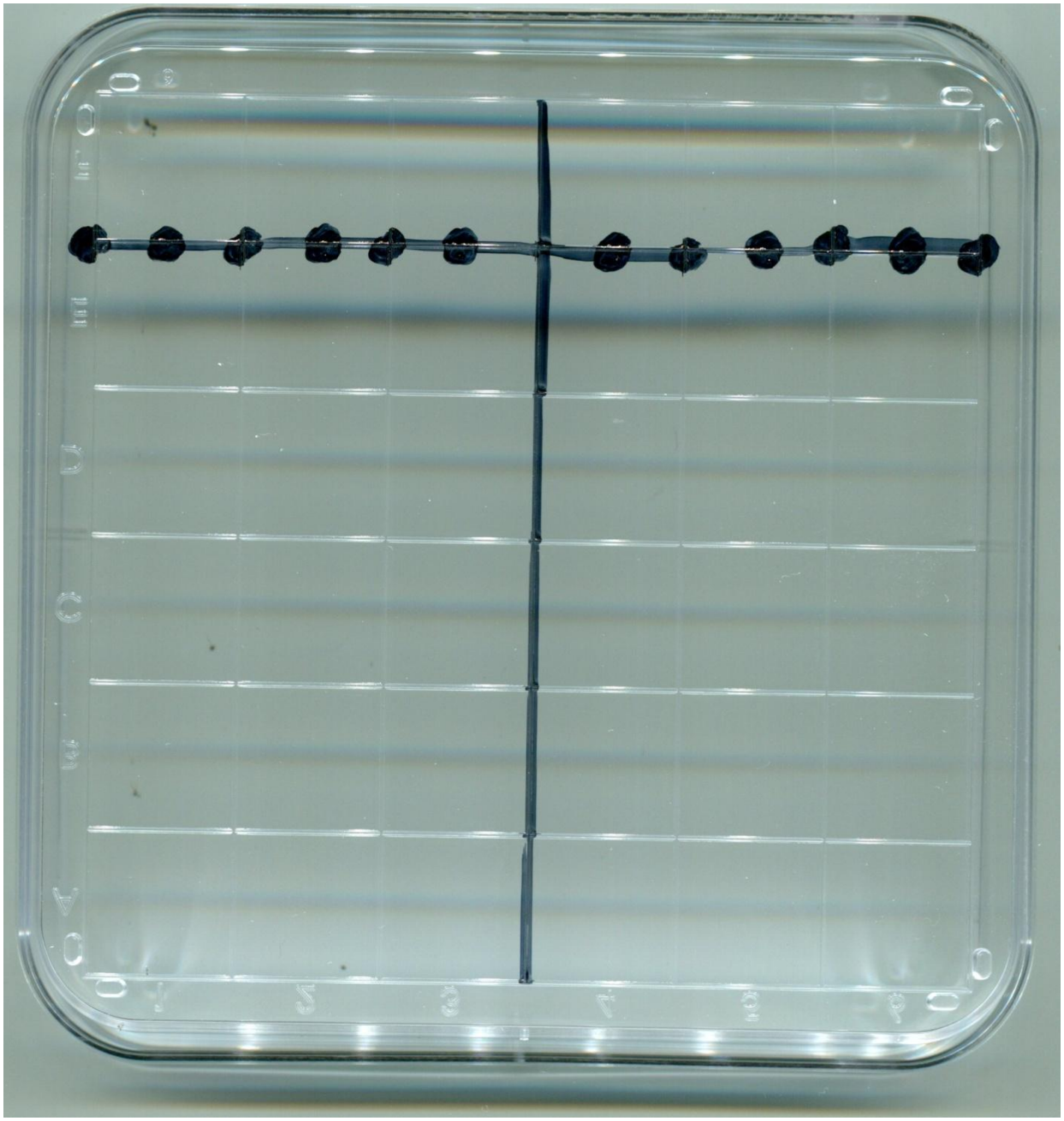


Figure 5.3 Seed distribution on vertical plates

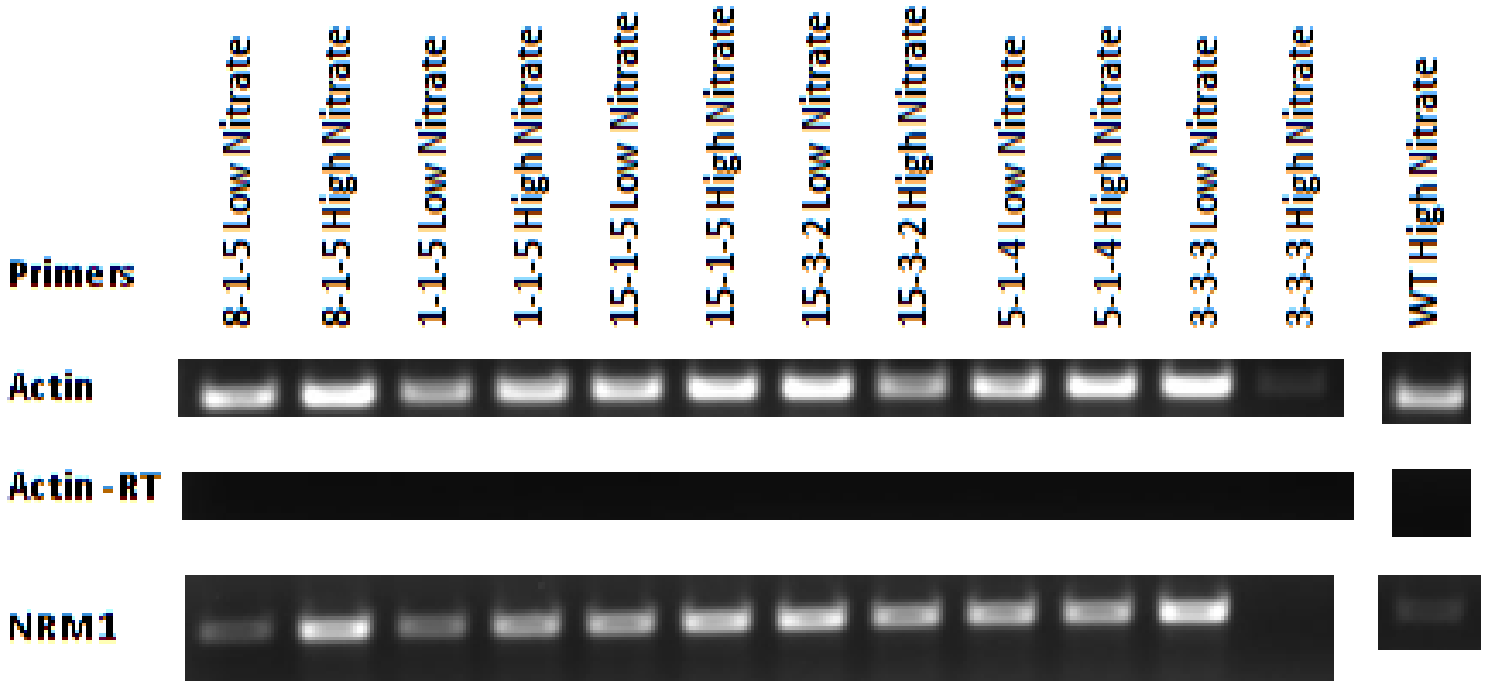


Figure 5.5 RT-PCR or NRM1 Expression in Overexpressor Lines

RNA extracted from roots of 8 day old transgenic seedlings.

-RT denotes lack of reverse transcriptase enzyme to check for genomic DNA contamination.

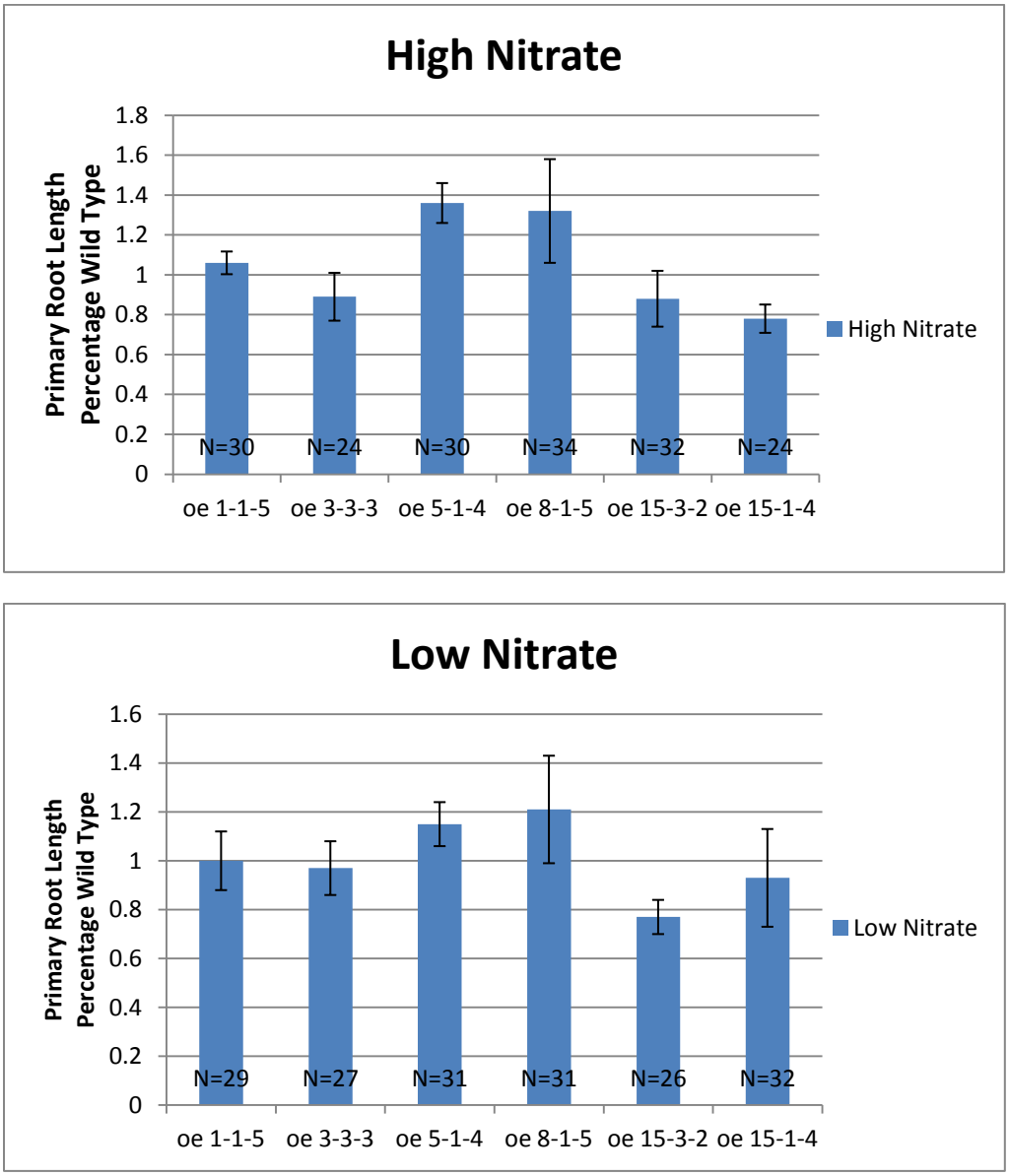


Figure 5.6 Overexpressor Root Architecture

Primary root length of Overexpressor represented as a percentage of Wild Type. Seedlings grown on split plates with complete media and low nitrate media for 6 days. Overexpressors were compared to wild type plants grown on the same plates. Roots were measured using Image J software. Each plate of each treatment was averaged for overexpressor and wild type seedlings.

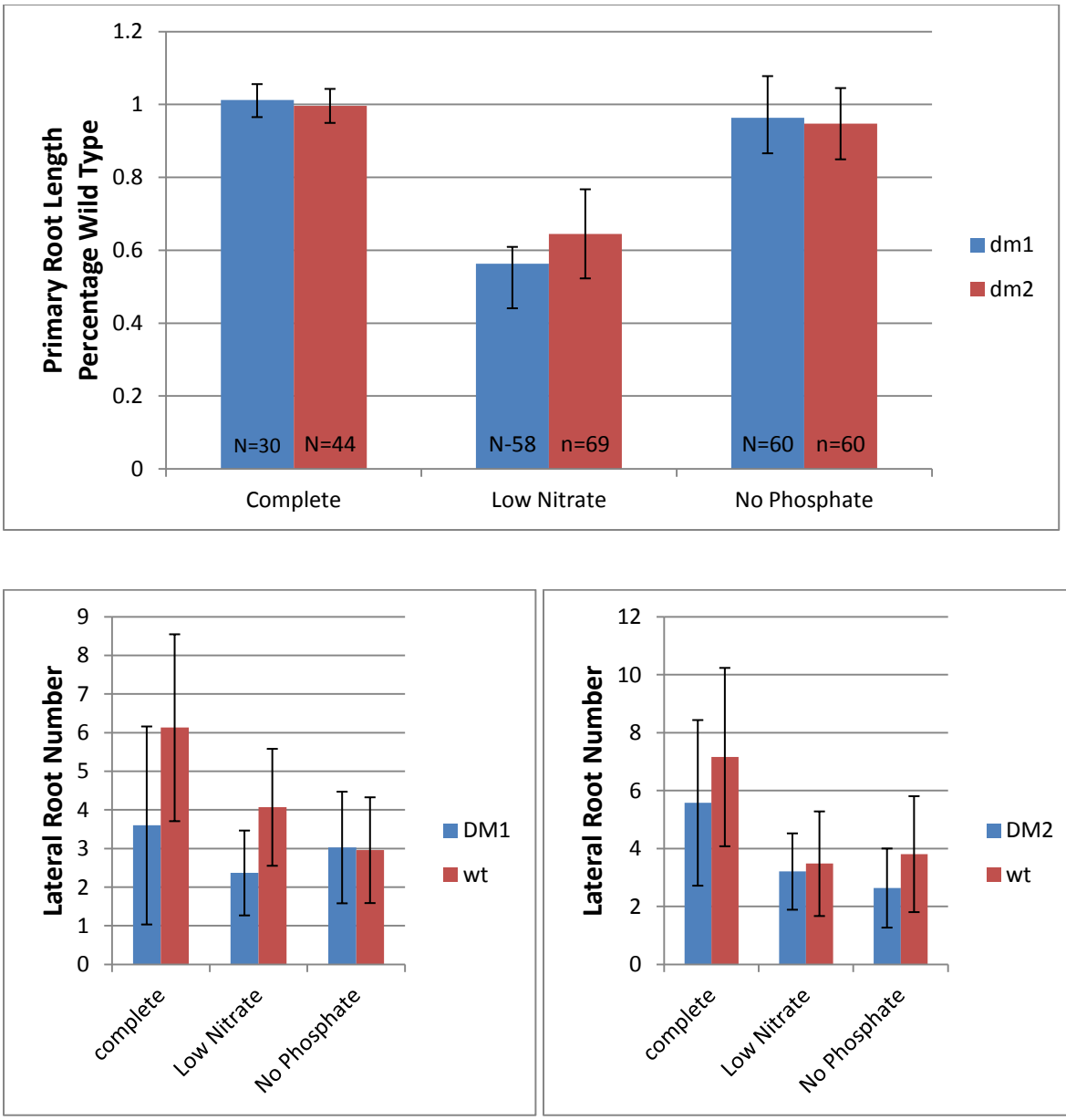


Figure 5.7 NRM1-NRM2 Double Mutant Root Architecture

Primary root length of Overexpressor represented as a percentage of Wild Type and lateral root number of Double Mutants and Wild Type seedlings grown on split plates with complete media, low nitrate media and no phosphate media for 8 days. Double mutants were compared to wild type plants grown on the same plates. Roots were measured using Image J software. Each treatment was averaged for double mutant and wild type seedlings.

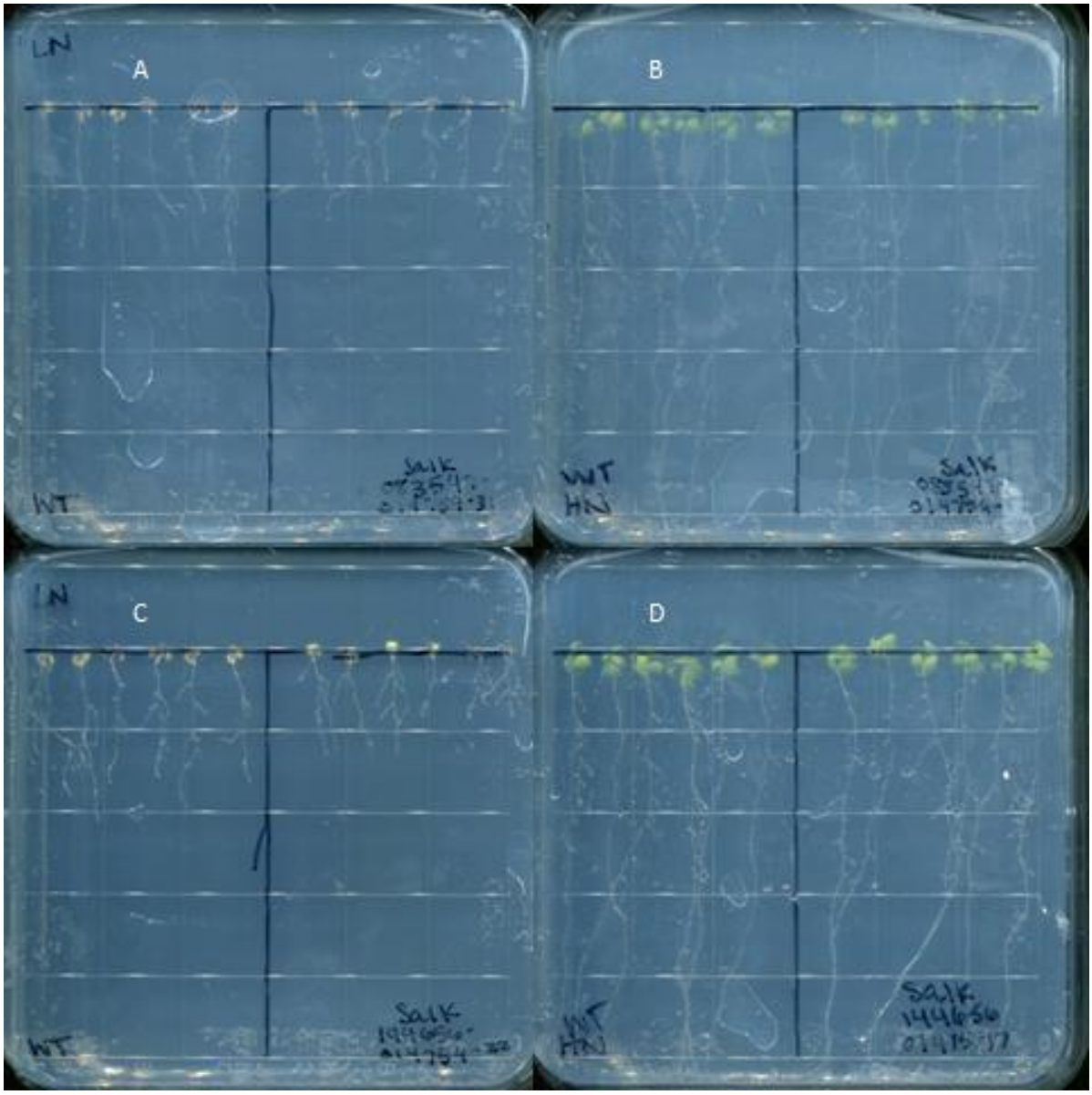


Figure 5.7 Scanned Images of Vertical Plates Used for Root Measurements

Typical scanned Images of plants grown on vertical plates to be used for root measurement in Image-J software. Double mutants and wild type plants are grown on the same plate to compare growth of plants under identical conditions. Images were optimized for analysis not visual appeal.

A. Double Mutant 1 grown on Low Nitrate

B. Double Mutant 1 grown on High Nitrate

C. Double Mutant 2 grown on Low Nitrate

D. Double Mutant 2 grown on High Nitrate

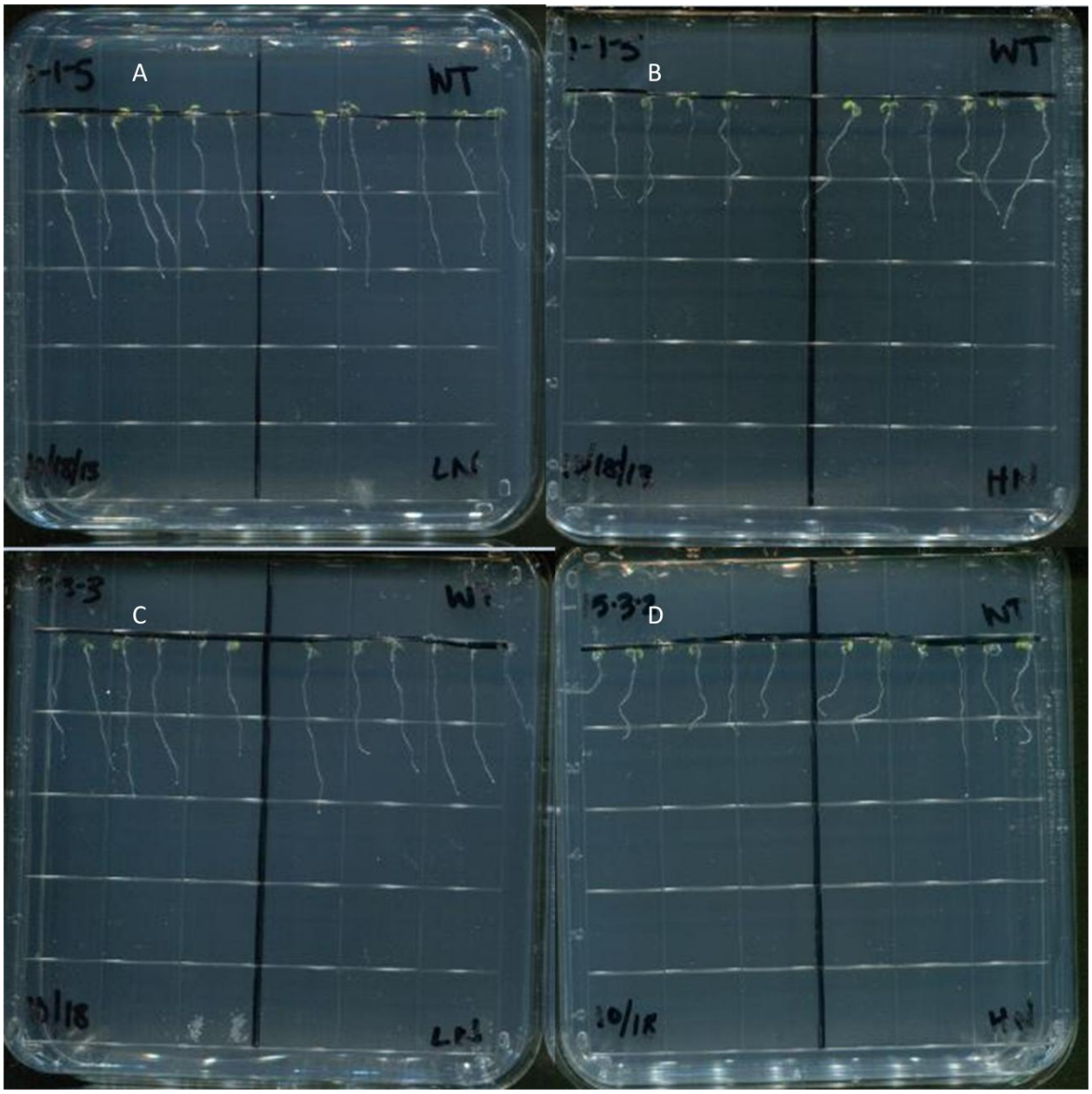


Figure 5.8 Scanned Images of Vertical Plates Used for Root Measurements

Typical scanned Images of plants grown on vertical plates for 6 days to be used for root measurement in Image-J software. Overexpressors and wild type plants are grown on the same plate to compare growth of plants under identical conditions. Images were optimized for analysis not visual appeal.

A. overexpressor 1-1-5 grown on Low Nitrate B. overexpressor 1-1-5 grown on High Nitrate

C. overexpressor 3-3-3 grown on Low Nitrate D. overexpressor 15-3-2 grown on High Nitrate

Discussion

Altering the expression of NRM1 has an effect on the root architecture of *Arabidopsis*. Double mutants of NRM1 and NRM2 showed the clearest result with primary roots that were significantly shorter than wild type plants on low nitrate media. Lateral root number was lower but variable in most treatments. There were also differences in primary root length in some of the overexpressor lines. This suggests that NRM1 may play a role in root elongation and lateral root formation. NRM1 or NRM2 single knockouts did not show an altered phenotype alone but when both genes were knocked out an altered phenotype was observed. This indicates that both genes have a role in root architecture and have some redundancy.

Overexpressing lines had variable root phenotypes. This could be the result of silencing of NRM1 in response to the abnormal expression. This could cause some of the lines to have less expression than wild type plants and mimic the phenotype of knockout plants.

Plants transformed with micro RNA constructs against NRM1 and NRM3 will be very useful in conjunction with the data obtained from the overexpressor plants and double mutants. Because NRM1 and NRM3 are very similar, eliminating expression of these two genes will be very useful. These plants were designed late in my work in response to temporary problems with double mutants, so they are not ready for nutrient starvation experiments.

The plants with altered expression of NRM1 could be tested on other media in the future to determine if there was an altered root architecture phenotype. Media lacking calcium, sulphate and other nutrients could be tested to look for phenotypes. Previous GUS

experiments showed altered expression in response to these nutrients and it is possible that there would be an altered root phenotype.

Chapter 6

Recombinant Expression of NRM1

Protein in *E. coli*

Introduction

One valuable tool for studying proteins is antibodies specific for a protein of interest. Antibodies can be used in several ways to help understand the role of a protein in an organism. Antibodies can be used to help monitor the levels of NRM1 protein under different nutrient conditions and at different developmental stages. This information could be used in conjunction with information about transcript levels to obtain a more complete understanding of the control of NRM1. Information about the level of transcription of a gene can be obtained through the use of RT-PCR or microarrays. This information is very valuable and can be used to infer the level of NRM1 expression but it cannot account for possible post-transcriptional gene regulation. To accurately measure the level of NRM1 protein, antibodies can be used for western blotting.

NRM1-specific antibodies would also be useful for chromatin immunoprecipitation experiments. In this kind of experiment the endogenous NRM1 protein will be cross linked with DNA. The NRM1 protein will then be bound to the antibodies and this complex would be purified. The DNA will then be sequenced and analyzed to determine what sequence NRM1 is binding. The optimal result from this experiment would be to find a group of genes and a conserved binding sequence that NRM1 interacts with. This will hopefully provide more insight into which pathways are regulated by NRM1.

Antibodies could be used for other applications to provide insight about NRM1. Localization of NRM1 protein could be further confirmed by the use of immunofluorescence.

After binding of an NRM1 antibody to NRM1 in cells a florescent secondary antibody can give accurate information about sub cellular localization of the protein.

Antibodies can be made using recombinant proteins or with synthetic peptides. The recombinant proteins are difficult and time-consuming to produce. Synthetic peptides, however, can be made by the company producing the antibodies, and while they are expensive, the overall cost can be significantly less. The synthetic peptides are short segments and have to be chosen very carefully to maximize utility of the antibodies. If the amino acids recognized by the antibodies are located in the interior of the native protein, the antibodies will not be able to bind the native protein. Recombinant proteins can be much bigger than synthetic peptides and when used as antigens will likely lead to the production of antibodies against multiple parts of the protein. Therefore, use of larger proteins as antigens will have much better chance of producing antibodies that will bind native protein effectively.

In order to produce recombinant proteins, many organisms and strategies can be used. Because we are working with a eukaryotic protein it could be problematic to produce recombinant protein in a prokaryote such as *E. coli.* Problems with protein folding and post translational modification can occur in prokaryotes. To combat this problem eukaryotes such as yeast can be used. If yeast does not express the protein properly, a higher level eukaryote such as insect tissue culture from *Spodoptera frugiperda* can be used. The more complex the organism used to produce the recombinant protein the more expensive and complicated the production becomes. For this reason *E. coli* is tested first and then other organisms are used if needed.

Some recombinant proteins are insoluble when produced in large amounts in other organisms. To combat this problem, a protein tag can be used. Tags such as thioredoxin and GST are highly soluble peptides that can increase the solubility of the recombinant protein greatly when produced as a chimeric protein. These tags also contain motifs that can be helpful in purifying recombinant protein, such as the 6x histidine motif. This motif can be added to the recombinant protein directly without the peptide tag but will not increase the solubility of the protein as much as large protein tags. These peptide tags generally contain a protease cleavage sequence that can be recognized by a protease such as enterokinase. This cleavage sequence is very useful for removing the peptide tag after purification so antibodies are not produced against the tag peptide.

Materials and Methods

Bacterial Lines and Media

To facilitate maximum translation of NRM1 constructs Rosetta 2 the strain of *E. coli* named was used (Novagen). These cells are codon optimized for eukaryotic gene expression. Rosetta 2 cells contain a plasmid with tRNAs for 7 codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) that are rarely expressed in *E. coli* but are common in plants. These tRNAs are coded on a plasmid that contains a choloramphenicol resistance gene for selection. All cells were grown on Luria Broth media (green 2012). To select for the tRNA plasmid, chloramiphenicol was added at 34µg/mL. To select for the expression plasmids either 50ug/mL kanamycin (pET 41) or 100µg/mL carbenicillin (pET 32) was added to the media. For solid media, 8g per liter bactoagar was added to the media before autoclaving.

Bacterial Expression Constructs

Nine NRM1 expression constructs were made and tested. The full length gene along with two truncated versions (Figure 6.1) were inserted into either pET-32a downstream of the thioredoxin (TRX) peptide tag or in the pET-41a vector both upstream and downstream of the GST peptide tag (Figure 6.2). The combination of 3 peptides in 3 positions of 2 vectors resulted in a total of 9 tagged bacterial expression constructs.

NRM1 coding regions were amplified from cDNA. For the TRX:NRM1 fusion (Figure 6.3b), the full length NRM1 coding sequence was amplified using primers with restriction sites for EcoRV and Sal I (primers Vs5trx1 and Vs5trx2a) . The PCR product was ligated into pDRIVE using the Qiagen cloning kit (Qiagen ref) and the sequence was verified using M13 forward and reverse primers. The EcoRV-SalI fragment was then excised and ligated into an appropriately digested pET-32a expression vector.

For the GST:NRM1 construct (Figure 6.3c), the full length NRM1 coding sequence minus the stop codon was amplified using primers containing NdeI sites (primers Vs3gst1 and Vs3gst2a). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The NdeI fragment was then excised and ligated into appropriately digested pET-41a expression vector.

A.

NRM1 Full Length

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETM

TRDESMRKDPMVNGGEGRKREAEKDGGGGRKQRRCWSSQLHRRFLNALQH

LGGPHVATPKQIREFMKVDGLTNDEVKSHLQKYRLHTRRPRQTVPNNGNS

QTQHFVVVGGLWVPQSDYSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQW

PSHSNYRPSIIVDEGSGSHSEGVVVRCSSPAMSSSTRNHYVKNN

B.

NoMYB

MIKKFSNMDYNQKRERCGQYIEALEEERRKIHVFQRELPLCLDLVTQAIE

ACKRELPEMTTENMYGQPECSEQTTGECGPVLEQFLTIKDSSTSNEEEDE

EFDDEHGNHDPDNDSEDKNTKSDWLKSVQLWNQPDHPLLPKEERLQQETM

TRDESMRKDPMVNGGEGRKREAEKDGGGGRK

C.

End

SDYSTGKTTGGATTSSTTTTTGIYGTMAAPPPPQWPSHSNYRPSIIVDEGSGSHSEGVVVRCSSPAM
SSSTRNHYVKNN

Figure 6.1 Amino Acid Sequence of NRM1 Constructs

The Leucine zipper like domain is highlighted in blue and the MYB like DNA binding domain is highlighted in red.

A. **Full length** amino acid sequence

B. **NoMYB** Peptide: NRM1 with the leucine zipper domain and without the MYB like DNA binding domain.

C. **END** peptide

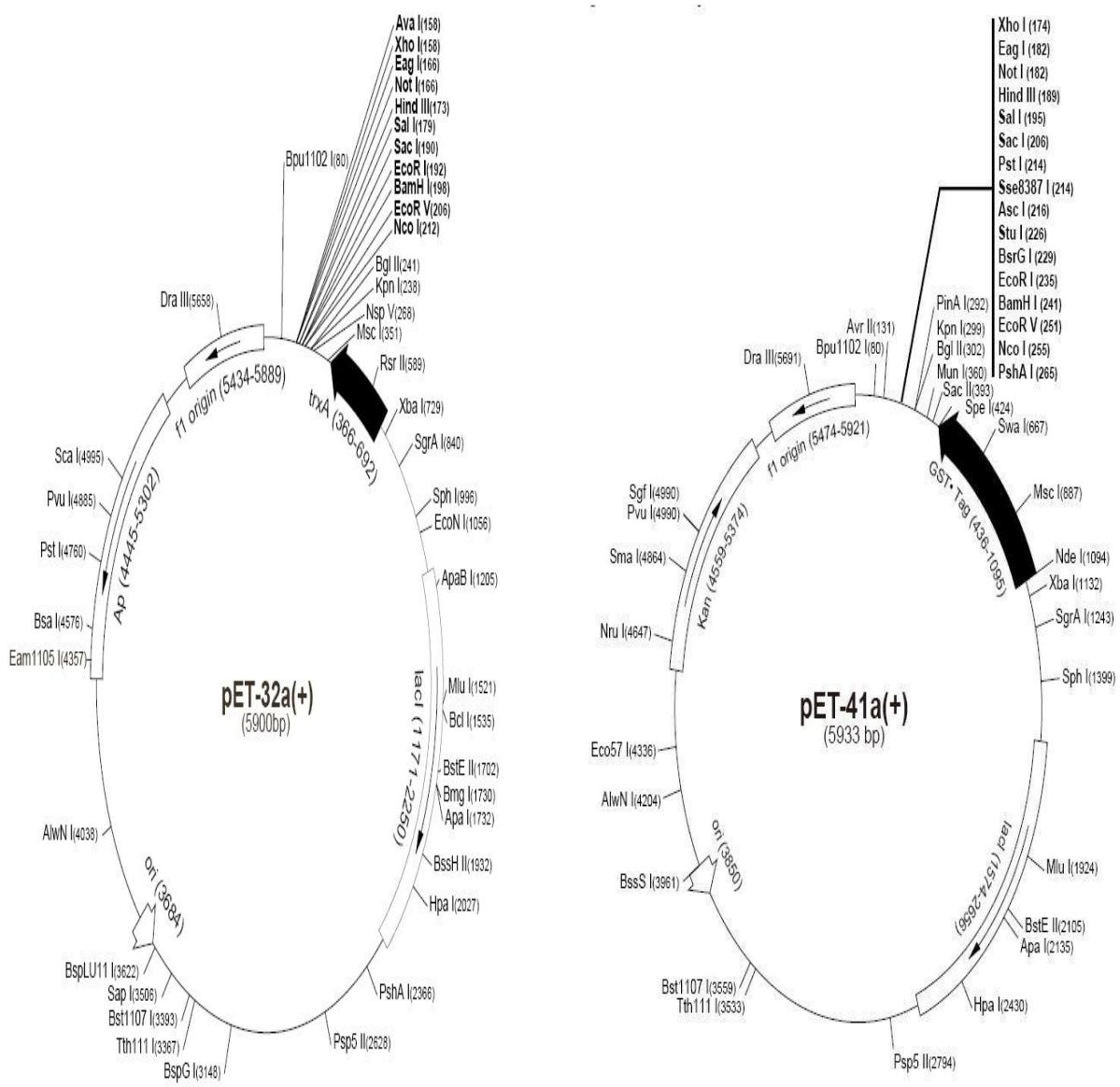


Figure 6.2 Plasmid Maps of Protein Expression Vectors

A. pET-32a plasmid with Thioredoxin peptide tag

B. pET-41a plasmid with GST peptide tag

For the NRM1:GST construct (Figure 6.3d), the full length coding sequence was amplified using primers with restriction sites for BamHI and Stu I (primers Vs5gst1 and Vs5gst2a). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-41a expression vector using the stated restriction enzymes.

For the TRX:Nomyb construct (Figure 6.3e), a partial NRM 1 coding sequence was amplified using primers with EcoRV and Sal I sites (primers Vs5trx1 and Vs5trx2t). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers. The EcoRV and Sal I fragment was excised and ligated into the pET-32a expression vector using the stated restriction enzymes.

For the NoMYB:GST construct (Figure 6.3f), a partial NRM1 coding sequence minus the stop codon was amplified and restriction sites for Nde1 were added with the primers Vs3gst1 and Vs3gst2t . The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-41a expression vector using the stated restriction enzymes.

For the GST:NoMYB construct (Figure 6.3g), a partial NRM coding sequence was amplified using primers with restriction sites for BamH1 and Stu 1 (primers Vs5gst1 and Vs5gst2t). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-41a expression vector using the stated restriction enzymes.

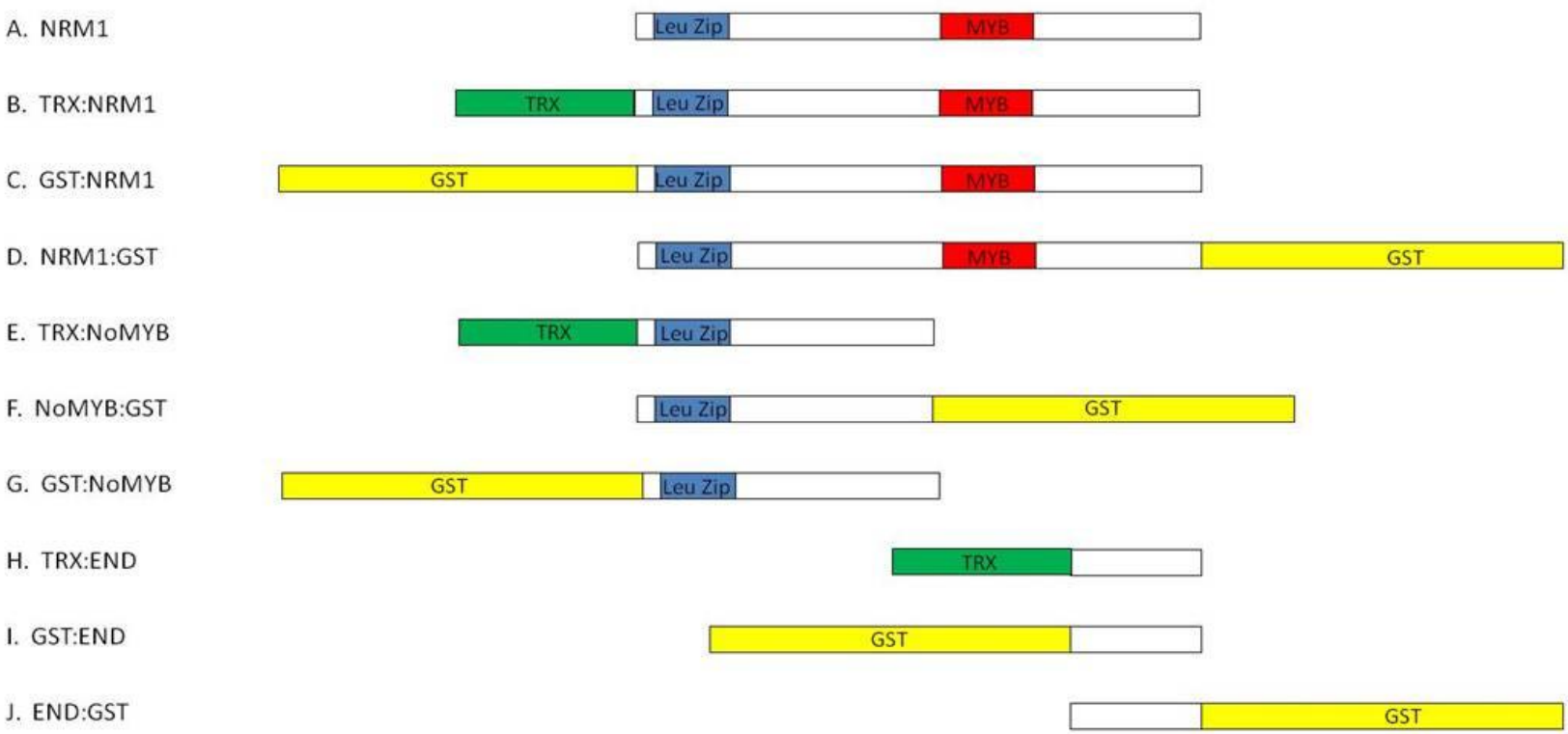


Figure 6.3 NRM1 Bacterial expression constructs

For the TRX:END construct (Figure 6.3h), a partial NRM1 coding sequence was amplified using primers with restriction sites for EcoRV and Sal I (primers Vs5trx0 and Vs5trx2a). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-32a expression vector using the stated restriction enzymes.

For the GST:END construct (Figure 6.3i), the End coding sequence was amplified using primers with restriction sites for BamHI and Stu I (primers Vs5gst0 and Vs5gst2a). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-41a expression vector using the stated restriction enzymes

For the END:GST construct (Figure 6.3j), a partial NRM1 coding sequence minus the stop codon was amplified using primers containing restriction sites for Nde1 (primers Vs3gst0 and Vs3gst2a). The PCR product was ligated into pDRIVE and the sequence was verified using M13 forward and reverse primers then excised and ligated into the pET-41a expression vector using the stated restriction enzymes.

Plasmids were verified by sequencing and introduced into Rosetta 2 cells according to manufacturer's protocol.

Protein Induction Test

Overnight cultures of Rosetta cells containing each vector were grown in 3 mL Luria broth with 50ug/mL kanamycin plus 100 µg/mL chloramphenicol. A 750µL aliquot of the

overnight culture was transferred into 50mL Luria broth with 50ug/mL kanamycin and 100 µg/mL chloramphenicol. The 50 mL culture was incubated at 37^0C for 3 hours with constant shaking. The $O.D._{600}$ was measured and noted. To induce production of the recombinant protein IPTG was added to a final concentration of 0.4mM. Samples of 500 µL were taken before induction and at 1, 2 and 4 hours post induction. The $O.D._{600}$ was measured and noted for each time point. The samples were centrifuged for 1 minute at maximum speed in a benchtop centrifuge and the supernatant was discarded. Sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue) was added to the pellet using the formula (O.D. / O.D. t=0) x 50 µL. The samples were incubated at 100^0C for five minutes then centrifuged for 1 minute at maximum speed. A 10% Tris-HCl precast gel (BioRad) was loaded with 10 µL sample and the empty lanes were loaded with 10 µL sample buffer. The gel was then run at 15 mAMP until the leading dye front reached the bottom of the gel. The gel was then removed and placed in 0.025% Coomassie Brilliant Blue, 40% methanol, 7% acetic acid solution overnight with gentle agitation. The stain was then removed and replaced with 40% methanol, 7% acetic acid with gentle agitation. To aid in destaining, paper towels were placed in the destain solution and changed intermittently. The gels were then examined visually to compare recombinant protein expression levels.

Partial Purification of TRX:NoMYB

Rosetta 2 cells containing the TRX:NoMYB construct were grown in a 20 mL culture of LB media plus 50 µg/mL carbenicillin and 100 µg/mL chloramphenicol overnight at 37^0C. The culture was transferred to one liter of LB media plus 50 µg/mL carbenicillin and 100 µg/mL

chloramphenicol. The culture was incubated at 37^0C with vigorous shaking until the OD_{600} was 0.6. To induce expression IPTG was added to a final concentration of 1 mM. The culture was incubated for an additional 4 hours at room temperature then the cells were harvested by centrifugation at 4000 x g for 20 minutes. The cell pellet was then stored at -80^0C until use.

Protein was extracted under native conditions according to the protocol in the QIAexpressionist handbook(Qiagen 2003). Briefly, cell pellets were resuspended in 5 mL per gram lysis buffer (supplemented with 1 mg/mL lysozyme) and incubated for 30 minutes on ice. Cells were disrupted with a sonicator in six 10 second bursts with 10 second breaks between bursts. The lysate was centrifuged at 10,000 x g for 30 minutes at 4^0C and the supernatant was collected. NiNTA resin was added to the supernatant at a 1 to 4 ratio and mixed on a shaker at 200 rpm for 60 minutes at 4^0C. The lysate slurry mixture was then added to a column and allowed to drain into a collection tube. The column was washed twice with 4 mL wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole) and each wash was collected separately. The protein was finally eluted with 4 separately collected fractions of 0.5 mL elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole). Samples were taken for analysis on SDS page gels at each step to monitor purification efficiency.

To remove the thioredoxin and 6x His tag on the recombinant protein the Novagen Recombinant Enterokinase and Capture Kit was used according to manufacturer's protocol (Novagen 1998). Briefly, 5 mg of protein was incubated at room temperature for 16 hours in 2.5 mL buffer with 50 units of enterokinase. To remove the enterokinase enzyme, 2500 µl capture slurry was added and the suspension was centrifuged at 1000 x g for five minutes. The supernatant was discarded and replaced with 12500 µl 1X rEK Cleavage/Capture Buffer and

centrifuged at 1000 x g for five minutes. The supernatant was removed and replaced with 1250 µl 1X rEK Cleavage/Capture Buffer. This slurry was divided into four 2-mL Spin filters provided in the kit. The cleavage reaction was added to each of the four filters, mixed to resuspend the resin, and the mixture was incubated at room temperature for five minutes. The spin filters were then centrifuged at 1000 x g for five minutes. The cleaved protein was present in the flow through and the resin was discarded.

To remove the cleaved thioredoxin 6x His tag from the NRM1 protein, the protein solution was passed over a NiNTA column for a second time. In the second purification the tag is bound to the column and the protein of interest is collected in the flow through and the first wash. The flow through from the enterokinase capture column was mixed with NiNTA resin at a 1 to 4 ratio and mixed on a shaker at 200 rpm for 60 minutes at 4^0C. The lysate slurry mixture was then added to a column and allowed to drain into a collection tube. The column was washed four times with 2 mL wash buffer and each wash was collected separately. The protein tag was finally eluted with 4 separately collected fractions of 0.5 mL elution buffer. Samples were taken for analysis on SDS page gel at each of the steps in this procedure to monitor purification efficiency. The solution was dialyzed against phosphate buffered saline pH 7.4 solution (Green 2012). Thermo Scientific Slide-A-Lyzer dialysis cassettes were used for dialysis.

Results

In order to generate NRM1 protein for antibody production, 3 different NRM1 peptides in combination with 2 different protein tags were expressed in *E. coli* using the pET vector system (McDowell et al. 2004). Vectors pET 32 and pET 41 (Figure 6.2) allow proteins to be

expressed as thioredoxin (TRX) or Glutathione S-transferase (GST) fusions, respectively. Fusion of proteins with TRX and GST has been shown to enhance protein solubility as well as expression. Because it was difficult to predict which tag would work best with our protein, we tested both. Each protein is also expressed with a 6x histidine motif to facilitate purification.

The 3 different NRM1 peptides that were used were the full length protein (NRM1), an N-terminal peptide lacking the DNA binding region (noMyb), and a C-terminal peptide lacking the putative leucine zipper protein interaction region (End) (Figure 6.4). The noMYB protein includes the first 181 amino acids of NRM1. This peptide lacks the MYB like domain and antibodies generated against this portion of the protein may avoid the possibility of transcriptional interference because they would not interfere with binding of DNA through the MYB domain. In addition, the smaller size of the noMYB protein (181 AA vs 341 AA) may allow it to be synthesized by the bacteria more efficiently. The End protein, which lacks both the Leucine zipper and MYB domains, is also much smaller than the full-length protein (80 AA), and was chosen because it lacks all conserved protein domains, and may therefore lead to the production of very NRM1 specific antibodies.

In order to maximize protein expression in *E. coli*, Rosetta 2 cells were chosen (Harris, Rouiller et al. 2006). These cells are codon optimized for eukaryotic gene expression. Specifically, they contain a plasmid encoding tRNAs for 7 codons that are rarely expressed in *E. coli* but are common in plants and they have been reported to enhance synthesis of plant proteins in *E. coli*.

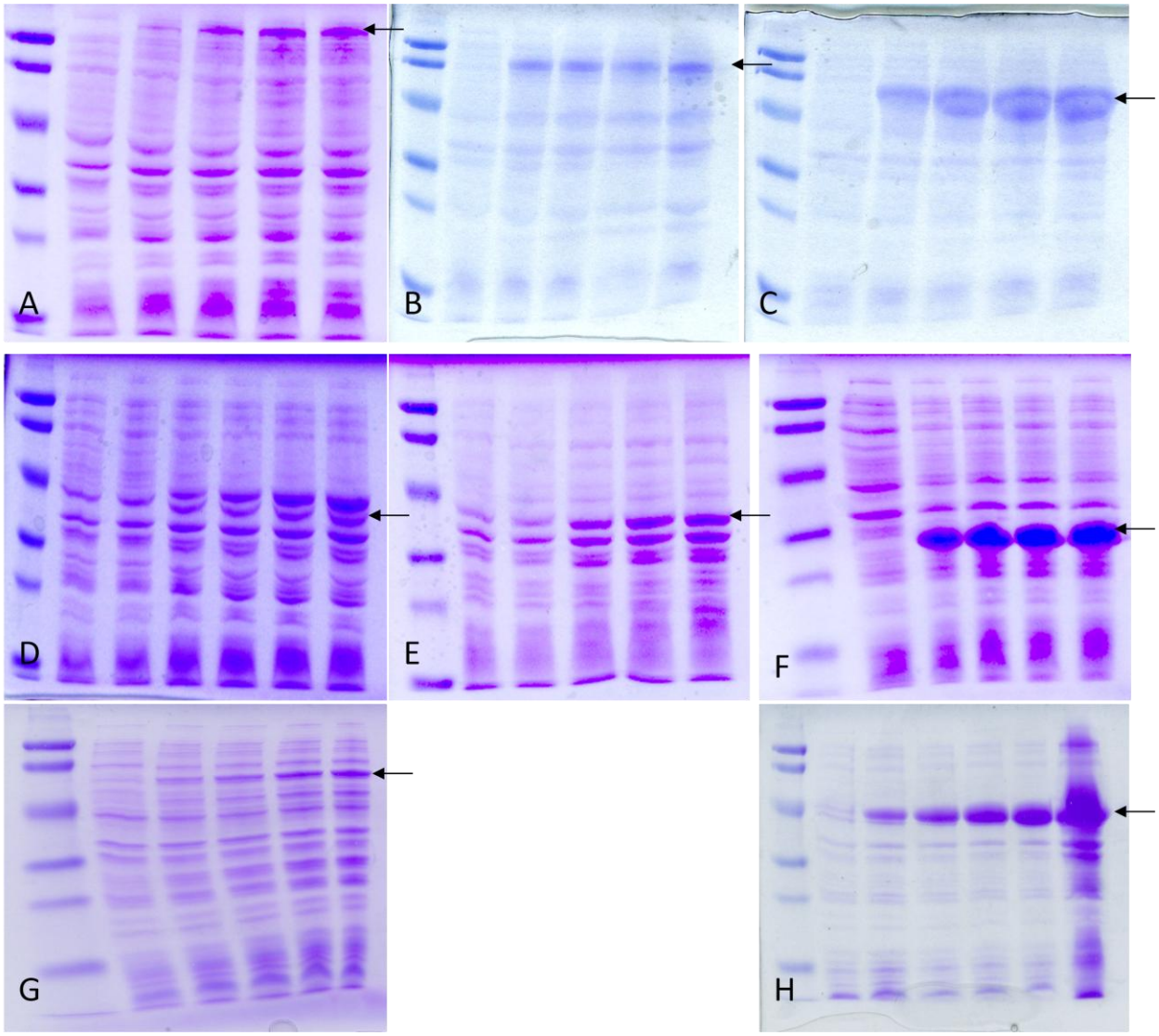


Figure 6.4 Expression of Recombinant NRM1 in *E. coli*

Induction Test of NRM1 protein expression. Each gel is loaded with a molecular weight ladder and time points 0, 1, 2, 4 and 8 hours after IPTG induction.

A. pET-41a GST:NRM1
B. pET-41a NRM1:GST
C. pET-32a TRX:NRM1
D. pET-41a GST:End N
E. pET-41a End:GST C
F. pET-32a TRX:End
G. pET-41a GST:NoMYB
H. pET-32a TRX:NoMYB

The nine NRM1 constructs were first tested for protein expression**.** Protein induction tests showed a wide variety of expression levels. The results of the induction test are shown in Figure 6.4. The expression of all three full length constructs was detectable but low. The constructs containing the END portion of NRM1 expressed at low to moderate levels as N or C-terminal GST fusions, and higher expression was seen for the TRX:END fusion. The NoMYB portion of NRM1 failed to express or was expressed weakly as N- or C-terminal GST fusions. The TRX:NoMYB construct expressed at high levels. Because, in general, the TRX fusions expressed much better than the GST fusions, the NoMYB:GST construct was not tested further.

Because it was expressed at high levels, the TRX:NoMYB construct was chosen for full scale protein purification. This protein was determined to be the best candidate for antibody production since it lacks the conserved MYB domain, which would reduce the chance that the antibodies would recognize other MYB domain proteins. Furthermore, having antibodies that recognize the N-terminus of the protein would be advantageous for certain applications such as Chip because they would not bind the same regions as NRM1-DNA interaction. Large scale purification of TRX:NoMYB was performed using 1 liter of culture. The protein was expressed well at this scale (Figure 6.5a). TRX:NoMYB was purified from *E. coli* using a NiNTA column and then subjected to enterokinase digestion to cleave the TRX tag. Following removal of enterokinase, the cleaved protein was passed over a second NiNTA column to remove the TRX tag. As expected, most of the cleaved NoMYB protein was present in the first flow through with only a small amount remaining in the second flow through (Figure 6.5c). The TRX tag was retained on the column and was able to be eluted using imidazole. Fractions containing NoMYB were concentrated and dialysed against phosphate buffered saline in preparation for antibody

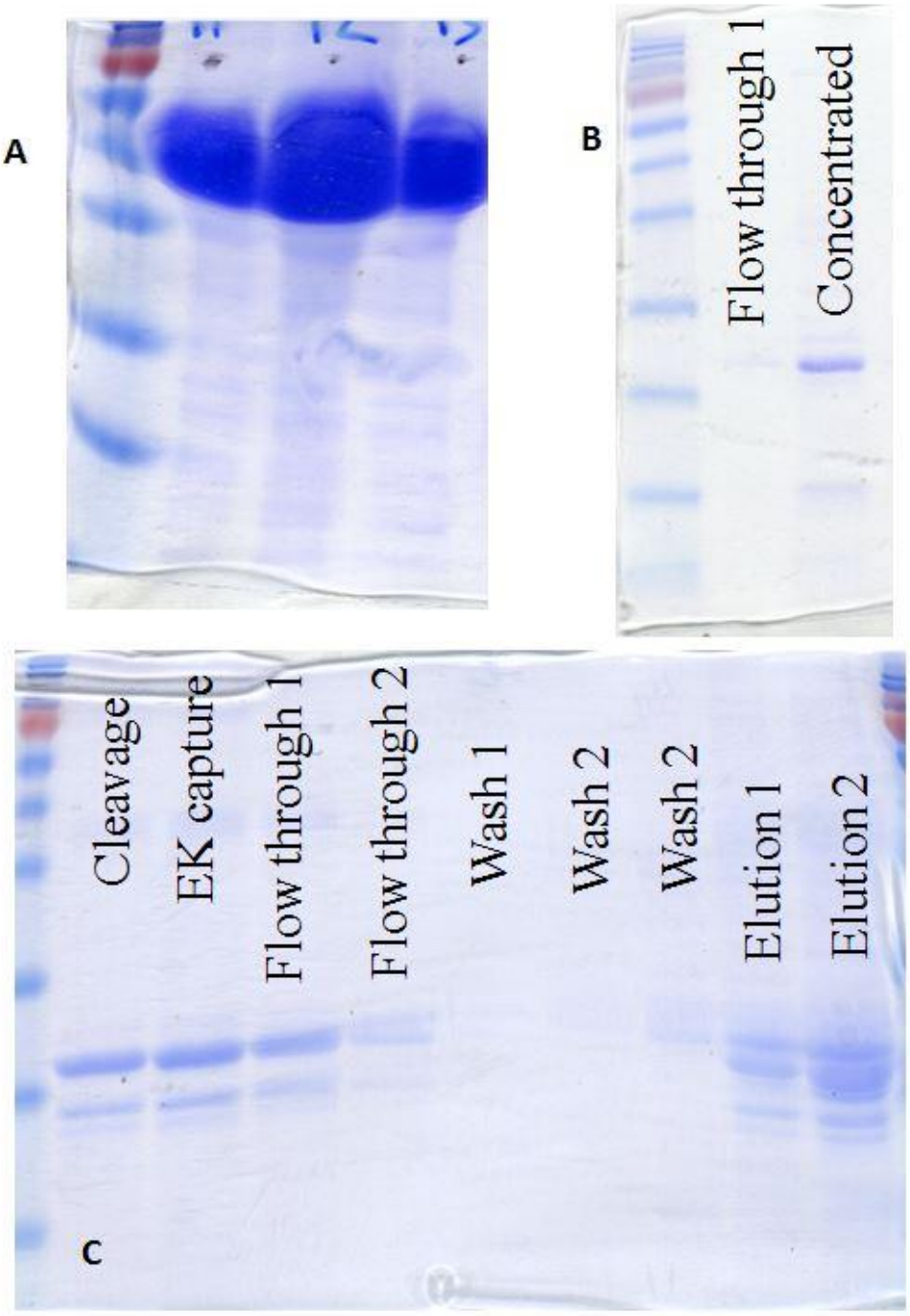


Figure 6.5 Expression, Cleavage and Purification of Recombinant NRM1 in *E. Coli*

A. Large scale production of pET-32a TRX:NoMYB 24 hours after induction with ITPG

B. Dialysis of flow through 1 and 2 with phosphate buffered saline solution

C. Enterokinase Cleavage, Capture and NiNTA purification flow through 1 and 2 contained NRM1 recombinant protein and elutions contained cleaved TRX protein.

generation and the final protein preparation was run on SDS-PAGE to assess purity and concentration. It was determined that the protein preparation was not of sufficient purity (85% minimum purity) to use for antibody generation as evidenced by the presence of numerous contaminating bands (Figure 6.5b).

Discussion

Although we were not able to generate antibodies against NRM1 using bacterially expressed protein, we were able to show that NRM1 can be successfully expressed in *E. coli*, which is notable because transcription factors are notoriously hard to express. This is most likely due to the use of Rosetta 2 cells. Expression of partial NRM1 constructs (NoMyb and END) were better than full-length. This was possibly due to the size of the chimeric peptide. Smaller peptides can be produced more efficiently. With the full length protein and the peptide tag the overall size of the recombinant protein is quite large. The truncated NRM1 segments combined with the peptide tag are similar in size to the full length protein. The thioredoxin tagged recombinant protein showed much higher levels of expression than ones tagged with GST. This difference in expression could be the result of many factors. It is hard to predict which peptide tag will produce the best results with a specific protein. So it is important to test several tags empirically. Indeed, differences in expression levels that we observed justified testing multiple protein tag combinations were tested.

Purification via metal affinity chromatography was insufficient to obtain >90% pure protein. Greater purity may be achieved by using size exclusion, ion-exchange, or hydrophobicity interaction chromatography. The contaminating proteins were likely purified

along with the target protein because they had a histidine rich region that bound to the NiNTA column or interacted with part of the chimeric protein. Using an additional purification step is likely to exclude many of the contaminants. While the contaminating protein had this in common with the target protein they are very unlikely to have other similar properties that would cause co-purification at a second step.

Chapter 7

Conclusion

NRM1 was discovered in a microarray experiment seeking genes that respond to starvation and readdition of nitrate. Microarray experiment showed NRM1 expression increased quickly after adding back nitrate to plants grown on a nitrate free medium for 4 days. Based on our initial observations concerning nitrate induction of NRM1 gene expression, and the presence of leucine zipper and MYB domains, we hypothesized that NRM1 was a transcription factor that plays a role in the plants response to nitrate availability. Because NRM1 contains a MYB-domain associated with known transcription factors (Ording et al. 1994) we speculated it plays a role in regulating changes in gene expression associated with nitrate availability.

NRM1-GFP fusions showed that NRM1 was localized to the nucleus of the cell in onion epidermal cells and *Arabidopsis* protoplasts. This is consistent with the hypothesis that NRM1 may be a transcription factor. Transcription factors have to be localized to the nucleus at least a portion of the time to interact with promoters of genes they control. Transcription factors can change cellular localization based on changing conditions (Gorner et al. 2002). We did not see any evidence of this for NRM1 when examining its location under changing levels of nitrate availability. It is possible that NRM1 does change localization in response to nitatrate but the GFP fusion protein changes or obscures parts of NRM1 because of the GFP fusion. It is also possible that under other conditions it could change localization.

NRM1 contains only a single MYB like domain, but it is thought that two are needed for specific DNA binding (Ording et al. 1994). If this is the case, to be an effective transcription factor NRM1 would need to interact with a second MYB-domain containing protein to create

the binding site required for sequence specific binding to DNA (Ryu et al. 2005). The yeast two hybrid system was utilized to test if NRM1 interacts with itself which would be required for NRM1 to form a homodimer or with another protein to from a heterodimer needed for DNA binding. No interactions were found with our experiments nor are there reports of NRM1 interactions in the publicly available database of interactions. This could be because NRM1 interacts with a large complex which is not assembled in the 2-hybrid experiment or the protein with which it interacts may require a conformational change that is driven by a third protein component. Alternatively, NRM1 may participate in another type of signaling system that does not require it to interact directly with DNA.

Antibodies directed against NRM1 could be used to identify the DNA sequence that NRM1 interacts with. Chromatin immuno precipitation uses antibodies to purify DNA cross linked to proteins (Collas 2010). The DNA recovered from this method is sequenced and compared to the genome sequence. The data obtained from this could reveal what genes NRM1 influence and might be helpful in finding a conserved binding sequence. Antibodies to NRM1 need to be produced because none are currently available. The first steps in the production of the antibodies have been completed. The recombinant expression plasmids have been designed and the protein expression has been examined. The purification of recombinant protein needs to be improved in order to be used for antibody production. Once the pure protein is obtained antibodies will be made and chromatin immunoprecipitation experiments can be performed to provide insight into which DNA sequences NRM1 might interacts with.

After another group showed that NRM1 responded to phosphate (Liu et al. 2009) the original hypothesis was re-examined. Because NRM1 was responsive to another nutrient it was necessary to test the possibility that changes in NRM1 gene expression are not specific to nitrate, but may be part of general response to nutrient deficiency. Experiments performed with NRM1 promoter::GUS plants confirmed that NRM1 had reduced expression in response to nitrate and phosphate starvation. The experiments also showed NRM1 had reduced expression in response to calcium and sulphate starvation.

In order to generate a detailed spatial and temporal expression pattern for the gene. An NRM1 promoter::GUS transgenic *Arabidopsis* was made. The spatial expression pattern showed that NRM1 was mostly expressed in areas of lateral root formation and in the elongation zone of the root. In young seedlings (5-9 days) NRM1 showed expression in a large portion of the root except the root tip. In older plants (10-18 days) NRM1 was expressed in the lower portion of the root except for the root tip. Expression was not observed in older root tissue where lateral roots were well established, but expression was also observed in lateral roots except in the root tip. These results suggest that NRM1 is responsive to a wide range of nutrients and is expressed in regions where root architecture changes could occur, since the areas where NRM1 expression was visualized were areas of root elongation and lateral root emergence. This suggests that NRM1 may play a role in root elongation and lateral root emergence. NRM1 has also been implicated in seed germination and early ABA signaling (Wu et. al. 2012), these processes could also be involved at later stages of root development.

To determine what role NRM1 might play in root architecture, NRM1-NRM2 double mutants, NRM1 overexpressors and NRM1-NRM3 microRNA plants were made. When these plants were examined on nutrient deficient media, changes in root architecture were observed under some treatments. The changes in root architecture were not dramatic but in some cases were statistically significant. NRM1-NRM2 double mutant plants showed shorter primary roots than wild type plants when grown on low nitrate media. These plants also produced statistically fewer lateral roots when grown on complete, low nitrate and low phosphate media (some of these did not show significance at the 0.05 significance level). Plants designed to overexpress NRM1 did show differences in root architecture but these changes were not consistent between lines. At this point these plants do not provide any clear insight into the function of NRM1. Further experiments will need to be performed to deduce exactly how these changes in root architecture are related to the function of NRM1.

To summarize the experiments that I performed support the hypothesis that NRM1 is a transcription factor. NRM1 is localized to the nucleus, based on NRM1-GFP fusion experiments and its MYB like domain. More experiments such as chromatin immuno-precipitation will be needed to more conclusively demonstrate that NRM1 binds DNA. My data also shows that NRM1 responds to multiple nutrients including nitrate, phosphate, sulphate and calcium, because in plants containing GUS gene reporter, GUS staining is greatly reduced in plants grown on media deficient in these nutrients. Additional experiments indicated that changes in expression of NRM1 changed the root architecture response to nutrient starvation. NRM1-NRM2 double mutants showed shorter primary roots on low nitrate media. NRM1 overexpressor plants showed varied changes in root architecture that were inconsistent,

possibly because of gene silencing. Further experiments would be needed with the overexpressor plants and the recently generated mico RNA (Ossowski et al. 2008) plants to determine more precisely what role NRM1 plays in root architecture.

In future work, it would be interesting to study NRM2 in more detail. This gene also responds to nitrate starvation and may respond to other nutrient deficient conditions as well. In the publicly available yeast two hybrid database (Mukhtar et al. 2011) NRM2 shows several interactions with other proteins. NRM2 could possibly interact with different proteins in different tissues and cause growth changes in tissues other than roots because it is expressed throughout the plant and could possibly produce changes in shoot morphology when misexpressed. The information obtained from studying NRM2 would also be useful to help understand NRM1 and the other NRM family members.

This work has limited potential applications on its own, but when combined with a vast collection of similar projects will lead to a more complete understanding of the functioning of the model plant species *Arabidopsis thaliana*. More direct applications of this work and similar work studying nutrient response and root architecture adaptations could lead to the production of plants that can more efficiently utilize increasingly limited resources.

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Appendix 1 Primers

Name	Restriction Enzyme	Sequence	note
VSPACTFL-L	Nco1	GAATTCTGATGATTAAAAAGTTCAGCAATAT	Yeast 2 Hybrid Bait
VSPACTFL-R	BglII	GATCTATTAATTATTCTTGACGTAATGATT	Yeast 2 Hybrid Bait
VSPACT0-R	BglII	GATCTACTTTCTCCCTCCTCCTCCGTCTTT	Yeast 2 Hybrid Bait
VSNMY1	SalI	GTCGACCATGATTAAAAAGTTCAGCAATAT	Yeast 2 Hybrid prey
VSNMY2	BglII	AGATCTTAATTATTCTTGACGTAATGATT	Yeast 2 Hybrid prey
VSNMT1	SalI	GTCGACCTCGGACTACTCTACGGGCAAGACT	Yeast 2 Hybrid prey
VSNMY01	BglII	AGATCTTACTTTCTCCCTCCTCCTCCGTCTTT	Yeast 2 Hybrid prey
Pact F	none	CTATCTATTCGATGATGAAG	Yeast 2 Hybrid sequencing primer
Pact R	none	ACAGTTGAAGTGAACTTGCG	Yeast 2 Hybrid sequencing primer
PACT 3'	none	GTTGAAGTGAACTTGCGGGG	Yeast 2 Hybrid sequencing primer
PACT 5'	none	TACCACTACAATGGATGATG	Yeast 2 Hybrid sequencing primer
35Sleft	hindIII	GGTCAGAAGCTTATGTTACGTCCTGTAGAAACCC	pCAMBIA 2300 plant primer
35Sright	sphI	GCATGCTCATTGTTTGCCTCCCTGCTGCG	pCAMBIA 2300 plant primer
NRMLeft	SalI	GTCGACATGATTAAAAAGTTCAGCAATAT	pCAMBIA 2300 plant primer
NRMnostop	bamHI	GGATCCATTATTCTTGACGTAATGATTAC	pCAMBIA 2300 plant primer
NRMstop	bamHI	GGATCCTTAATTATTCTTGACGTAATGAT	pCAMBIA 2300 plant primer
RPleft	hindIII	AAGCTTTATGAACCAAGTTTCTTGTTCTT	pCAMBIA 2300 plant primer
RPright	sphI	GCATGCATTTTCAGAACCTAAGATTTTTTGCA	pCAMBIA 2300 plant primer
NRMPleft	hindIII	AAGCTTCGAAGATAAAAACTACATTTAT	pCAMBIA 2300 plant primer
NRMPright	pst I	CTGCAGGATGATACTTTAGGGACTTAATTT	pCAMBIA 2300 plant primer
FOR_1	none	TCCAGTATGGACGATTCAAGG	pCAMBIA 2300 sequencing primer
FOR_2	none	AGGAAAGGCCATCGTTGAAG	pCAMBIA 2300 sequencing primer
FOR_3	none	GGAGTTACCGGAGATGACGA	pCAMBIA 2300 sequencing primer
FOR_4	none	CGCAATTGCATAGACGCTT	pCAMBIA 2300 sequencing primer
FOR_5	none	GGTCGTGGTCCGGTGTAG	pCAMBIA 2300 sequencing primer
REV_1	none	TCCATGGCCTTTGATTCAGT	pCAMBIA 2300

			sequencing primer
REV_2	none	TTGGAACGTCTTCTTTTTCCA	pCAMBIA 2300 sequencing primer
REV_3	none	TTAGAAACTGCTCCAAGACCG	pCAMBIA 2300 sequencing primer
REV_4	none	CCATCAACCTTCATAAACTCCC	pCAMBIA 2300 sequencing primer
REV_5	none	TGTATATCTCCTTGGGATCCATT	pCAMBIA 2300 sequencing primer
gfp2 for 1	sal 1	GTCGACACATGATTAAAAAGTTCAGCAATATG	pgfp2-ga5 II primer
gfp2 re 1	sma 1	CCCGGGTTAATTATTCTTGACGTAATGATTACGG	pgfp2-ga5 II primer
ga5 gfp seq L	none	GTCCACACAATCTGCCCTTT	pgfp2-ga5 II sequencing primer
ga5 gfp seq R	none	AAGACCGGCAACAGGATTC	pgfp2-ga5 II sequencing primer
CA013	none	CATTTCATTTGGAGAGGACACGCTG	pTA7002 sequencing primer
CA014	none	GTCGAAACCGATGATACGGACG	pTA7002 sequencing primer
Vs5gst1	BAMH1	GGATCCATCATGATTAAAAAGTTCAGCAATAT	Bacterial expression primer
VS5gst2A	STU1	AGGCCTTTAATTATTCTTGACGTAATGATTACG	Bacterial expression primer
Vs3gst1	NDE1	CATATGATCATGATTAAAAAGTTCAGCAATAT	Bacterial expression primer
Vs3gst2a	NDE1	CATATGATTATTCTTGACGTAATGATTACGGGT	Bacterial expression primer
Vs5trx1	ECORV	GATATCATCATGATTAAAAAGTTCAGCAATAT	Bacterial expression primer
Vs5trx2a	SAL1	GTCGACTTAATTATTCTTGACGTAATGATTACG	Bacterial expression primer
Vs5gst0	BAMH1	GGATCCTCGGACTACTCTACGGGCAAGACTACC	Bacterial expression primer
Vs3gst0	NDE1	CATATGTCGGACTACTCTACGGGCAAGACTACC	Bacterial expression primer
Vs5trx0	ECORV	GATATCTCGGACTACTCTACGGGCAAGACTACC	Bacterial expression primer
VS5gst2T	STU1	AGGCCTTTACTTTCTCCCTCCTCCTCCGTCTTT	Bacterial expression primer
Vs3gst2T	NDE1	CATATGTTGCTTTCTCCCTCCTCCTCCGTCTTT	Bacterial expression primer
Vs5trx2T	SAL1	GTCGACTTACTTTCTCCCTCCTCCTCCGTCTTT	Bacterial expression primer
S1LP	none	ATTTGTGATTTGCGTTTCAGG	SALK_067195 test primers
S1RP	none	CTTCTTTTTCTTCCACTTGCG	SALK_067195 test primers
S2LP	none	TTTGGGGTTGAATTGAAACAG	SALK_014754 test primers
S2RP	none	ATTCTTTAATCTCCGATCCGC	SALK_014754 test primers

S3LP	none	GCTCAGAACATTCTGACTGGC	SALK_083547 test primers
S3RP	none	TTTTGTCGCCAACCAATTTAG	SALK_083547 test primers
S4LP	none	GCTCAGAACATTCTGACTGGC	SALK_144656 test primers
S4RP	none	GAAAAGAGGCGAGTAGGTTGG	SALK_144656 test primers
S5LP	none	GCTCAGAACATTCTGACTGGC	SALK_083545 test primers
S5RP	none	TTTTGTCGCCAACCAATTTAG	SALK_083545 test primers
miRNA-a	none	CTGCAAGGCGATTAAGTTGGGTAAC	micro RNA primer
miRNA-b	none	GCGGATAACAATTTCACACAGGAAACAG	micro RNA primer
13b I	none	GATGTTGAACAGACTTACGCCATTCTCTCTTTTGTATTCC	micro RNA primer
13b II	none	GAATGGCGTAAGTCTGTTCAACATCAAAGAGAATCAATGA	micro RNA primer
13b III	none	GAATAGCGTAAGTCTCTTCAACTTCACAGGTCGTGATATG	micro RNA primer
13b IV	none	GAAGTTGAAGAGACTTACGCTATTCTACATATATATTCCT	micro RNA primer
13d I	none	GATTAAGAGCGTTCAACAAGCGTTCTCTCTTTTGTATTCC	micro RNA primer
13d II	none	GAACGCTTGTTGAACGCTCTTAATCAAAGAGAATCAATGA	micro RNA primer
13d III	none	GAACACTTGTTGAACCCTCTTATTCACAGGTCGTGATATG	micro RNA primer
13d IV	none	GAATAAGAGGGTTCAACAAGTGTTCTACATATATATTCCT	micro RNA primer
3a I	none	GATCCGTATAAGTTATGCGTCGATCTCTCTTTTGTATTCC	micro RNA primer
3a II	none	GATCGACGCATAACTTATACGGATCAAAGAGAATCAATGA	micro RNA primer
3a III	none	GATCAACGCATAACTAATACGGTTCACAGGTCGTGATATG	micro RNA primer
3a IV	none	GAACCGTATTAGTTATGCGTTGATCTACATATATATTCCT	micro RNA primer
3b I	none	GATGTAACTCTTGCGCCTAACACTCTCTCTTTTGTATTCC	micro RNA primer
3b II	none	GAGTGTTAGGCGCAAGAGTTACATCAAAGAGAATCAATGA	micro RNA primer
3b III	none	GAGTATTAGGCGCAACAGTTACTTCACAGGTCGTGATATG	micro RNA primer
3b IV	none	GAAGTAACTGTTGCGCCTAATACTCTACATATATATTCCT	micro RNA primer