IDENTIFYING MUTANTS OF *ARABIDOPSIS THALIANA* WITH ALTERED NITRIC OXIDE HOMEOSTASIS

An Honors Thesis

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ABSTRACT

Title: IDENTIFYING MUTANTS OF ARABIDOPSIS THALIANA WITH ALTERED NITRIC OXIDE HOMEOSTASIS
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Nitric oxide (NO) is an essential signaling molecule for almost all organisms. In plants, NO was found to regulate many essential metabolic functions such as plant immunity and regulation of abiotic stress. Despite the importance of NO as a signaling molecule, understanding of genes involved in NO homeostasis is limited. Screening for mutants with altered NO response under NO stress and isolating the responsible genes through genome sequencing could reveal more information about NO homeostasis in plants.

The goal of the research described here is to identify genes of the model plant *Arabidopsis thaliana* involved in NO homeostasis. Ethyl methanesulfonate (EMS)-treated *A. thaliana* Columbia-0 (Col) first-generation mutant (M_1) seeds were used to produce an M_2 generation of seeds to use for mutant identification based on aberrant root elongation. Methods to conduct successful root elongation assays, establishing the conditions to expose seedlings to the effect of diethylenetriamine/nitric oxide (DETA/NO) as an NO source, transferring selected seedlings to soil, and other logistics for performing this research were first established. With the established methods, M_2 seedlings were exposed to 1.0 mM DETA/NO during growth to identify seedlings with mutations that allow them to survive NO stress. The root lengths of mutagenized and unmutagenized *A. thaliana* Col-0 seedlings were compared, and the mutagenized seedlings with longer root length were selected and transferred to soil for further M_3 generation analysis.

A total number of 12,528 M_2 seeds were screened, and 96 of them were selected as mutant candidates for retesting in the M_3 generation. Out of those 96, only 7 were ready to be screened in the M_3 generation during this project period. 3,000 seeds from the 7 M_3 plants were further screened, and only one of them appeared to be the possible mutant candidate.

With established methods of screening mutants from this project, more M_2 mutagenized seeds can be screened in effective manner, and the remaining M_3 candidates from the screening already performed can be re-screened to confirm their phenotypes. Once an M_3 candidate that demonstrates inheritance of the longer root length phenotype in the M_3 generation is found, subsequent genetic experiments can be performed to determine how many genes confer the phenotype and if the mutation is recessive or dominant. Mutant M_3 plants can be backcrossed to WT *A. thaliana*, and progeny used for whole genome sequencing to locate the mutation.

Acknowledgments

Coming to UMass, I was a lost freshman who did not know what I wanted to study or what I wanted to do both during and after college. As an immigrant in this country and having no parents who could give me advice on the American college system, I felt lost and alone during the process of figuring out how to build my college career.

My college career was full of mistakes and failures. However, looking back at it gives me hope, because as I am standing here four years later, my life has turned out to be okay.

The education and the experiences that I have gained through UMass and Commonwealth Honors College have made me realize in which direction I want my life to take me to. This piece of writing represents only a very small portion of the knowledge that I have gained during my time at UMass.

I want to take a moment to recognize Dr. Elizabeth Vierling's continuous support and patience throughout my time in the Vierling lab. I thank Dr. Ludmila Tyler for all the work she has done for me to make this accomplishment come true. I also want to thank Dr. Patrick Treffon for patiently training me from when I first came into lab with no experience until now. Lastly, I thank my family and friends for all of their support. All that I have accomplished would not have been possible without the endless support that I received from the UMass community and I cannot thank everybody enough.

As I walk out of this institution, I am going to maintain my pride in successfully surviving the last arduous four years at UMass and continue to do my best in my future endeavors.

Esther Oh
May 14, 2019

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Abbreviations

ABBREVIATION	FULL NAME
AtGSNOR1	*Arabidopsis thaliana* S-nitrosogluthathine reductase
cGMP	Cyclic guanosine monophosphate
CLV1	CLAVATA 1
CN^-	Cyanide
Col-0	Columbia-0
DAF-FM	4-Amino-5-methylamino-2′,7′-difluorofluorescein
DETA	Diethylenetriamine
DETA/NO	Diethylenetriamine/nitric oxide
DMSO	Dimethyl sulfoxide
EDRF	Endothelium-derived relaxing factors
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMS	Ethyl methanesulfonate
Fe	Iron
GP	Germination percentage
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
GTPase	Guanosine triphosphate-ase
LRR	Leucine-rich repeat
MES	2-(N-morpholino)ethanesulfonic acid
MS	Murashige and Skoog (medium)
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
ROS	Reactive oxygen species
SiAR	Simulated acid rain
SN-SiAR	Sulfate-and-nitrate-mixed simulated acid rain

SNP	Sodium nitroprusside
SNO	S-nitrosothiol
TD	Transmitted light
WT	Wild-type

Chapter 1. Introduction and Review of Literature

Section 1: Nitric Oxide and Its Significance

Nitric oxide (NO) is a free radical signaling molecule that has physiological and pathophysiological roles in almost all biological systems (Lee et al., 2008). NO was discovered in 1772 by Joseph Priestly, and at first, it was only known as a colorless gas with a half-life of six to ten seconds. Scientific knowledge of NO was limited until the late 1900s when researchers began to understand the significance of NO (Yetik-Anacak and Catravas, 2006).

In 1980, Dr. Robert Furchgott's discovery of endothelium-derived relaxing factors (EDRF) sparked a dramatic increase in NO-related research (Yetik-Anacak and Catravas, 2006). Furchgott discovered that endothelial cells release EDRF to relax smooth muscles, and EDRF could be used to treat cardiovascular diseases. In 1986, Dr. Salvador Moncada and Dr. Louis Ignarro concluded that EDRF is NO, and in the following year, L-arginine was identified as a biological precursor of NO (Yetik-Anacak and Catravas, 2006). In 1992, the cover of *Science* magazine featured NO as the molecule of the year. Viagra is a drug developed in 1998 to treat erectile dysfunction through the NO-cyclic guanosine monophosphate (NO-cGMP)

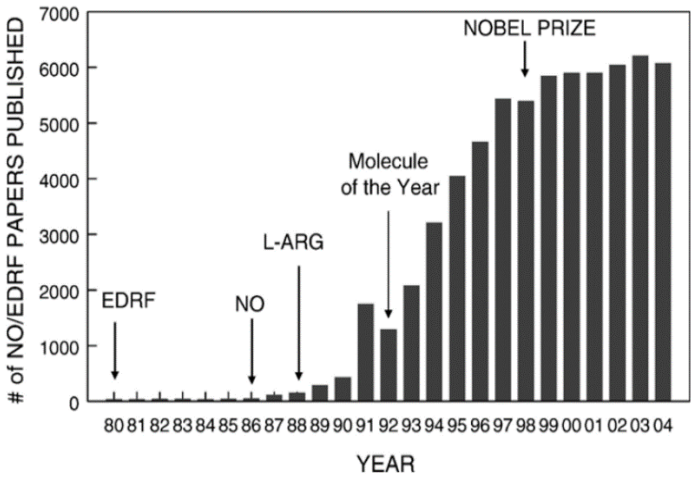


Figure 1. Number of NO-Related Publications Between 1980 and 2004. The number of NO-related publications dramatically increased between the 1990s and early 2000s. (Figure adapted from Yetik-Anacak and Catravas, 2006).

system. In the same year, the Nobel Prize in Physiology and Medicine was awarded to three scientists who discovered NO as a signaling molecule in the cardiovascular system (Yetik-Anacak and Catravas, 2006). The number of NO publications dramatically increased between 1990 and 2000 (Figure 1), and as more interest in NO-related research sparked, more applications of NO research were realized.

NO was found to be relevant in the field of cardiovascular research, but its role expands vastly to other mammalian functions such as: vasodilation, neurotransmission, smooth muscle contraction and relaxation, apoptosis, innate immune response, egg fertilization, embryo development, and more (Kim et al., 2004; Siddiqui et al., 2011; Zhou and Zhu, 2009). As much as NO is a critical signaling molecule in mammalian physiology, it plays essential roles in plant physiology as well. NO can adapt to a variety of roles in both mammals and plants, because NO is abundant in cells and can form various reactive nitrogen species such as $ONOO^-$, NO_2, N_2O_3, and other NO_X species (Lindermayr and Durner, 2015). The versatility of NO allows it to indirectly regulate the activity of various intracellular target enzymes (Cooper, 2000) by reacting with oxygen species, hemes, thiols, and proteins (Siddiqui et al., 2011). In plant metabolism, NO regulates many different functions as well, and to highlight a few, NO's impact on plant immunity and regulation of abiotic stresses will be discussed here.

Many pieces of evidence indicate that NO is an essential signaling molecule in plant immunity. In one study, the relationship between the *Arabidopsis thaliana* S-nitrosoglutathione reductase (*AtGSNOR1*) gene and plant immunity was investigated. The *AtGSNOR1* gene encodes an enzyme AtGSNOR1, which is the primary regulator of cellular S-nitrosothiol (SNO) concentration. SNO is an organic compound that contains an NO moiety on a reactive cysteine thiol (Kwon et al., 2012). When *AtGSNOR1* was silenced, an increased cellular concentration of

SNO was observed. The change in SNO concentration decreased plant immunity by disabling the distinct *Resistance* (*R*) gene-mediated protection and multiple other forms of plant disease resistance. (Feechan et al., 2005). The same study also demonstrated that AtGSNOR positively regulated the salicylic acid (SA) signaling network (Feechan et al., 2005). SA is an important signaling molecule which is essential to plant immunity (Chen et al., 1993; Kumar and Klessig, 2003; Slaymaker et al., 2002). From this study, AtGSNOR1 was found to be involved with regulating the activities of SNO and SA, which are both involved with the regulation of plant immunity; thus, this proves how closely related NO is to the regulation of plant immunity.

Stressor	NO-mediated effect	Species of induced NO
Drought/osmotic stress	Involving in ABA signaling, stomatal closure induction of ABA synthesis, LEA expression	*Nicotiana tabacum* *Pisum sativum*
Salinity	Increased osmotic tolerance; induce expression of Na^+/H^+ antiporter gene	*N. tabacum* maize
Heavy metal toxicity	Increased the root elongation; reduced the NOS activity	*Hibiscus moscheutos* (Al^{3+}) (Reduced NO level)
Herbicide	Promoted the activity of antioxidant enzymes	*Scenedesmus obliquus* *Chlamydomonas reinhardtii*
High temperature	Increased tolerance of seedlings; rapid NO release	*Medicago sativa* *N. tabacum*
Low temperature	Decline the ROS level	*S. obliquus*
Mechanical injury	NO burst result in cell death	*Arabidopsis thaliana* *Taxus brevifolia*
Nurient deficiency		*S. obliquus*
UV-B radiation	Induced the expression of *CHS* gene	*A. thaliana*

ABA, abscisic acid; LEA, late embryogenesis abundant; ROS, reactive oxygen species.

Table 1. Reports of NO Induction by Abiotic Stress and NO-Mediated Effect. (Adopted from Qiao and Fan, 2008)

NO was found to be closely related to abiotic stress regulation as well (Siddiqui et al., 2011). Examples of abiotic stresses are drought, salinity, heavy metal toxicity, herbicide, high or low temperature, mechanical injury, nutrient deficiency, UV-B radiation, aluminum and more (Table 1) (Qiao and Fan, 2008; Yu et al., 2014).

In peas (*Pisum sativum*) for example, an increased level of salt triggers S-nitrosation, which changes the activities of mitochondrial proteins to regulate abiotic-response-related respiratory and photorespiratory pathways (Camejo et al., 2013). It was also found in *Arabidopsis* that *Atnoa1* mutants with reduced NO levels were more sensitive and less resistant to salt stress when compared with WT *Arabidopsis* (Guo et al., 2003; Zhao et al., 2007).

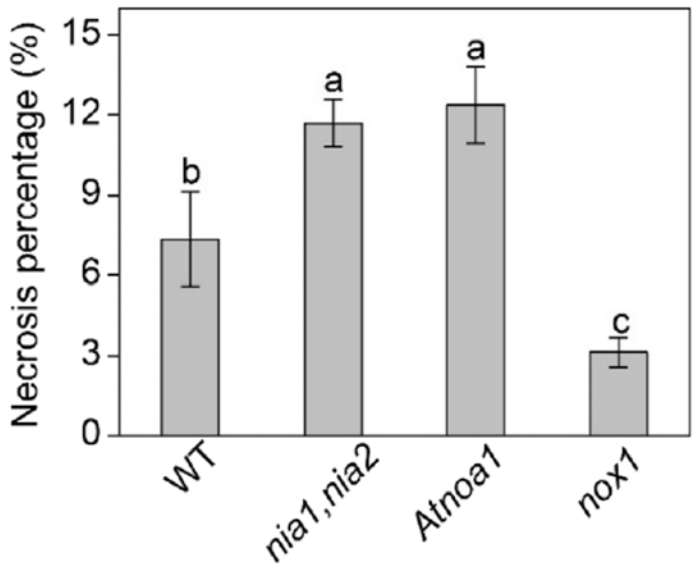


Figure 2. Necrosis percentage of *Arabidopsis* wild-type, *nia1nia2, atnoa1* and *nox1* mutants under SN-SiAR treatment for 5 days. Data are mean values ± SE of three independent experiments and different uppercase letters indicate significant differences between different types of SiAR treatment ($p < 0.05$) (Figure and legend adapted from Qiao et al., 2018).

In another study, it was concluded that an elevated NO level in *A. thaliana* enhances resistance to damage by simulated acid rain (SiAR) (Qiao et al., 2018). The researchers exposed four *A. thaliana* mutants, *nia1*, *nia2*, *atnoa1*, and *nox1*, to sulfate and nitrate solutions, mixed to simulate acid rain, for five days. The *nia1* and *nia2* mutations reduce endogenous NO levels by reducing the function of nitrate reductase (NR), while the *atnoa1*

mutation affects the AtNOA1 protein, which also reduces endogenous NO levels. The *nox1* mutant over-produces NO at the cellular level. At the end of the trial, the Zheng lab observed a significantly lower percentage of necrosis on the leaves of *nox1* mutants (Figure 2), while mutant seedlings with lower NO content showed a much higher percentage of necrosis compared to WT. These investigators concluded elevated NO concentrations could enhance the cellular immune response of plants under acid rain, a form of abiotic stress.

Besides plant immunity and resistance to different types of abiotic stresses, NO regulates induction of seed germination, stomatal movement, leaf senescence, photosynthesis, mitochondrial function, plant defense, photomorphogenesis, fertilization, and flowering (Baudouin and Hancock, 2014; Siddiqui et al., 2011; Wang et al., 2012; Wendehenne and Hancock, 2011; Yu et al., 2014).

Section 2: Chemistry of NO

NO synthesis occurs both nonenzymatically and enzymatically (Domingos et al., 2015), yet the pathways of NO synthesis in mammals and plants differ. In mammals, NO is synthesized by the enzyme nitric oxide synthase (NOS), which is a homodimer that converts L-arginine to L-citrulline and NO (Figure 3) (Wendehenne and Hancock, 2011). There are three different isoforms of NOS in mammals, and these isoforms function at different organs (Zhou and Zhu, 2009). One nonenzymatic method of NO synthesis is through denitrification or nitrification cycles, where NO is the by-product of nitrous oxide oxidation (Cooper, 2000; Domingos et al., 2015). This mechanism exists in plant metabolism as well. Another nonenzymatic synthesis of NO in plants

occurs via conversion of nitrogen dioxide to NO in the presence of carotenoids in the light (Cooney et al., 1994).

Enzymatic NO synthesis in plants involves nitrate reductase (NR), which is an enzyme that reduces nitrate to nitrite (Yamasaki and Sakihama, 2000). Although NR is named after its function of reducing nitrate, this enzyme can reduce nitrite to NO, as well, via a mitochondrial electron transport-dependent reductase (Figure 4) (Astier et al., 2018; Crawford, 2006; Planchet et al., 2005).

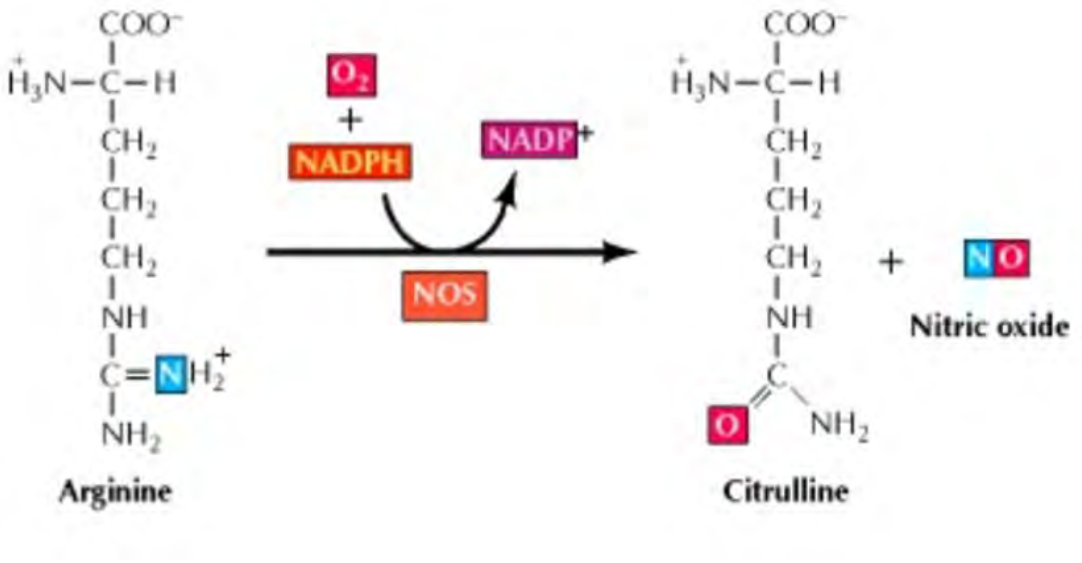


Figure 3. Synthesis of Nitric Oxide. Nitric oxide is synthesized from arginine through catalysis by NOS in animals (Figure adapted from Cooper et al., 2000).

Once NO is synthesized, it can diffuse directly across the plasma membrane and locally affect other nearby cells (Crawford, 2006). NO itself can only act locally due to its instability; it has a half-life of only a few seconds (Cooper, 2000). Therefore, S-nitrosoglutathione (GSNO) is vital in NO homeostasis; GSNO is a reservoir that stores and transports NO in cellular systems

(Figure 5) (Lindermayr, 2017). The level of GSNO is regulated through production or degradation by the enzyme GSNO reductase (GSNOR) (Barnett and Buxton, 2017). Deficiency in GSNOR in plants can lead to growth defects, impaired disease response, heat sensitivity (Lee et al., 2008), defects in stem and trichrome branching, and increased susceptibility to apoptosis (Xu et al., 2013). Plant GSNOR contains solvent-accessible cysteines, and it is a ubiquitously-expressed protein localized in the cytosol. GSNO can be formed through the reaction of NO and glutathione (GSH), which is a coenzyme that is responsible for oxidation-reduction reactions in cells. When GSNO is reduced by GSNOR in the presence of NADH, an unstable intermediate, GSNHOH, is formed, which can be further oxidized to form GSSG (Figure 5) (Barnett and Buxton, 2017). Many chemical reactions reduce or oxidize NO, making NO homeostasis a non-trivial process requiring further investigation.

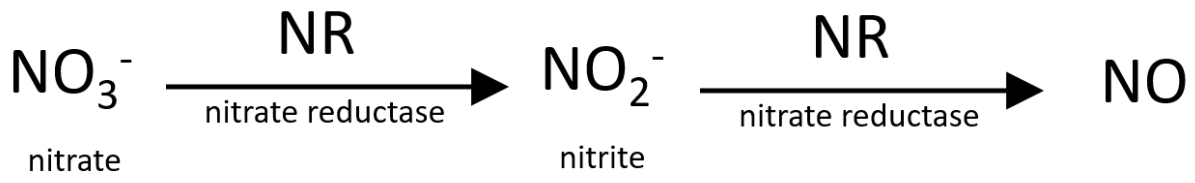


Figure 4. Nitrate Reductase Reduces Nitrate and Nitrite to NO. Nitrate reductase (NR) is an enzyme that reduces nitrate to nitrite in plants. NR can also reduce nitrite to NO via mitochondrial electron transport-dependent reductase.

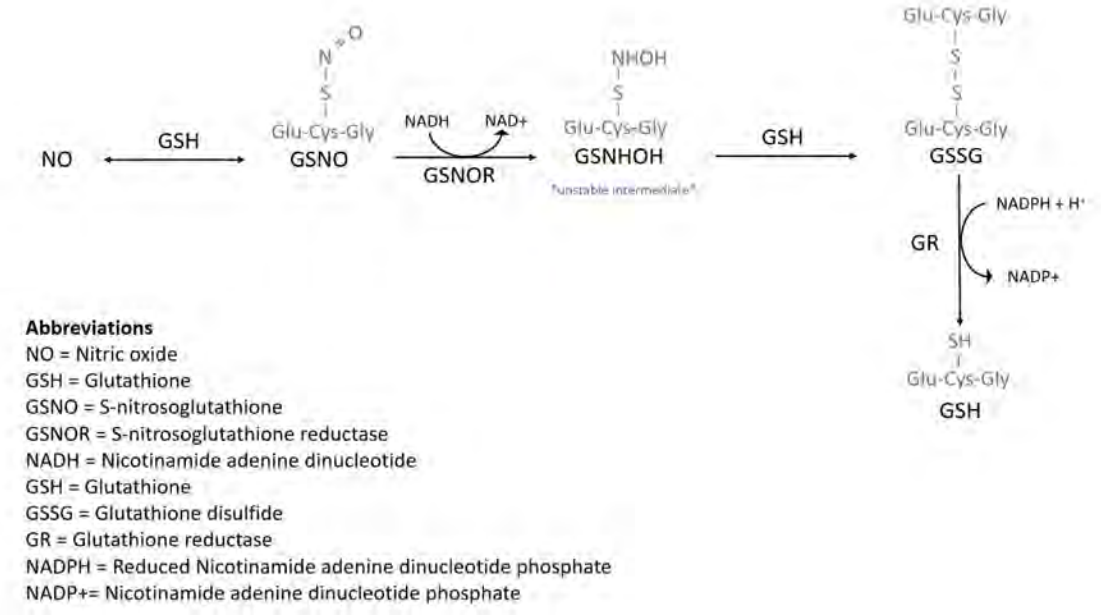


Figure 5. NO-Related Reactions Involved in NO Homeostasis and the Role of GSNOR. NO is a free radical with a short lifetime, so it is primarily found as GSNO, which is metabolized by GSNOR (Barnette and Buxton, 2017).

Section 3: Research Objectives

Though it provides many benefits to the plant, NO causes damage at high concentrations. Excess levels of NO become a stress factor by causing oxidative and nitrosative damage to the cell membrane, resulting in problems such as impairment of leaf expansion, changes in thylakoid viscosity, inhibition of root or shoot growth, and DNA fragmentation (Siddiqui et al., 2011). Therefore, the positive or negative effects of NO are determined by its location and concentration.

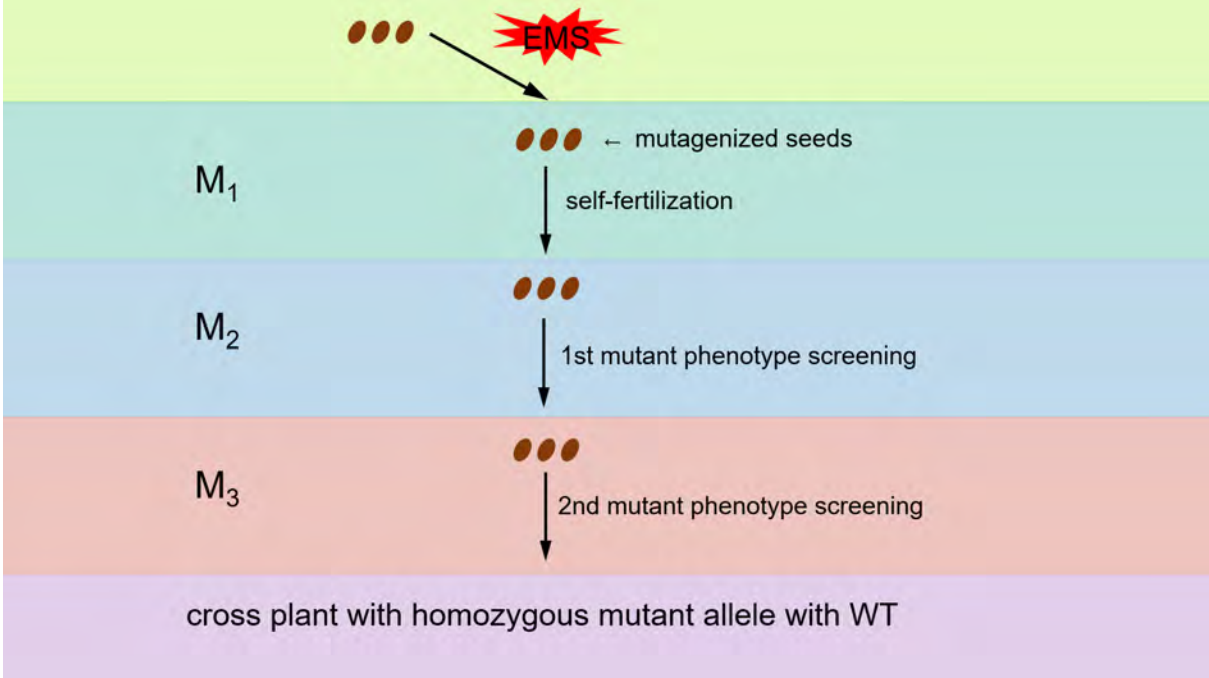


Figure 6. Diagram of mutagenesis of screening relative to the M_1, M_2, and M_3 generations. EMS-treated M_1 seeds are self-fertilized and M_2 seeds collected. M_2 seedlings are screened and those that show the desired mutant phenotype, which is longer root length in comparison to unmutagenized seedlings, are grown and self-fertilized to obtain the M_3 generation. The collected M_3 seeds will be screened to confirm the phenotype observed from the M_2 generation. M_3 seeds can be backcrossed with WT in order to obtain information about whether the mutation is dominant or recessive.

The goal of this research was to identify new mutants of *A. thaliana* involved in NO homeostasis. Although understanding of NO metabolism in plants has increased dramatically, the knowledge of other genes involved in NO homeostasis is limited. Knowledge about genes involved in NO homeostasis could reveal novel information about plant metabolism, especially because NO homeostasis is complex and involves multiple oxidation-reduction reactions and enzyme catalysis.

The research strategy included exposing unmutagenized and M_2 mutagenized *A. thaliana* seeds to a high concentration of NO and comparing root lengths as they germinate and elongate on agar medium in Petri dishes. M_2 seedlings with longer roots compared to unmutagenized

seedlings were selected and transferred to soil. Seeds from those selected plants were harvested at the end of their life cycle, and the collected M_3 seeds were exposed to the high concentration of NO, in parallel with unmutagenized seeds, to confirm if the longer root length phenotype observed from the previous generation is inherited in the third generation. By observing the percentage of plants that present the mutant phenotype (longer roots in comparison to unmutagenized seedlings), whether the mutation is dominant or recessive can be determined (Figure 6).

As of now, the project only progressed up to the point of screening the M_3 seeds. Once the phenotype observed from the M_2 generation is confirmed in the M_3 generation in the future, the genetic location of mutations that suppress the effects of the high NO concentration stress can be located through positional cloning and whole genome sequencing. For positional cloning, the homozygous mutant plant is crossed with a wild-type (WT) of a different ecotype and progeny used for genetic mapping of the mutant allele. When two different ecotypes are crossed, segregating polymorphisms function as genetic markers. When the plant homozygous for mutant alleles (with genotype C/C) is crossed with the WT of another ecotype (e.g. with genotype L/L), if the markers are unlinked, when those two plants cross, the frequency of C and L alleles will be close to equal. However, if the markers are linked, then rather than having a similar frequency, the frequency of the C allele will be significantly higher than the frequency of L alleles (Weigel and Glazebrook, 2002).

Alternatively, whole genome sequencing can be used to locate the mutation of interest. In this case, the mutant can be backcrossed to WT of the same ecotype and mutants reselected from the selfed, backcrossed progeny. DNA from multiple mutants is pooled and sequenced and compared to the WT parent and the WT progeny. The region with the mutation should carry the

mutation in all of the mutant progeny, while other EMS mutations will be segregating (Weigel and Glazebrook, 2002).

Longer root length was chosen as the parameter and the mutant phenotype of interest because of two reasons. First, longer root length of mutant seedlings under NO stress implies that existing mutations increase the plant's ability to compensate for NO stress, and let the mutant plants elongate better than the WTs. Second, longer root length is an efficient parameter to use in the process of screening for mutants because it is relatively fast and easy to plant many seeds at the same time to identify the candidate. More about using longer root length as a parameter is written in the Discussion section.

Section 4: Ethyl Methanesulfonate Mutagenesis

Mutagenesis is the process of inducing changes in the genetic material, and if the germline is mutated, then the mutations get inherited by subsequent generations. This can be done either using chemical mutagens or other mutagenic treatments (Meyerowitz and Somerville, 1994). As explained in the previous section, the goal of this research can be achieved through comparing the root lengths of WT and mutagenized *A. thaliana* seedlings exposed to high NO stress. In order to create mutagenized seeds, WT seeds were treated with the mutagenic compound ethyl methanesulfonate (EMS).

EMS is a monofunctional ethylating agent that is widely used for mutagenesis in both mammalian and plant systems (Sega, 1984). EMS causes random point mutations in the genome through chemical modification of nucleotides that results in misparing and base changes (Figure 7). When genetic material is treated with EMS, guanine, one of the four nucleotides in DNA, is

alkylated, resulting in O^6-ethylguanine. This pairs with thymine (T) on the opposite strand of DNA, rather than its normal partner, cytosine (C). Subsequently, during DNA replication, the original G/C pair is replaced with an A(adenine)/T pair, causing point mutations at every O^6-ethylguanine position. EMS can sometimes generate G/C to C/G or G/C to T/A transversions at low frequency; however, about 99% of the time, EMS induces C-to-T changes that result in C/G to T/A transition substitutions (Salinas and Sánchez-Serrano, 2006).

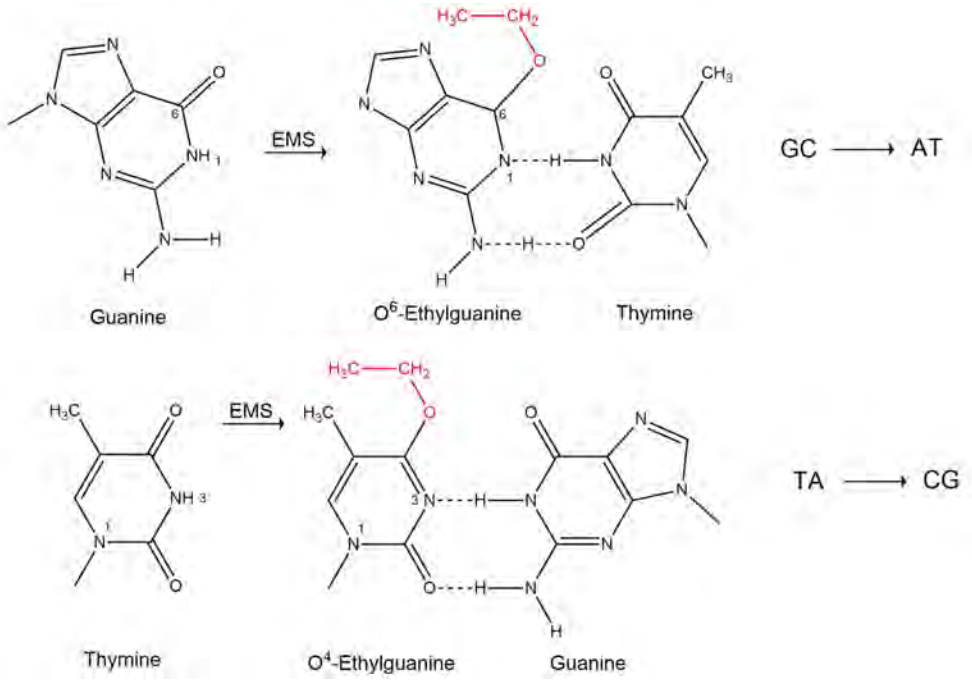


Figure 7. Alkylation-Induced Specific Mispairing. The alkylation (in this case ethylation generated by EMS) of the O-6 position of guanine and also the O-4 position of thymine can lead to direct mispairing with thymine and guanine, respectively, as shown here. In bacteria, where mutations have been analyzed in great detail, the principal mutations detected were GC → AT transitions, indicating that the O-6 alkylation of guanine is most relevant to mutagenesis (Figure and legend adapted from Suzuki et al., 1986).

Treating genetic material with EMS is a relatively simple process. In order to mutagenize *Arabidopsis thaliana*, seeds are soaked overnight in 0.4% EMS, 100 mM phosphate buffer (pH

7.5) and dried (Salinas and Sánchez-Serrano, 2006). This simple process of soaking seeds results in an entire batch of seeds with different point mutations (Meyerowitz and Somerville, 1994). These seeds are designated as M_1 seeds and produce M_2 seeds. M_1 seeds can be planted and screened for dominant mutations, or more typically seeds from the next generation (M_2 seeds) are used for screening for both recessive and dominant mutations (Salinas and Sánchez-Serrano, 2006).

Section 5: Diethylenetriamine/Nitric Oxide (DETA/NO) as an NO Donor

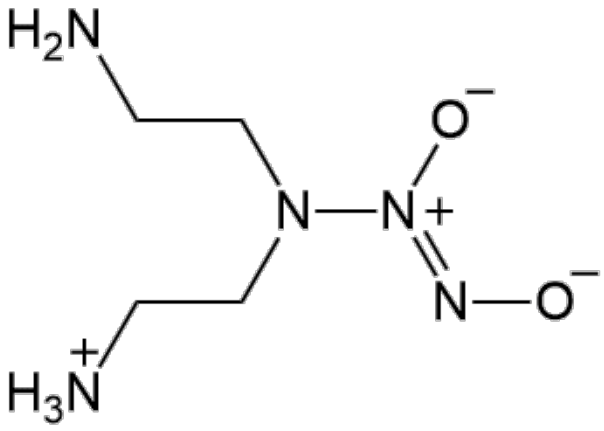


Figure 8. Chemical Structure of DETA/NO. Diethylenetriamine/nitric oxide (DETA/NO) was selected as an NO donor for the experiment. DETA/NO liberates two moles of NO per mole of parent compound.

Diethylenetriamine/nitric oxide (DETA/NO) (Figure 8) was chosen as the NO donor for this work because it relatively slowly releases NO. At pH 7.4 and 37°C, the half-life of DETA/NO was 20 hours. However, other NO donors such as spermine/NO (SPER/NO), propylamine/NO (PAPA/NO), diethylamine/NO (DEA/NO), methylamine/NO (MAHMA/NO) and 1-[2-(carboxylate)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) showed half-lives of 39, 15, 2, and 1 minutes, or 2 seconds, respectively (Figure 9) (Fitzhugh and Keefer, 2000).

Because this mutant screen requires multiple days of NO exposure of seedlings during root elongation, DETA/NO, which showed the longest half-life, was chosen as the NO donor for this work.

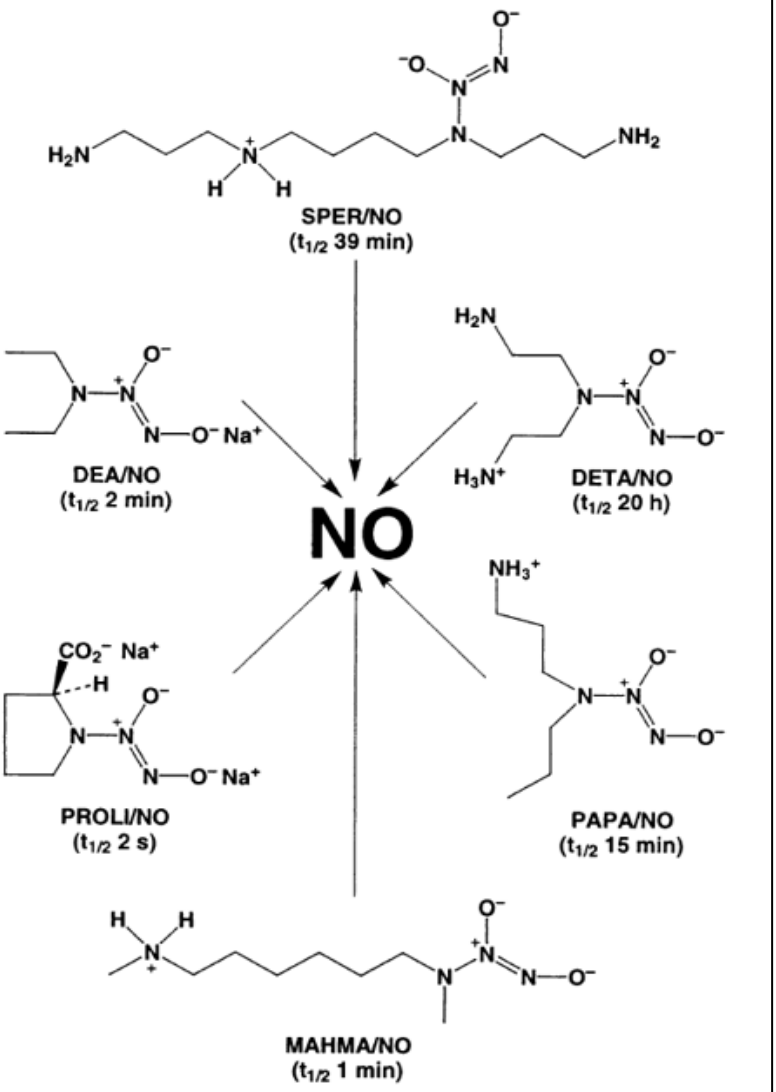


Figure 9. Structures of Compounds that Release NO and Their Measured Half-Lives at pH 7.4 at 37°C. (Figure and legend adapted from Fitzhugh and Keefer, 2000).

Another reason for selecting DETA/NO as an NO donor is because it only releases NO. One mole of DETA/NO releases two moles of NO. Another very commonly used NO source in life science research is sodium nitroprusside (SNP). Although SNP is significantly more affordable than DETA/NO, SNP releases cyanide (CN^-) and iron (Fe), in addition to NO in solution. When cyanide content in DETA/NO and SNP solutions were compared, the SNP solution showed about four times higher cyanide content, while the DETA/NO solution had a higher NO content. The DETA/NO solution also contained lower ROS levels, meaning that there was less reactive oxidative stress (Keisham et al., 2019). Thus, DETA/NO is a better source of NO even though SNP is very commonly used, and for those reasons, DETA/NO was selected as an NO donor for this experiment.

Chapter 2. Materials and Methods

Unless otherwise noted, all equipment, instruments, and reagents were purchased from Thermo Fischer Scientific (Fair Lawn, NJ, USA).

Preparation and Use of Nylon Mesh

Nylon mesh with a pore size of 70 µm [Sigma-Aldrich (St. Louis, MO)] was cut into strips 1.3 cm by 8.7 cm. The nylon mesh was autoclaved for 15 minutes on dry cycle before each use.

After each use, if no contamination occurred in the plate throughout the experiment, nylon mesh was reused after thorough cleaning. To clean used nylon mesh, the strips were soaked in an ethanol solution overnight, handwashed gently using deionized water, dried overnight using paper towels, and autoclaved for 15 minutes. If contamination occurred, the nylon mesh was discarded as a biohazard.

Seed Preparation and Sterilization

For unmutagenized seeds, WT *A. thaliana* Columbia-0 (Col-0) seeds were used. For mutagenized seeds, *A. thaliana* Col-0 seeds treated with ethyl methanesulfonate (EMS) were used. WT Col-0 seeds were harvested and provided courtesy of Dr. Patrick Treffon from the Vierling lab. The mutagenized seeds were prepared by Dr. Damian Guerra in 2014, a previous member of the Vierling lab and by Dr. Patrick Treffon in 2017 using the same protocol.

The mutagenesis procedure is summarized here: WT Col-0 seeds were soaked in 0.2% (v/v) EMS-solution overnight. The EMS-treated M_1 seeds were dried and planted on soil for production of M_2 seeds. The screening done here was conducted using the M_2 seeds that were collected from

the first-generation M_1, EMS-treated Col-0 plants (see Figure 6). Multiple batches of M_2 EMS-treated Col-0 seeds were prepared.

For sterilization, seeds were placed in a 1 mL microcentrifuge tube, and a solution of deionized H_2O with 20% (w/v) Clorox, and 0.5% (w/v) Tween 20 was added. The tube was vortexed and mixed using a thermomixer at 22°C at 1,000 RPM for 7 minutes. Under the sterile biosafety hood, the seeds were rinsed seven times using sterile H_2O.

Preparation of Murashige and Skoog Agar Medium

Murashige and Skoog (MS) agar medium for growth of seedlings contained: 0.5X Murashige and Skoog Basal Medium [Sigma-Aldrich (St. Louis, MO)]; 0.5% (w/v) sucrose, 0.8% (w/v) plant-cell-culture-tested agar [Sigma-Aldrich (St. Louis, MO)], and 1.0% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES) [Sigma-Aldrich (St. Louis, MO)].

In a clean beaker, 0.5X Murashige and Skoog Basal Medium, 0.5% (w/v) sucrose, and 1.0% (w/v) MES were added. The beaker was filled with deionized H_2O to 80% of the final volume of the agar medium. The components in the beaker were mixed using a stir bar at 350 rpm until everything dissolved.

The pH of the medium was measured and adjusted to 5.7 using a pH meter and 10, 5, and 1 M potassium hydroxide (KOH) solutions. Once the pH was adjusted, the solution was transferred to a graduated cylinder and brought to the final volume with additional deionized H_2O. The final solution was added to a glass bottle containing 0.8% (w/v) plant-cell-culture-tested agar. The bottle was autoclaved on a liquid cycle for 15 minutes.

Seed Stratification and Germination

Before placing sterilized seeds on plates, three nylon mesh strips were placed directly on top of solidified MS agar medium without DETA/NO inside the plates using sterile forceps. Sterilized seeds were then planted along the mesh for the stratification and germination period. Plated seeds were stratified in the dark at 4°C for 3 days. After stratification, plates were moved to a growth chamber on a 22/18°C, 12-hr day/night cycle, and incubated for 5 days, which is when the average root length of each seedling was about 1 cm. Plates were placed in a 90˚ vertical position in the growth chamber throughout the experiment. Every 2 days, the location of each plate in the growth chamber was switched to ensure that each plate received a similar amount of light. This was done because it had been noted that plates located closer to the side of the growth chamber showed longer root length than the ones which were located more in the center of the chamber due to the location of light bulb inside the chamber.

After 5 days of germination and growth on MS agar medium, seedlings were transferred to newly made plates containing 1 mM DETA/NO by moving the nylon mesh with the seedlings using sterile forceps in a biosafety hood. Each DETA/NO plate received only a single nylon mesh of seeds to allow for growth of roots. Seedlings were left to elongate on DETA/NO-containing plates for about 5 to 7 days, depending on the average length of the seedlings (Figure 10).

At the end of the stratification, germination, and root elongation, EMS-treated seedlings with a longer root length in comparison to WT seedlings were selected and transferred to soil, individually to pots.

Details about the preparation of plates and other methods are covered in the following sections.

Preparation of Plates – Stratification and Germination

All the plates that were prepared throughout this experiment were made in the biosafety hood to reduce the chance of contamination. All the equipment used for the experiment in the biosafety hood were sterilized as well.

Polystyrene square, 8.5 x 8.5 cm, integrid petri dishes were used for these experiments. The sterilized MS agar medium was reheated using a microwave for 2 or more minutes until the gel was fully liquified and became transparent. Once the MS agar medium was fully liquified, the bottle was sprayed with 70% ethanol and then placed in the biosafety hood.

Liquified MS agar medium was poured into petri dishes as a preparation. Using 25 mL disposable, plastic biosafe pipettes [Sigma-Aldrich (St. Louis, MO)] and Pipet-Aid Automatic Pipette Pumps [Sigma-Aldrich (St. Louis, MO)], 20 mL of the MS agar medium was transferred into each plate, which was then lightly swirled to ensure an even layer of MS agar medium; plates were left in the biosafety hood for about 10 to 15 minutes at room temperature until the MS agar medium fully solidified.

Once the MS agar medium had solidified, the prepared nylon mesh strips were placed on the surface of the medium using sterile forceps. Three nylon mesh stripes were placed horizontally on each plate, and the first nylon mesh strip was placed about 0.5 cm away from the top of the plate to provide enough space for the leaves to grow. The second nylon mesh strip was placed about 2 cm below the first nylon mesh strip, and the third nylon mesh strip was likewise placed about 2 cm below the second nylon mesh strip (Figure 10).

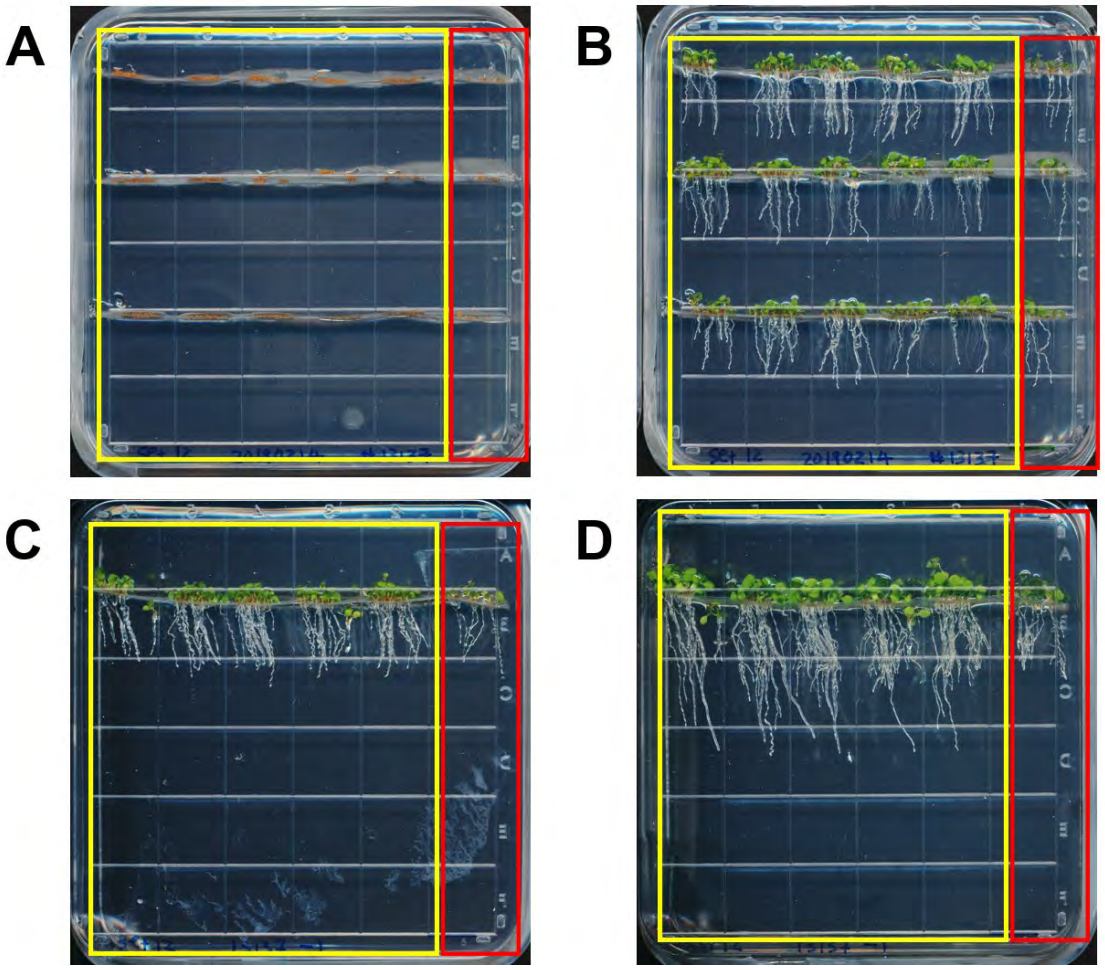


Figure 10. Examples of How Seeds on Nylon Mesh Strips Were Placed and Transferred. Pictures of plates were taken from Day 0 to Day 5 of the experiment to illustrate how each nylon mesh was located and transferred. **(A)** Picture of a plate taken on Day 0 of the experiment, right after the 3-day-stratification, before the plate was placed in the growth chamber. This plate contains three lanes of nylon mesh on the MS agar medium without DETA/NO. **(B)** Picture of a plate taken 5 days after time in the growth chamber. **(C)** Picture of a plate after one nylon mesh strip from **(B)** was transferred to a newly made plate containing DETA/NO. Only one strip of nylon mesh was placed in each plate to allow root elongation. **(D)** Picture of the plate on Day 10, 5 days after the nylon mesh was transferred to a DETA/NO-containing plate.

Seeds were plated directly on top of the mesh covering the solidified agar medium using a P20 micropipette with a pipette tip that had about 2 mm cut off. For each lane of nylon mesh, 15 unmutagenized *A. thaliana* seeds were planted along with mutagenized seeds on the rest of the

lane (Figure 10). In Figure 10, mutagenized seeds were planted on a plate inside of the yellow boxes, while the WT seeds were planted inside of the red boxes.

Preparation of Plates – Root Elongation

After 5 days of germination, seedlings on MS agar medium were transferred to newly made plates containing MS agar medium and 1 mM DETA/NO.

These 1 mM DETA/NO plates were prepared by using the 200 mM DETA/NO stock solution. The stock solution was made by dissolving DETA/NO in 10 mM NaOH. DETA/NO was a kind gift of Dr. Katrina Miranda from the Department of Chemistry and Biochemistry, University of Arizona. The prepared solution was mixed with liquified MS agar medium using sterile 50 mL Falcon tubes. A mixed solution of 1 mM DETA/NO and MS agar medium was poured directly onto new, sterile 8.5 cm by 8.5 cm polystyrene, square integrid petri dishes.

Once the agar medium had fully solidified, seedlings from previous control MS agar medium plates were transferred to newly made 1 mM DETA/NO plates by using forceps to move the nylon mesh strip (Figure 11). Individual seedlings that were left on control MS agar medium plates were transferred by hand using feather-light forceps. This method of transferring seedlings using nylon mesh strips was originally adapted from " Measurement of Uptake and Root-to-Shoot Distribution of Sulfate in *Arabidopsis* Seedlings" (Yoshimoto et al., 2016), but was modified for use in this experiment.

Figure 11. Use of Nylon Mesh Strips for Seed Planting and Transfer. In a sterile hood, 20 mL of liquified Murashige and Skoog (MS) agar medium was poured into each plate. When the medium was half-solidified, sterile nylon mesh strips were placed directly on the medium using sterile forceps. Seeds were planted directly onto the nylon mesh after the medium had fully solidified. The individual plates were then sealed with parafilm.

Selecting Seedlings and Transferring to Soil

Individual EMS-treated Col-0 seedlings with root lengths longer than the average for WT Col-0 seedlings were selected and transferred to soil. Feather-light forceps were used to select and transfer each seedling to an individual pot (5.5 x 6 x 6 cm) containing soil, which contained 1 spike of Insect Control Plus Fertilizer [Bayer Advanced (Whippany, NY)]. Transferred seedlings in pots were put into a growth chamber on a 22/18°C, 12-hr day/night cycle, and the phenotypes of the plants were observed throughout the rest of their six-week life cycle. Each pot was watered with deionized water (DI H_2O). Once the transferred plants started to bolt and produce flowers, Aracons [Arasystems (Belgium)] were placed to prevent cross-contamination.

Acquiring and Analyzing Root Length Data

Plates were scanned multiple times throughout the experiment using a standard scanner at the resolution of 600 dpi, with black paper in the background to improve visibility of the roots.

Pictures of plates were analyzed using ImageJ software (https://imagej.net/Welcome) to quantify and record root length over time.

DAF-FM Staining and Microscopy

Seeds were washed using the same method from "Seed Stratification, Germination, and Root Elongation" section above. Washed seeds were plated on sterile petri dishes containing 20 mL MS agar medium. Seeds were stratified for 3 days at 4°C in the dark. After stratification, plates were moved to a growth chamber on a 22/18°C, 12-hr day/night cycle, and grown for 6 days. All the plates were placed vertically inside the growth chamber. If the plated seeds were being screened without DETA/NO treatment, they were imaged on Day 6 of the experiment after being grown on a plate that only contained MS agar medium. However, if the seeds were being screened after DETA/NO treatment, they were grown on a plate with MS agar medium only for the first 3 days and then transferred to newly made plate containing 1 mM DETA/NO for 3 days. All seeds were imaged on Day 6 of the experiment. All the seeds were plated without nylon mesh to limit damage to roots.

To visualize the NO content in the roots of the seedlings, 4-Amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate was used to stain the roots. On Day 6 of the experiment, roots were selected from the plate and placed in a sterile 6-well tissue culture plate [Becton Dickinson Labware (Franklin Lakes, NJ)]. Roots were stained using 5 mM stock solution of DAF-FM diacetate, which was made by dissolving 1 mg of DAF-FM diacetate in 0.4 mL of high-quality anhydrous dimethyl sulfoxide

(DMSO). The solution was vortexed and centrifuged for 10 seconds. In the tissue culture plate, 10 mM Tris-HCl and the 5 mM stock DAF-FM solutions were mixed. Roots were stained for 10 minutes. As soon as staining was complete, the DAF-FM solution was removed, and roots were washed using 1 mL 100 mM Tris-HCl for 5 minutes. Washing was repeated 2 more times with the same time interval, meaning that all the roots were washed 3 times for 5 minutes each.

All steps of staining and treating the roots were conducted in the dark because DAF-FM diacetate is sensitive to light. Tissue culture plates were covered in tin foil at all times to decrease light exposure.

NO content was visualized using confocal microscopy. Roots were imaged with a 20X objective using Nikon A1SP confocal microscope with a laser frequency at 487.7 nm. Excitation wavelength was 495 nm, and the emission wavelength was 515 nm.

Chapter 3. Results

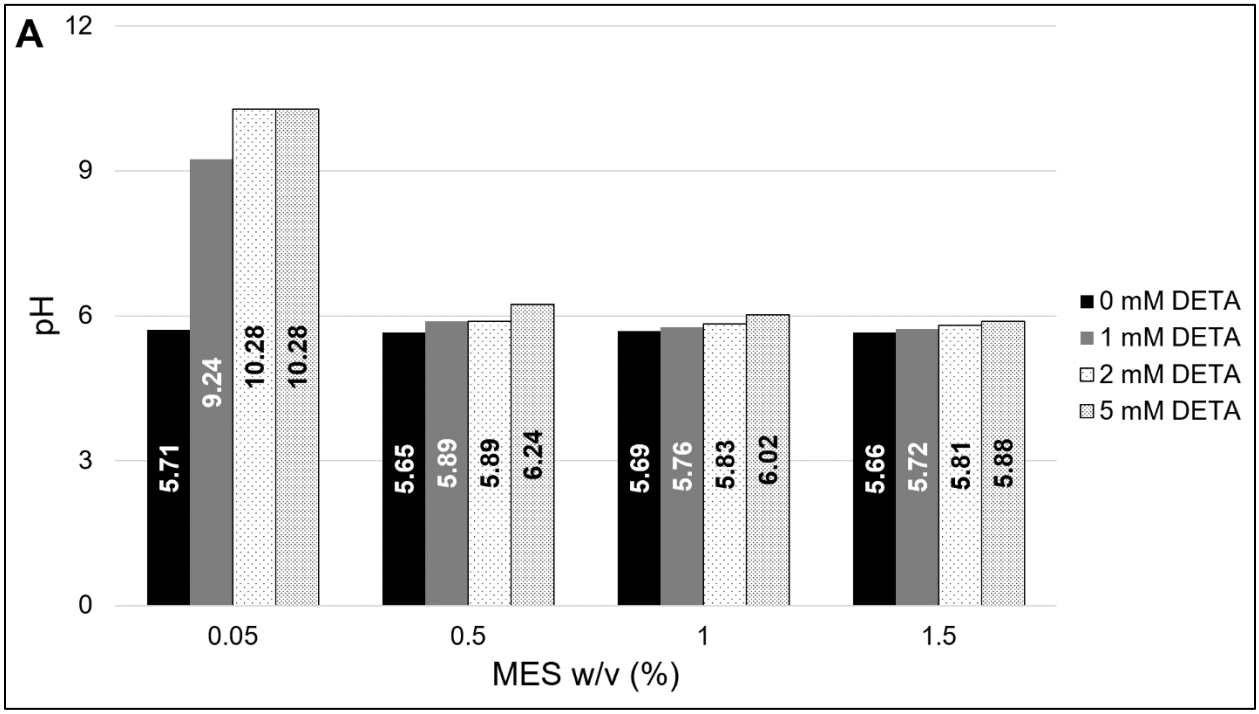
Before starting to screen for mutants, many logistical issues regarding the conditions of the experiment had to be determined. Examples include: testing different concentrations of the buffer 2-(N-morpholino)ethanesulfonic acid (MES), determining the optimal concentration of DETA/NO, and testing the optimal density for seed planting. Standardized conditions were established in the following experiments.

Effects of DETA on the pH of MS Medium

To ensure that NO was the only abiotic stress on *A. thaliana* seedlings during the screening, other types of abiotic stresses had to be minimalized. One abiotic stress that could have impacted the roots was a change in pH due to the addition of DETA/NO, as MS medium is unbuffered. To stabilize the pH of unbuffered MS agar medium, the buffering agent MES was chosen to be added.

In order to determine a suitable concentration, different concentrations of MES were mixed into MS solutions containing 0, 1, 2, or 5 mM diethylenetriamine (DETA). The pH level of each mixture was measured and recorded (Figure 12A). The two concentrations of MES that resulted in the most stable pH level with varying concentrations of DETA were 1.0 and 1.5%. When 1.0% MES was mixed into 0, 1, 2, 5 mM DETA, the pH was 5.69, 5.76, 5.83, and 6.02, respectively. Similarly, the pH was 5.66, 5.72, 5.81, and 5.88 when 1.5% MES was added (Figure 12A). Either 1.0 or 1.5% MES could be used to buffer the pH level of MS agar medium; however, in the interest of saving resources, 1.0% MES was chosen as the optimal concentration.

Once it was decided that 1.0% MES would be optimal for buffering DETA, the experiment was repeated one more time using 0, 1, 2, 5 mM DETA/NO. The varying pH levels of four different concentrations of DETA/NO mixed into 1.0% MES were measured and recorded (Figure 12B). The data on DETA/NO solution pH levels were compared with pH levels obtained using DETA solutions from Figure 12A. As is shown in the graph, no significant difference in the pattern of pH levels was observed. Therefore, it was concluded that 1.0% would be the optimal concentration of MES, despite the fact that the concentration of DETA/NO to be used throughout the experiment had not been determined yet.



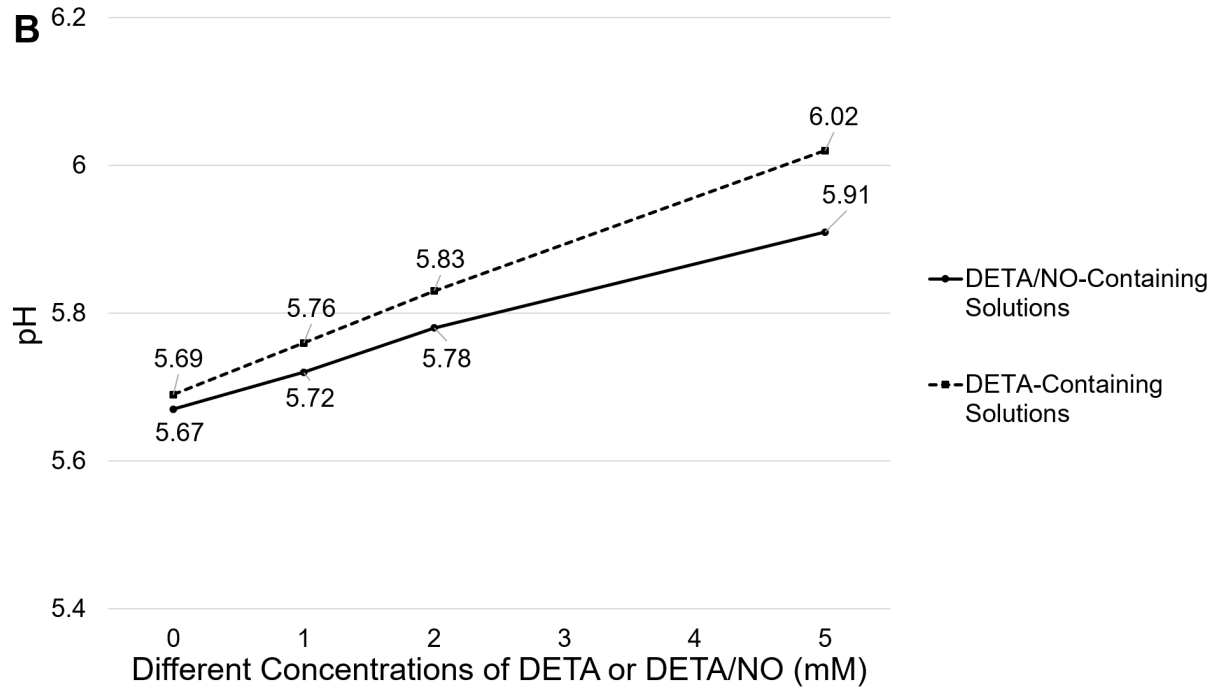


Figure 12. pH Levels of MS Solutions Containing Different Concentrations of MES and DETA. (A) To decide which concentration of MES to use as a buffer, 0.05, 0.5, 1.0, 1.5% MES were mixed into 0, 1, 2, 5 mM DETA solutions. Solutions containing different concentrations of DETA showed the most stable pH levels with 1.0 or 1.5% MES. 1.0% was chosen as the concentration of MES to be used throughout the experiment to save resources. **(B)** 1.0% MES was mixed into solutions containing different concentrations of DETA/NO to confirm that the pH stability observed with DETA was the same with DETA/NO. 0, 1, 2, 5 mM DETA/NO were mixed with 1.0% MES. The data obtained using DETA solutions is from **(A)**. The pH level differences observed using DETA/NO were similar to those observed using DETA.

Determination of DETA/NO Concentration

In the beginning phase of the experiment, the optimal ceonctration of DETA/NO had to be determined to ensure that the roots can be stressed in the presence of DETA/NO, but that the stress would not be severe enough to permananetly damage the roots.

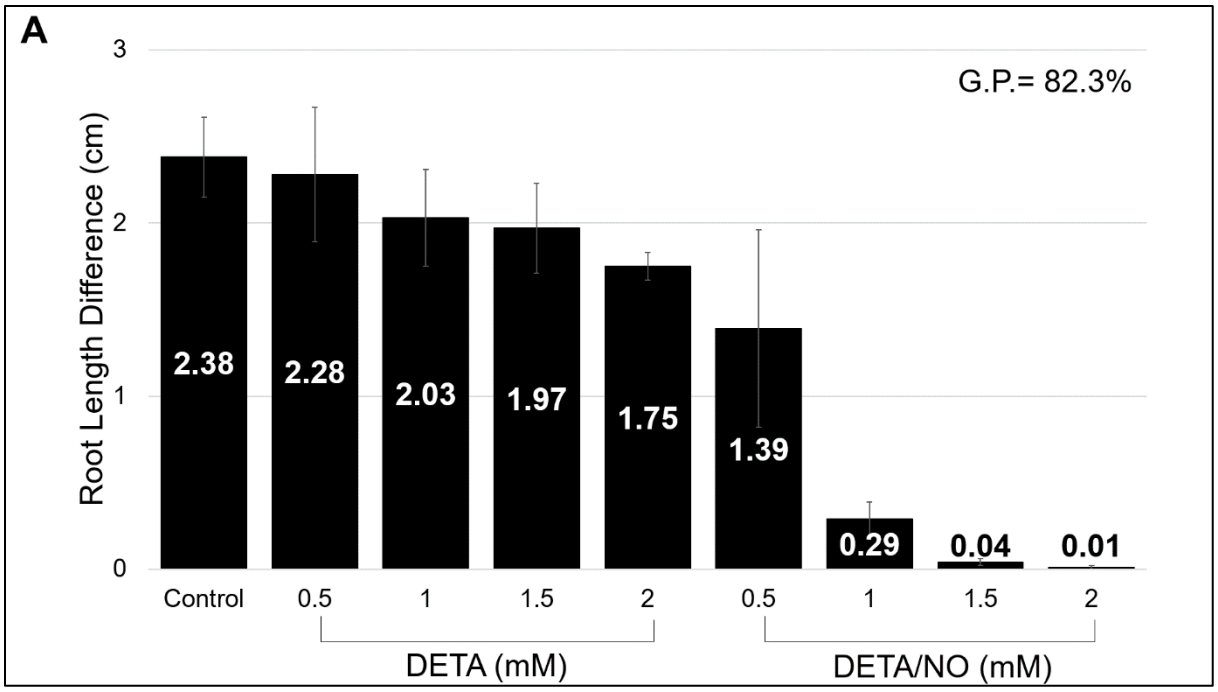
To achieve this goal, four different concentrations of DETA/NO were tested, 0.5, 1, 1.5, and 2.0 mM. As described in the Methods section, WT Col-0 *A. thaliana* seeds were planted along strips of nylon mesh on square integrid petri dishes containing MS agar medium with 1.0% MES without DETA/NO during stratification and germination periods. At the end of the germination period, seedlings were transferred to newly made plates that contained 0.5, 1.0, 1.5, or 2.0 mM DETA or DETA/NO, and the seedlings were left to grow for 5 days in the growth chamber.

The root length of each seedling was then measured, and the lengths in each condition were averaged; the overall germination percentage was also calculated. This experiment was repeated three times, and results were averaged (Figure 13). Seedling roots on control plates without DETA or DETA/NO were 2.38 cm ± 0.23 long. Roots on 0.5, 1.0, 1.5, and 2.0 mM DETA showed additional growth of 2.28 cm ± 0.39, 2.03 cm ± 0.28, 1.97 cm ± 0.26, and 1.75 cm ± 0.08, respectively. Roots on DETA/NO-containing plates showed a dramatic decrease in growth, because 0.5, 1.0, 1.5, and 2.0 mM DETA/NO plates showed the average root length growth of 1.39 cm ± 0.57, 0.29 cm ± 0.1, 0.04 cm ± 0.02, and 0.01 cm ± 0.01, respectively (Figure 13A).

Although increasing DETA concentration resulted in decreased root growth, the changes were more dramatic when the roots were treated with DETA/NO. Seedlings treated with 1.5 or 2.0 mM DETA/NO showed almost no root growth, implying that the concentration of DETA/NO was too high for the goal of the experiment. Therefore, it was concluded that 0.5 or 1.0 mM DETA/NO would be appropriate, and to further analyze this, root growth over time for the seedlings on 0, 0.5, and 1.0 mM DETA/NO plates during the NO stress treatment was plotted (Figure 13B).

During the DETA/NO treatment, seedling roots on 0, 0.5, and 1.0 mM DETA/NO-containing plates elongated in a similar pattern, without any significant difference in slope.

However, at the end of the 5-day treatment, seedlings on 0 mM DETA/NO plate grew 2.25 cm ± 0.16, while the ones on 0.5 and 1.0 mM DETA/NO plates grew 1.08 cm ± 0.2 and 0.19 cm ± 0.16 (Figure 13B). Because the root length difference between seedlings treated with 0 mM and 1.0 mM DETA/NO was more significant than 0 mM and 0.5 mM and no permanent damage from high NO-stress was observed, it was concluded that 1.0 mM DETA/NO would be the optimal concentration to test for mutants with resistance to the growth inhibition caused by NO.



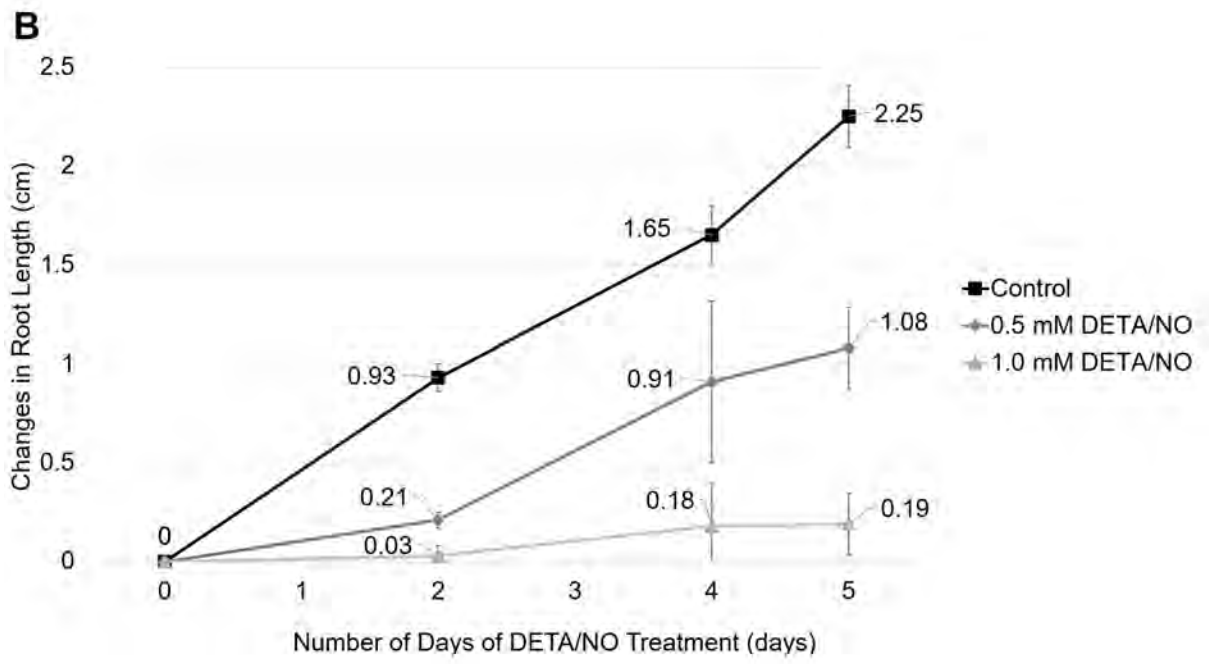


Figure 13. Average Root Length Changes Observed in the Roots of WT (Unmutagenized) *A. thaliana* Seeds Over Multiple Days of Different [DETA] and [DETA/NO] Treatment. (A) WT *A. thaliana* seeds were plated on MS agar medium plates without DETA or DETA/NO during stratification (3 days) and germination (5 days) and were transferred to newly made plates which contained DETA or DETA/NO at varying concentrations. Values represent the average root length difference of seedlings between the Day 1 and Day 5 of the DETA or DETA/NO treatment. The tested concentrations of DETA and DETA/NO were 0.5, 1.0, 1.5, 2.0 mM. The y-axis represents the changes in the average root length of seedlings since they were transferred to DETA- or DETA/NO-containing plates. Germination percentage (G.P.) was 82.3%. Standard error was calculated for each plate. **(B)** Growth over time of WT seeds treated with 0.5 and 1.0 mM DETA/NO during the 5 days of DETA or DETA/NO treatments. The x-axis represents the number of days since the seeds were transferred to DETA/NO-containing plates. The y-axis represents the root length observed since the transfer. Standard errors were calculated and are shown.

Determining Optimal Planting Density

Once it was confirmed that 1.0mM DETA/NO would be the optimal concentration for screening, the number of mutagenized seeds to be planted on each nylon mesh had to be

determined. This was to ensure that as many candidates can be scanned in efficient manner while making sure that each root can be identified individually and transferred to soil.

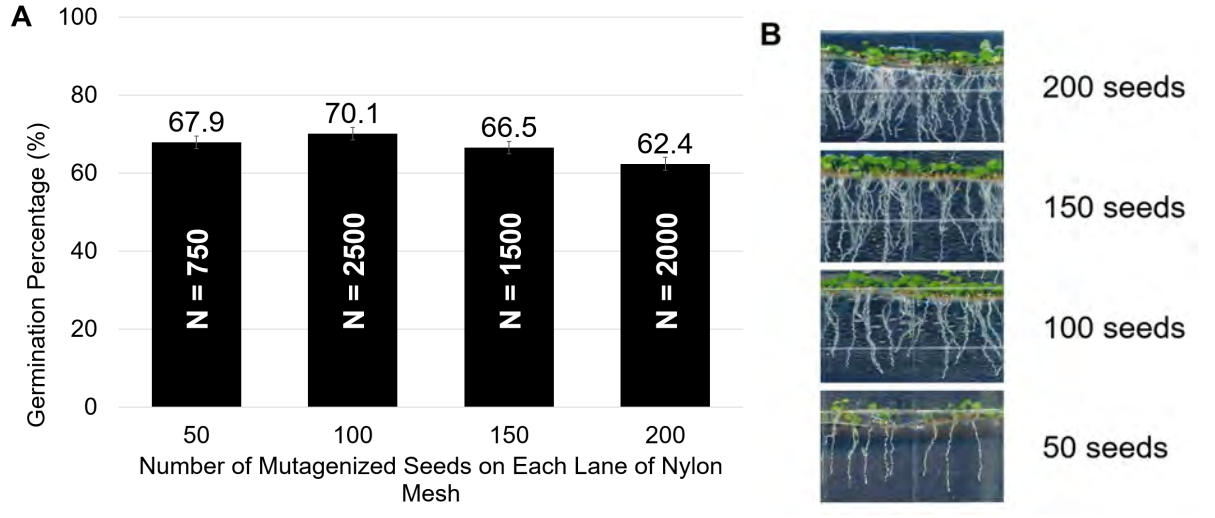


Figure 14. Germination Percentages of EMS-treated Col-0 (Mutagenized) *A. thaliana* Seeds. Different numbers of EMS-treated Col-0 *A. thaliana* seeds were planted on each lane of nylon mesh. Seeds were stratified for 3 days, germinated and grown for 5 days on MS agar medium plates without DETA/NO. After the 5 days of growth, each seedling was transferred to new plates containing MS agar medium and 1 mM DETA/NO. **(A)** Germination percentages were collected 5 days after seeds were transferred to DETA/NO-containing plates. The x-axis indicates the number of mutagenized seeds planted on each nylon mesh. The y-axis shows the germination percentage. Error bars signify standard error. **(B)** Example pictures of each lane of nylon mesh with different numbers of mutagenized seeds.

Four different numbers of mutagenized seeds (50, 100, 150, 200) were planted along the nylon mesh strips for testing. The germination percentages of were estimated to be substantially similar for all samples (Figure 14A). However, a higher number of seeds led to crowding of seedlings, making it challenging to identify individual roots because of crowding and tangling. This made it difficult to count roots, measure root length, or select seedlings for transfer to soil for

the later steps of the experiment (Figure 14B). Because all four different numbers of seeds planted on the nylon mesh strips resulted in similar germination percentages and it was easier to identify the individual seedling roots when 50 seeds were planted, this number was selected as the optimal planting density.

Screening M_2 Mutagenized Seeds

Once the concentration of MES, DETA/NO, and the planting density were determined, the core part of this project, mutant screening, was initiated. As explained in the introduction, the *A. thaliana* mutagenized seeds screened were the M_2 generation (Figure 6). EMS-treated, mutagenized M_2 seeds of *A. thaliana* were screened using the conditions that were established in the experiments described above (1.0% MES, 1.0 mM DETA/NO, 50 mutagenized seeds on each nylon mesh lane). Candidate mutants for M_3 confirmation screening were selected by comparing mutagenized seedling root length with WT root length (Figure 15). The mutagenized seedlings with significantly longer roots than WT were selected and rescued to soil. The selected candidates were grown on soil for the rest of their lifecycle and allowed to self-fertilize.

M_3 Screening

At the end of the lifecycle of candidate mutants that were selected from M_2 screening, their self-fertilized, M_3 progeny seeds were collected. The newly collected M_3 generation seeds were then rescreened using the same conditions used for screening the M_2 generation with DETA/NO stress. This was to test if the phenotype observed in M_2 generation is present in M_3 generation.

Out of 63 rescued plants, only 7 were ready to be used for M_3 generation screening before completion of this thesis. However, one of the 7 plants did not produce seeds, therefore, only seeds

from 6 different rescued M_2 plants were collected. Those seeds were labeled as #13222, 13223, 13223, 13224, 13225, 13226, and 13228. Out of these 6, 5 did not show any root length difference compared to WT. Therefore, it was concluded that they were false positives from the M_2 generation screening. Only M_3 seeds from batch #13226 showed potential to be a mutant altered in response to NO. Some of roots of seedlings grown on 1.0 mM DETA/NO showed significantly longer root length compared with WT seedlings on the same plate (Figure 16). In addition, on some replicates of the assay, some M_3 seedlings showed more root branching than WT. Retesting of #13226 M_3 seeds is warranted to confirm that this line shows altered response to NO treatment.

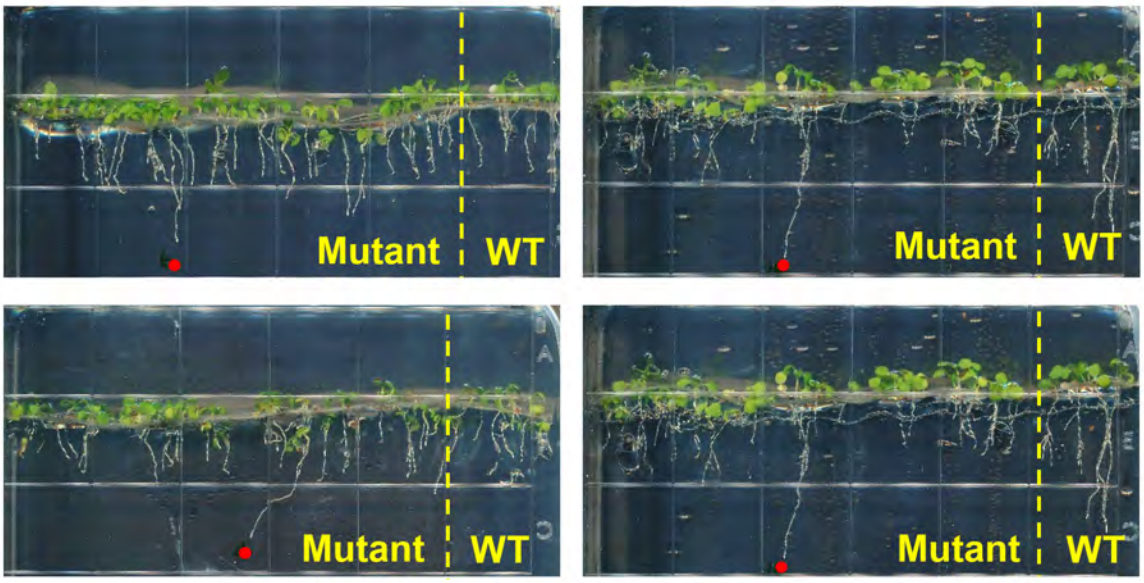


Figure 15. Selected Mutant Candidates from Screening M_2 Seeds. M_2 seeds were grown on 1.0 mM DETA/NO for 5 days, and their root lengths were compared with WT unmutagenized seedlings on the same plate. The seedlings with significantly longer roots compared to WT seedlings were rescued and transferred to soil for confirmation of the phenotype in the M_3 generation. This experiment was repeated multiple times, and out of 6,750 M_2 seedlings that were screened, 63 were selected and rescued for M_3 screening.

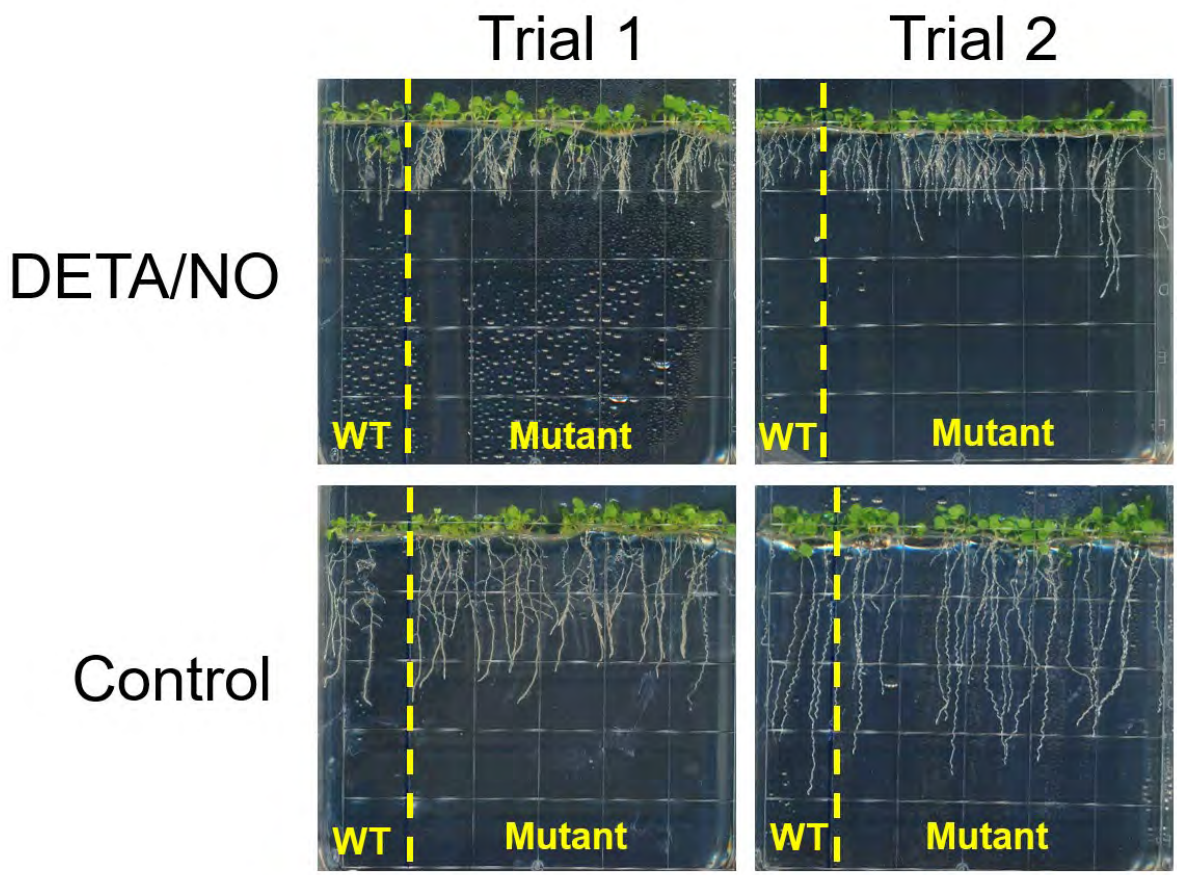
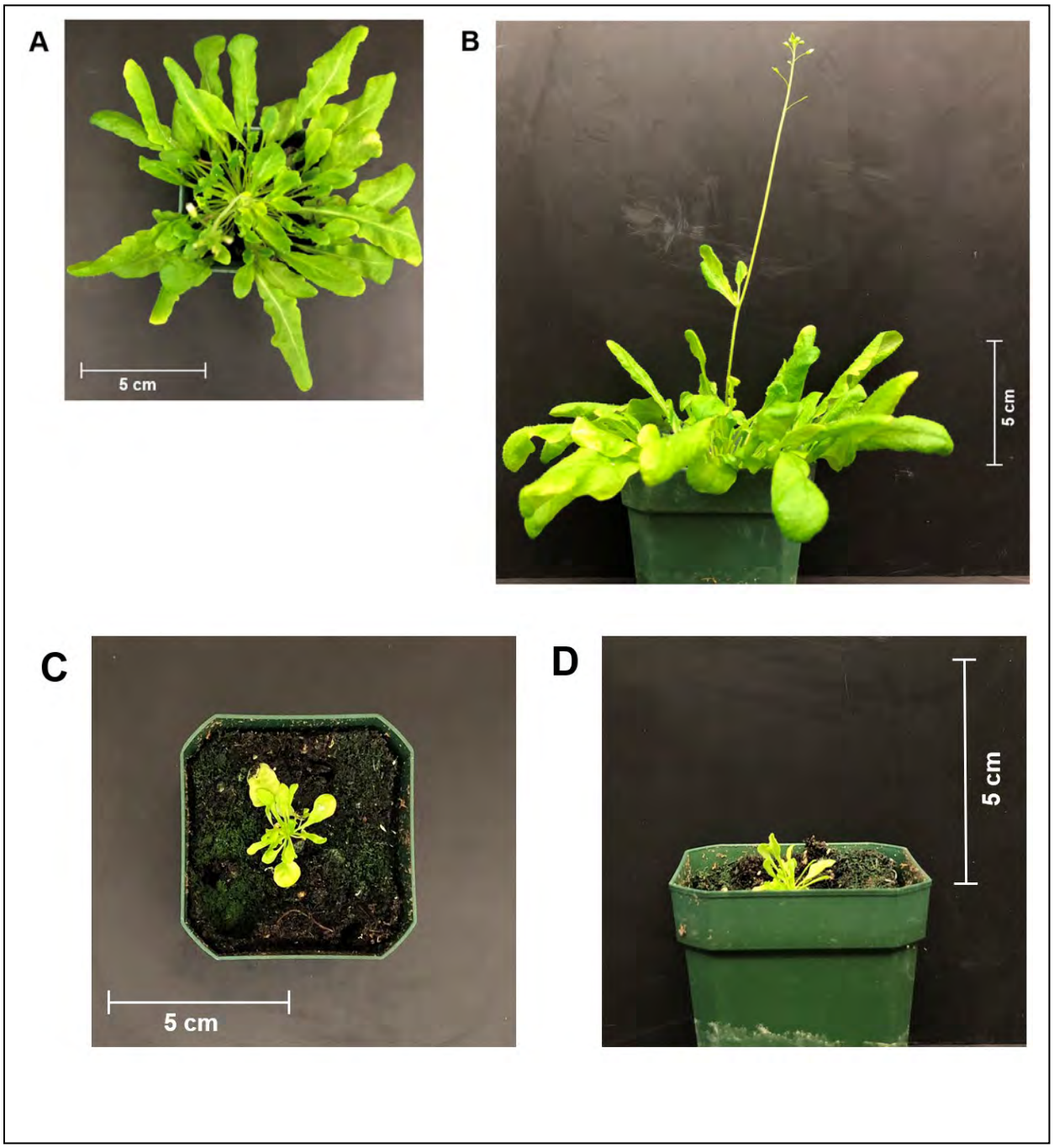


Figure 16. Results of M_3 Screening of Seed Batch #13226. Seed batch #13226 was harvested from one of the 7 rescued, self-fertilized M_2 seedlings that showed increased root length on DETA/NO. Seeds from #13226 were planted and grown on 1.0 mM DETA/NO to confirm the longer root length phenotype, observed in the M_2 generation. Seeds shown above on either control or DETA/NO plates were planted initially on MS agar medium without DETA/NO during stratification and germination. At the end of the stratification and germination periods, seeds on control plates were transferred to newly made MS-agar-media-containing plates, and the seeds on DETA/NO plates were transferred to newly made plates containing MS agar medium with 1.0 mM DETA/NO. This experiment was repeated one more time.

Observed Abnormal Phenotypes of Rescued M_2 Plants

After the rescued M_2 plants were transferred to soil in individual pots and fully grown, it was observed that 4 of 96 rescued plants showed abnormal phenotypes that were not directly related to the root length or height of the plant (Figure 17).



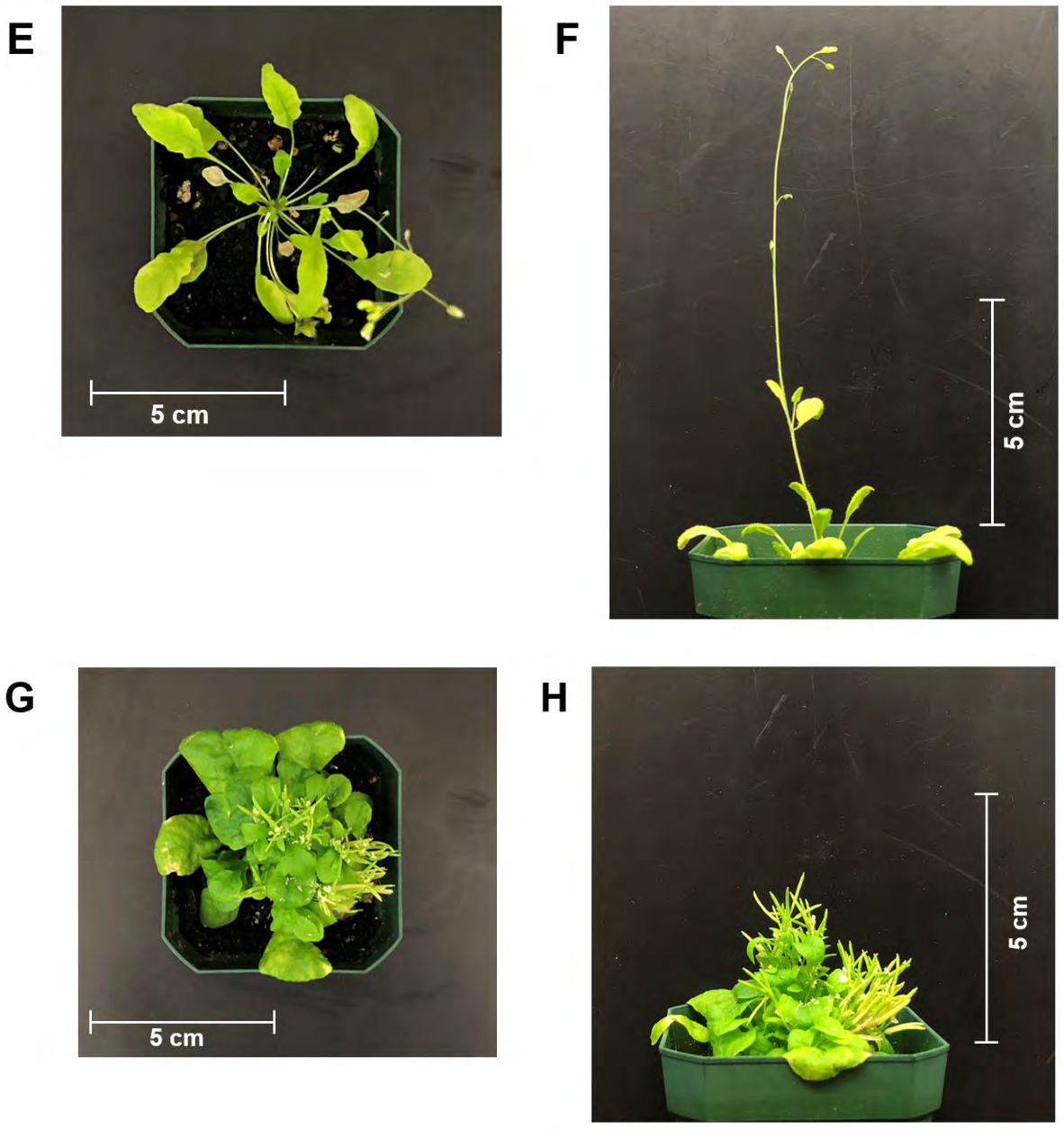


Figure 17. Interesting Phenotypes Observed from Rescued M_2 Plants. 4 out of 96 rescued M_2 plants from mutant screening after the 1 mM DETA/NO treatment showed abnormal phenotypes. Pictures were taken on May 8, 2019. **(A)** and **(B)** are pictures of the same plant, which was rescued transferred to soil on February 9, 2019. **(C)** and **(D)** are pictures of the same plant that was rescued transferred to soil on February 18, 2019. **(E)** and **(F)** are pictures of the same plant that was rescued transferred to soil on March 2, 2019. **(G)** and **(H)** are pictures of the same plant that was rescued transferred to soil on March 7, 2019.

Confocal Microscopy of NO Contents Using DAF-FM

The goal of this project was to screen for mutants that are involved in NO homeostasis through exposing mutagenized Col-0 *A. thaliana* seeds to 1 mM DETA/NO. For the purpose of confirming that the established experimental conditions were valid, the NO content of *Arabidopsis* roots in multiple conditions was analyzed using confocal microscopy.

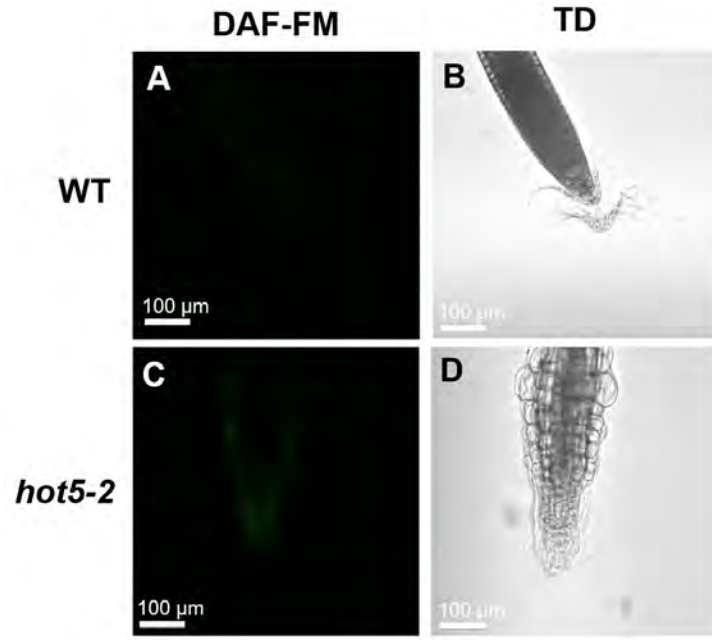


Figure 18. Pictures of WT and *hot5-2 A. thaliana* roots dyed with DAF-FM diacetate to visualize NO contents under the confocal microscope. WT and *hot5-2 A. thaliana* roots were planted on non-DETA/NO-containing MS agar medium plates for six days, treated with DAF-FM diacetate, and visualized using a confocal microscope. **(A)** Picture of the WT root in the DAF-FM channel. **(B)** Picture of the WT root in the transmitted light (TD) channel. **(C)** Picture of the *hot5-2* root in the DAF-FM channel. **(D)** Picture of the *hot5-2* root in the TD channel.

WT and *hot5-2* roots were imaged after staining the roots with DAF-FM (Figure 18). DAF-FM is a membrane permeable dye that can be hydrolyzed by intracellular esterases. It remains non-fluorescent until it reacts with intracellular NO to form a fluorescent heterocycle, and it fluoresces when excited under the microscope at excitation and emission wavelengths of 495 and 515 nm (Xie and Shen, 2012). By using DAF-FM, NO content in the roots of plants can be visualized by examining how much the roots fluoresce. WT and *hot5-2* (plant with complete loss-of-function mutation in the gene which encodes GSNOR, an enzyme that reduces NO (Lee et al., 2008)) were stained

with DAF-FM to visualize their NO content. It was expected that the NO content of *hot5-2* roots would be higher and show more fluorescence than WT. Both the WT and *hot5-2* roots were grown on MS agar medium without DETA/NO for six days, and in the DAF-FM channel, it can be observed that the NO content in the roots of *hot5-2* was significantly higher than in the roots of WT (Figure 17). Transmitted light (TD) channel at Figure 18B and 18D show structures of roots observed under the microscope.

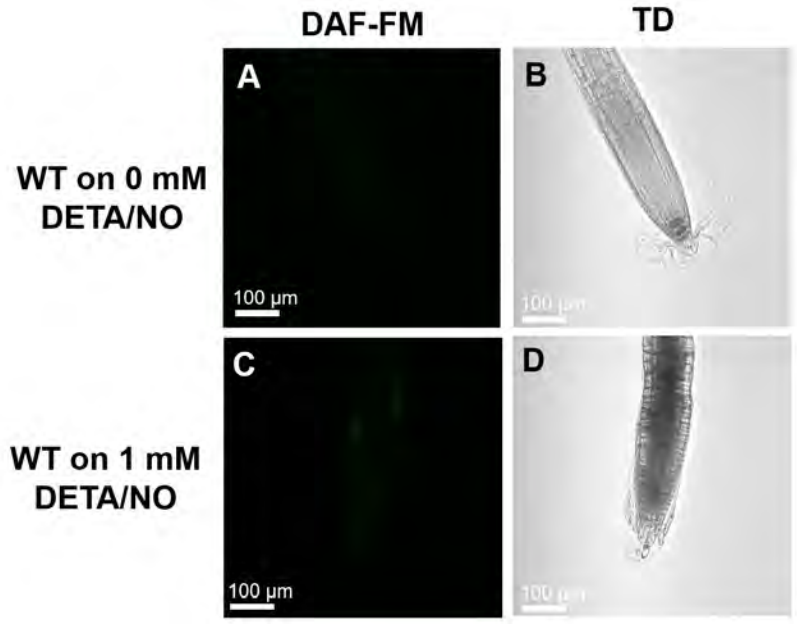


Figure 19. Confocal microscopy of WT *A. thaliana* roots grown on different concentrations of DETA/NO. WT *A. thaliana* roots were planted on 0 or 1 mM DETA/NO-containing MS agar medium plates for six days, treated with DAF-FM diacetate, and visualized using a confocal microscope. **(A)** WT root grown on 0 mM DETA/NO visualized in the DAF-FM channel. **(B)** WT root grown on 0 mM DETA/NO-containing MS agar medium visualized in the TD channel. **(C)** WT root grown on 1 mM DETA/NO-containing MS agar medium visualized in the DAF-FM channel. **(D)** WT root grown on 1 mM DETA/NO-containing MS agar medium visualized in the TD channel.

NO content in WT roots grown on 0 and 1.0 mM DETA/NO-containing MS agar medium was visualized as well (Figure 19). As it can be observed in the Figure 18A and 18C, NO content in the roots of WT grown on 1 mM DETA/NO is higher than the ones grown on 0 mM DETA/NO.

Summary of Screening Data

During this project, 10 different trials of M_2 screenings and 5 different trials of M_3 screenings were completed (Tables 2 and 3). A total of 12,528 M_2 seeds were screened, and 96 of them were selected as M_3 candidates. Out of those 96, 65 survived and 7 were ready to be screened in the M_3 generation before completion of this project. However, only 6 of the rescued plants produced seeds, therefore only 6 were screened for M_3 screening. 3,000 seeds from the 6 rescued plants were further screened, and only one (seed batch # 13226) appeared to be the possible mutant candidate (Figure 15).

Table 2. Summary of M_2 Screenings.

Trial #	Date	Seed Batch #	Generation	# of Mutagenized Seeds Screened	# of Rescued Plants	# of Survived Rescued Plants
4	2018/10/22-2018/10/27	Patrick EMS 2/8	M_2	540	0	
5	2018/10/31-2018/11/05	Patrick EMS 2/8	M_2	540	0	
6	2018/11/12-2018/11/18	Patrick EMS 2/8	M_2	540	0	
7	2018/11/20-2018/12/02	Patrick EMS 7/8 & Patrick EMS 8/8	M_2	108	7	7
8	2018/09/17-2018/10/12	#13133, 13134, 13135, 13136, 13137	M_2	~1,800	9	0
9	2019/01/28-2019/02/09	#13133, 13134, 13135, 13136, 13137	M_2	2,250	27	18
10	2019/01/31-2019/02/18	#13133, 13134, 13135, 13136, 13137	M_2	2,250	19	14
11	2019/02/04-2019/02/09	#13133, 13134, 13135, 13136, 13137	M_2	2,250	N/A. Discarded due to contamination.	4
12	2019/02/14-2019/03/02	#13133, 13134, 13135, 13136, 13137	M_2	1,500	20	12
13	2019/02/22-2019/03/07	#13133, 13134, 13135, 13136, 13137	M_2	750	14	10
Total Number of M_2 Seeds Screened and Rescued				**12,528**	**96**	**65**

Table 3. Summary of M_3 Screenings Throughout the Experiment.

Trial #	Date	Seed Batch #	Originally Rescued from Which M_2 Trial	# of Mutagenized Seeds Screened
16	2019/03/27-2019/04/04	#13222, 13223, 13224, 13225, 13226, 13228, 13229	7	600
17	2019/04/02-2019/04/15	#13222, 13223, 13224, 13225, 13226, 13228, 13229	7	600
18	2019/04/03–2019/04/16	#13222, 13223, 13224, 13225, 13226, 13228	7	600
19	2019/04/04–2019/04/17	#13222, 13223, 13224, 13225, 13226, 13228	7	600
20	2019/04/05–2019/04/18	#13222, 13223, 13224, 13225, 13226, 13228	7	600
Total Number of M_3 Seeds Screened				**3,000**

Chapter 4. Discussion

Before conducting mutant screenings of the M_2 generation mutant seeds, many conditions regarding the methods for screening had to be determined. The first part of this project focused on determing the MES and DETA/NO concentrations, and the number of seeds to plant on each nylon mesh strip. Once those conditions were determined, the second part of the project, M_2 and M_3 screenings, was performed.

1.0% MES Can Be Used to Buffer the pH Level of MS agar medium

A crucial step in a phenotypic assay is to establish a robust, reproducible protocol for the assay. Assay development in this case included the optimization of several variables. For example, MS agar medium is unbuffered, so the pH level can fluctuate significantly; therefore the addition of the buffering agent MES was tested to stabilize the pH level of the MS agar medium. Stabilizing the pH level of the MS agar medium is important because difference in pH level is a form of abiotic stress. This experiment's goal was to screen for mutants that are involved with NO homeostasis by exposing the mutagenized seeds to severe NO stress, therefore other forms of abiotic stress had to be eliminated.

In the beginning stage of the experiment, the concentration of DETA/NO had not yet been determined. Therefore, four concentrations of MES (0.05, 0.5, 1.0, 1.5%) were tested along with multiple concentrations of DETA/NO. The tested concentrations of DETA/NO were 0, 1, 2, and 5 mM. The reason for testing out multiple concentrations of DETA/NO during the process of determining the MES concentration is that the optimal concentration of DETA/NO to be added to the MS agar medium had not yet been determined. The appropriate amount of DETA/NO for the screen was initially unknown, because if the DETA/NO concentration was too low, the amount of

NO released would not affect root elongation, but if the DETA/NO concentration was too high, the seedlings would not have survived. In order to confirm that the addition of MES can stabilize the pH level of the MS agar medium regardless of DETA/NO concentration, MES concentrations were tested with different DETA concentrations.

However, before testing each MES concentration with DETA/NO, DETA was used (Figure 12A). The only structural difference between DETA and DETA/NO is the NO attached to DETA. Because the pH scale is affected by the concentration of H^+ or OH^-, the NO addition on DETA/NO would theoretically not affect pH. Because DETA is a significantly more affordable chemical compound than DETA/NO, DETA was used in this test to conserve materials. Once the optimal concentration of MES was determined, the experiment was repeated with DETA/NO to confirm the findings.

The 1.0 and 1.5% MES concentrations showed the most consistent pH with increasing concentrations of DETA (Figure 12A). However, for the purpose of minimizing resources used in future screenings, 1.0% MES was chosen as the concentration for the experiment.

Once it was confirmed with DETA that 1.0% MES would be a sufficient concentration for buffering (Figure 12A), 1.0% MES was tested again for how effective it can be in stabilizing pH levels using DETA/NO (Figure 12B). Both DETA and DETA/NO solutions showed similar patterns of pH level increase as their concentrations increased with no sigfinicant changes, therefore it was concluded that 1.0% MES could be used to efficiently buffer multiple concentrations up to 5 mM of either DETA or DETA/NO.

1.0 mM is the Optimal Concentration of DETA/NO

After the appropriate MES concentration was determined, the optimal concentration of DETA/NO for inhibition of root growth had to be determined. This was done by planting unmutagenized *A. thaliana* Col-0 WT seeds on MS agar plates with four different concentrations of DETA and DETA/NO (0.5, 1.0, 1.5, and 2.0 mM) (Figure 13A). DETA alone was used to confirm that any effects observed with DETA/NO were due to the released NO, not effects of DETA. The results showed that although the average root length of seedlings grown on 2.0 mM DETA was shorter compared to controls (Figure 13), this growth inhibition was minor compared to the effects of the same concentration of DETA/NO.

Based on the results observed from figure 13, it was concluded that 1.5 mM and 2.0 mM DETA/NO concentrations were too high, because not only did the roots barely elongate after transfer (0.04 and 0.01 cm based on Figure 13A), some of the leaves were bleached as well. Because the experiment requires the roots to still grow during the NO-stress, the concentration of DETA/NO could not be too high to the point that no additional root elongation happens or there is a permanent damage to the seedlings. Therefore, it was initially thought that either 0.5 or 1.0 mM DETA/NO could be used.

To decide whether 0.5 or 1.0 mM DETA/NO would be better for the screen, the average root length over time of seedlings since the beginning of DETA/NO treatment on both 0.5 and 1.0 mM DETA/NO plates from 3 different trials was plotted (Figure 13B). By comparing root elongation on 0.5 and 1.0 mM DETA/NO plates, it was concluded that 1.0 mM would be a better than 0.5 mM for two reasons. First, on 0.5 mM DETA/NO plates the seedlings grew relatively fast; suggesting that the concentration of DETA/NO did not provide significant stress. Second, 1.0 mM

DETA/NO impacted root elongation without killing or bleaching the plants. Because of these findings, it was concluded that 1.0 mM DETA/NO could be used for future screenings.

Increased Level of DETA is A Minor Form of Stress

It is also of note the DETA concentration alone can be a stress factor. As the concentration of DETA increased from 0.5 to 2.0 mM, the average root growth decreased from 2.28 cm to 1.75 cm (Figure 13A). This implies that increasing the concentration of DETA is a stress factor to the seedlings even in the absence of NO. However, this difference was disregarded for the rest of the experiment because its impact on root length was minor, compared to the effect of a similar concentration of DETA/NO.

Less Than 50 Mutagenized Seeds Should Be Planted on Each Nylon Mesh Strip

Once the concentrations of MES and DETA/NO were determined, the number of seeds to be planted on each nylon mesh lane had to be determined. This was done by planting 50, 100, 150, 200 mutagenized seeds with 15 unmutagenized seeds on each lane of nylon mesh and observing their germination percentage (Figure 14).

Measured germination percentages across different controls were relatively similar (Figure 14A). However, higher number of planted seeds on each lane did result in root crowding, making the identification of individual roots very challenging (Figure 14B). This was a prominent problem for multiple reasons. First, accurate germination percentages could not be calculated since individual roots could not be identified. This is also the reason why germination percentages from

Figure 14A are not accurate. Second, because each root could not be identified individually, selecting and transferring possible candidates to soil was difficult since the roots were entangled with each other (Figure 14B).

In contrast, it was easy to identify individual roots on nylon mesh strips that contained 50 seeds. Not only it was easy to identify an individual root, it showed an overall germination percentage of 67.9%, which was the second highest observed germination percentage among 4 different numbers of seedlings planted on a nylon mesh (Figure 14A). Therefore, it was decided that the optimal planting density was 50 mutagenized seeds with 15 unmutagenized seeds.

96 Candidates from M_2 Screening Were Selected

A total of 96 M_2 seedlings were selected as candidates for M_3 screenings. The candidates were individually transferred to soil and were grown fully. As it can be seed from Figure 14, candidates were selected by comparing their root lengths to WT seedlings that were planted on the same plate. Only the seedlings with significantly longer root length were selected for M_3 screenings.

This process of selecting candidates was easy and efficient because the parameter used for was root length only. Other parameters such as color or sizes of leaves, thickness of the roots, and number of lateral roots could have been used for selecting candidates with resistance to NO stress. However, root length was chosen because it is easy to spot seedlings with longer roots. If leaf color was chosen as the parameter for the experiment, a scale of how green the leaves are or a way of measuring the leaf pigment would have been necessary. Another reason is that even when seedlings are crowded candidates with long roots are spotted very easily. However, it is

recommended to screen a smaller number of seeds in one plate since that makes the process of transferring the candidate to soil easier (Figure 14).

Out of 96 selected candidate mutants, a total number of 58 survived on soil and reached the end stage of their lifecycle (Table 1). However, only 7 of the 58 rescued candidates had produced M_3 seeds by the end of this thesis project. Those M_3 seeds were screened under the same conditions as M_2 seeds treated with 1.0 mM DETA/NO, and only one of the 7 selected candidates showed potential for being the mutant of interest (Figure 16).

As it can be seen from Figure 15, two trials using the same M_3 seeds were conducted. Roots on control plates were significantly longer than those on 1 mM DETA/NO plates. Interestingly, in trial 2 seeds planted on the right side of the plate were much longer than those on the rest of the plate. This difference in root length was not observed in trial 1. One possible explanation of this result is that the 1 mM DETA/NO solution was not properly mixed with the MS agar medium when making this plate, therefore some parts of the plate contained less DETA/NO than the rest of the plate. However, this is unlikely because each DETA/NO-containing plates were made by mixing the DETA/NO solution and liquid MS agar medium in a Falcon tube before they were poured into the petri dish. The second possibility is that the DETA/NO-containing plate from trial 2 was placed in the growth chamber in a position where its right side received more light than the rest of the plate. As mentioned in the Methods, every two days the location of each plate was switched to ensure that all the plates get exposed to similar amounts of light ("Seed Stratification and Germination" section from the Methods).

Another thing to note in Figure 16 is the lateral root growth of DETA/NO containing plates in trial 1. Lateral root growth was not observed in trial 2, therefore whether the mutant candidate used for the screening impacts lateral root growth cannot be confirmed until more testing of the

same candidate is done. Previously, it was concluded that increased NO level inhibits the growth of lateral roots (Sun et al., 2017) Since the seedlings are exposed to elevated NO level stress, it is more likely for the lateral root growth to not be observed in this situation. Therefore, it is possible that the lateral root growth was observed because of the mutation, but this cannot be confirmed yet and more future screenings are necessary.

With the 96 rescued M_2 candidates that showed longer root lengths in comparison to other mutagenized seedlings, once they were transferred to soil and were left to grow, some of them showed interesting phenotypes such as abundant leaves or no or little shoot elongation (Figure 17). Whether these phenotypes are related to a mutation that effects root length is unknown and needs to be confirmed with further testing; however, these results prove that the M_2 seeds that are being used for this screening carry mutations.

NO Contents of Different Roots Demonstrate the Validity of the Experiment

This project focused on screening for mutants involved with NO homeostasis by exposing *A. thaliana* roots to NO stress. To confirm that the conditions established for this experiment were valid, NO content of differently treated *A. thaliana* roots were analyzed under the confocal microscope after being stained with DAF-FM.

Results in Figure 18 and 19 are significant for multiple reasons. First, they prove that when WT *A. thaliana* roots are not exposed to DETA/NO, the NO level in the roots are relatively low. The second point is that when the NO concentration of the root environment is increased, it results in an increased NO level in the roots. Roots of WT grown on 1 mM DETA/NO fluoresced significantly more than WT grown on 0 mM DETA/NO (Figure 19A and 19C). The last point is that *Arabidopsis* has an internal mechanism to regulate NO level. When GSNOR is absent, the

roots with that mutation (*hot5-2*) showed a significantly higher NO than the WT, even though both were grown in the absence of DETA/NO (Figure 18A and 18C). This reveals that plants do contain mechanism of NO regulation.

Future Directions and More Discussion

At this stage of the project, no mutant has been confirmed. Therefore, further screening of M_2 plants and screening the rest of the rescued M_3 plants needs to be continued to identify mutants altered in NO homeostasis.

For future screening of the M_3, two modifications are proposed. The first modification is to decrease the number of seeds being planted on each nylon mesh strip. Up to this point, M_3 screenings were conducted by planting 50 mutagenized seeds and 15 unmutagenized seeds. However, those numbers should be decreased to 9 mutagenized seeds being planted on half of the petri dish, and 9 unmutagenized seeds on the other half; less crowded seedlings should produce clearer results since the roots will be more significantly affected by NO stress. The second modification for M_3 screening is to not use nylon mesh strips. M_3 screening is the step where the phenotype observed from the M_2 screenings gets confirmed. Therefore, a closer look at how the roots react to NO stress is important. The primary purpose of using nylon mesh strips is to make it easier to transfer seedlings to new plates. However, a small number of seedlings are easy to transfer using feather-light forceps. Also, by not using the nylon mesh, less damage will be done to the roots, therefore a more controlled testing of the M_3 candidates.

Although mutants have not yet been confirmed in this study, it is interesting to consider what types of genes might be uncovered through this screen. Since mutants are being selected as

less sensitive to NO stress, there is a possibility that the mutated gene could impact the activity of GSNOR or the signaling pathway of NO. The mutation could impact the function of an enzyme, such as GSNOR, that is closely related to the reduction of NO. GSNOR is the enzyme which reduces GSNO, a form of chemical that stores and transfers NO in plant metabolism (Lee et al., 2008). If the mutation was a gain-of-function mutation causing the overexpression of GSNOR, then the level of GSNOR in the cell would increase, therefore decreasing NO inside the cell. If the roots with this mutation were stained with DAF-FM and imaged under the confocal microscope, then the NO-related fluorescence level would be similar to the WT roots grown on 0 mM DETA/NO, but significantly lower than the WT grown on 1 mM DETA/NO.

Another possibility is that a mutation impacts the signaling pathway of NO in cells. There is a very limited knowledge about the NO signaling pathway currently. However, a mutation that increases the NO resistance in plants could be a loss-of-function mutation that inhibits the expression of protein receptors receiving the NO signal. For example, one of the best-studied signaling receptors encoded by the *Arabidopsis* genome is CLAVATA 1 (CLV1), which is one of the 80 identified leucine-rich repeat (LRR) receptor kinases, embedded in the plasma membrane. When the gene encoding CLV1 was silenced, abnormal phenotypes such as production of flowers with extra floral organs or enlargement of both the shoot and floral meristems were observed. CLV1's ligand is a small protein called CLV3, and when the CLV3 is bound to CLV1, the cytosolic domain of CLV1 gets phosphorylated. This causes a conformational change and activates Rho-like guanosine triphosphate-ase (GTPase). There is a limited knowledge about further steps of signaling pathway, however, when the Rho-like GTPase is activated, proper growth of meristems and floral organs of *Arabidopsis* are observed (Alberts et al., 2002). Perhaps a loss-of-function mutation in the gene encoding an equivalent to CLV1 in the NO signaling pathway would

cause problems in NO responses. If this hypothesis is correct, then the mutant root containing this mutation will show high levels of NO under the confocal microscope after being stained with DAF-FM, because even though a high level of NO exists in the roots, it is not being recognized or processed through a properly working signaling pathway.

Observing the ratio of plants that carry or not carry the mutant phenotype (longer root length) in the M_3 seeds from an individual M_2 could reveal information about whether the mutation is dominant or recessive. For example, if the mutation is recessive and the mutant phenotype (longer root length) is caused by a single mutation, then 100% of the M_3 population is expected to show the mutant phenotypes. However, if the phenotype is caused by mutation in a single dominant gene, then either 75% or 100% of the M_3 population will exhibit the mutant phenotype. If 75% of the M_3 population show longer root length, then it can be assumed that the mutation is dominant. Whether 70%, 100%, or any other segregation in M_3 population is observed, the M_3 population will be backcrossed with WT *A. thaliana*. This is for the purpose of determining and confirming whether the mutation is dominant or recessive, but also to dilute out other mutations in the mutant genome that are irrelevant to the longer root length phenotype. If the mutation is dominant, then 100% of the backcross progeny will carry the mutant phenotype, while if it was recessive, then 0% of the backcross progeny will carry the mutant phenotype. However, if the mutant phenotype is caused by mutations located in two genes, then rather than observing the phenotype on 75 or 100% of the population, it will be observed at the 9:3:3:1 ration among M_3 plants due to dihybrid cross. As the number of mutations increase, the ratio of mutant to wild type phenotype gets more complicated. Therefore, whole-genome sequencing will be done using Illumina to efficiently locate the mutation in the entire genome of A. thaliana. Once locations of the mutations are identified, then the gene of interest can be specifically mutated In WT. Using the concept of reverse

genetics, the phenotypes related to the identified mutations can be studied, and more information about NO signaling pathway and other proteins relevant to NO homeostasis in plants will be revealed.

Chapter 5. Conclusion

Through many decades of research, it was concluded that NO is a significant signaling molecule that impacts many physiological functions of both animals and plants. There is still a very limited understanding of NO homeostasis and the NO signaling pathway, especially in plants, and methods of forward genetics applied in this project can broaden our understanding of how NO levels are regulated and perceived in plant metabolism.

This project initially focused on developing methods for screening mutant phenotypes of *A. thaliana* in a sterile environment. Findings from this project include: the benefits of using 1% MES as a buffer; the optimal concentration of DETA/NO for inhibition of root growth; how to utilize nylon mesh strips for growth and transfer seedlings on plates; and the optimal number of seeds to plant on each nylon mesh strips. These findings can be applied to other experiments where careful control of abiotic factors impacting the germination or growth of a plant is necessary.

Once these methods were established, mutagenized seeds from the M_2 generation were screened. 6 M_3 screening candidates were available during this project period, and only one of them could be the possible candidate of interest. However, more careful screenings of the selected M_3 candidate must occur with two modifications mentioned in the discussion sections: decreasing the number of seeds being planted on one plate and eliminating the usage of nylon mesh strips.

As the M_3 screening of the potential candidate continues, the remainder of the 58 M_3 seeds will be screened as well. If no candidates are identified from that pool of M_3 seeds, then M_2 screenings will be continued. Once a successful candidate is identified, its genome will be sequenced using Illumina to identify the location of the mutations which it carries. If there are

multiple mutations, then those mutations will be individually made on WT Col-0 and screened, in order to pinpoint which genetic mutation is the cause of the longer root length phenotype.

Identifying mutants with altered NO homeostasis can reveal information about other possible proteins that are involved with NO regulation, NO signaling pathways, and the unidentified correlation between mutated genotypes and their phenotypes. As this research continues, the modifications suggested above should be made to increase the quality of the M_2 screen and M_3 phenotyping.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Signaling in Plants. In Molecular Biology of the Cell, (New York: Garland Science), 831–906.

Astier, J., Gross, I., and Durner, J. (2018). Nitric oxide production in plants: an update. J. Exp. Bot. *69*, 3401–3411.

Barnett, S.D., and Buxton, I.L.O. (2017). The Role of S-nitrosoglutathione Reductase (GSNOR) in Human Disease and Therapy. Crit. Rev. Biochem. Mol. Biol. *52*, 340–354.

Baudouin, E., and Hancock, J. (2014). Nitric oxide signaling in plants. Front. Plant Sci. *4*, 1–3.

Camejo, D., Romero-Puertas, M. del C., Rodríguez-Serrano, M., Sandalio, L.M., Lázaro, J.J., Jiménez, A., and Sevilla, F. (2013). Salinity-induced changes in S-nitrosylation of pea mitochondrial proteins. J. Proteomics *79*, 87–99.

Chen, Z., Silva, H., and Klessig, D.F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science *262*, 1883–1886.

Cooney, R.V., Harwood, P.J., Custer, L.J., and Franke, A.A. (1994). Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. Environ. Health Perspect. *102*, 460–462.

Cooper, G.M. (2000). Signaling Molecules and Their Receptors. Cell Mol. Approach 2nd Ed.

Crawford, N.M. (2006). Mechanisms for nitric oxide synthesis in plants. J. Exp. Bot. *57*, 471–478.

Domingos, P., Prado, A.M., Wong, A., Gehring, C., and Feijo, J.A. (2015). Nitric Oxide: A Multitasked Signaling Gas in Plants. Mol. Plant *8*, 506–520.

Feechan, A., Kwon, E., Yun, B.-W., Wang, Y., Pallas, J.A., and Loake, G.J. (2005). A central role for S-nitrosothiols in plant disease resistance. Proc. Natl. Acad. Sci. U. S. A. *102*, 8054–8059.

Fitzhugh, A.L., and Keefer, L.K. (2000). Forum: Therapeutic Applications of Reactive Oxygen and Nitrogen Species in Human Disease. Elsevier Sci. Inc *28*, 1463–1469.

Guo, F.-Q., Okamoto, M., and Crawford, N.M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science *302*, 100–103.

Keisham, M., Jain, P., Singh, N., von Toerne, C., Bhatla, S.C., and Lindermayr, C. (2019). Deciphering the nitric oxide, cyanide and iron-mediated actions of sodium nitroprusside in cotyledons of salt stressed sunflower seedlings. Nitric Oxide *88*, 10–26.

Kim, B.H., Kim, C.H., Jung, K.Y., Jeon, B.H., Ju, E.J., and Choo, Y.K. (2004). Involvement of nitric oxide during in vitro fertilization and early embryonic development in mice. Arch. Pharm. Res. *27*, 86–93.

Kumar, D., and Klessig, D.F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. Proc. Natl. Acad. Sci. *100*, 16101–16106.

Kwon, E., Feechan, A., Yun, B.-W., Hwang, B.-H., Pallas, J.A., Kang, J.-G., and Loake, G.J. (2012). AtGSNOR1 function is required for multiple developmental programs in Arabidopsis. Planta *236*, 887–900.

Lee, U., Wie, C., Fernandez, B.O., Feelisch, M., and Vierling, E. (2008). Modulation of Nitrosative Stress by S-Nitrosoglutathione Reductase Is Critical for Thermotolerance and Plant Growth in Arabidopsis. PLANT CELL ONLINE *20*, 786–802.

Lindermayr, C. (2017). Crosstalk between reactive oxygen species and nitric oxide in plants: Key role of S -nitrosoglutathione reductase. Free Radic. Biol. Med.

Lindermayr, C., and Durner, J. (2015). Interplay of Reactive Oxygen Species and Nitric Oxide: Nitric Oxide Coordinates Reactive Oxygen Species Homeostasis. Plant Physiol. *167*, 1209–1210.

Meyerowitz, E., and Somerville, C. (1994). Arabidopsis (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Planchet, E., Jagadis Gupta, K., Sonoda, M., and Kaiser, W.M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. Cell Mol. Biol. *41*, 732–743.

Qiao, W., and Fan, L.-M. (2008). Nitric Oxide Signaling in Plant Responses to Abiotic Stresses. J. Integr. Plant Biol. *50*, 1238–1246.

Qiao, F., Zhang, X.-M., Liu, X., Chen, J., Hu, W.-J., Liu, T.-W., Liu, J.-Y., Zhu, C.-Q., Ghoto, K., Zhu, X.-Y., et al. (2018). Elevated nitrogen metabolism and nitric oxide production are involved in Arabidopsis resistance to acid rain. Plant Physiol. Biochem. *127*, 238–247.

Salinas, Julio, and Jose J. Sánchez-Serrano, eds. *Arabidopsis Protocols*. 2nd ed. Methods in Molecular Biology 323. Totowa, N.J: Humana Press, 2006.

Sega, G.A. (1984). A review of the genetic effects of ethyl methanesulfonate. Mutat. Res. Genet. Toxicol. *134*, 113–142.

Siddiqui, M.H., Al-Whaibi, M.H., and Basalah, M.O. (2011). Role of nitric oxide in tolerance of plants to abiotic stress. Protoplasma *248*, 447–455.

Slaymaker, D.H., Navarre, D.A., Clark, D., del Pozo, O., Martin, G.B., and Klessig, D.F. (2002). The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. Proc. Natl. Acad. Sci. U. S. A. *99*, 11640–11645.

Sun, C.-H., Yu, J.-Q., and Hu, D.-G. (2017). Nitrate: A Crucial Signal during Lateral Roots Development. Front. Plant Sci. *8*, 485.

Wang, Y.-H., Li, X.-C., Zhu-Ge, Q., Jiang, X., Wang, W.-D., Fang, W.-P., Chen, X., and Li, X.-H. (2012). Nitric oxide participates in cold-inhibited Camellia sinensis pollen germination and tube growth partly via cGMP in vitro. PloS One *7*, e52436.

Weigel, D., and Glazebrook, J. (2002). Arabidopsis: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Wendehenne, D., and Hancock, J.T. (2011). New frontiers in nitric oxide biology in plant. Plant Sci. Int. J. Exp. Plant Biol. *181*, 507–508.

Xie, Y., and Shen, W. (2012). In vivo Imaging of Nitric Oxide and Reactive Oxygen Species Using Laser Scanning Confocal Microscopy. Methods Mol. Biol. Clifton NJ *913*, 191–200.

Xu, S., Guerra, D., Lee, U., and Vierling, E. (2013). S-nitrosoglutathione reductases are low-copy number, cysteine-rich proteins in plants that control multiple developmental and defense responses in Arabidopsis. Front. Plant Sci. *4*.

Yamasaki, H., and Sakihama, Y. (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett. *468*, 89–92.

Yetik-Anacak, G., and Catravas, J.D. (2006). Nitric oxide and the endothelium: History and impact on cardiovascular disease. Vascul. Pharmacol. *45*, 268–276.

Yoshimoto, N., Kataoka, T., Maruyama-Nakashita, A., and Takahashi, H. (2016). Measurement of Uptake and Root-to-Shoot Distribution of Sulfate in Arabidopsis Seedlings. BIO-Protoc. *6*.

Yu, M., Lamattina, L., Spoel, S.H., and Loake, G.J. (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. New Phytol. *202*, 1142–1156.

Zhao, M.-G., Tian, Q.-Y., and Zhang, W.-H. (2007). Nitric Oxide Synthase-Dependent Nitric Oxide Production Is Associated with Salt Tolerance in Arabidopsis. Plant Physiol. *144*, 206–217.

Zhou, L., and Zhu, D.-Y. (2009). Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric Oxide Biol. Chem. *20*, 223–230.