Exploring The Potential Of AtENOD93 And *Arabidopsis thaliana* As A Model System To Study ENOD93-like Proteins

by

Nathaniel Secord

A thesis

presented to

The University of Guelph

In partial fulfillment of requirements

for the degree of

Masters of Science

in

Molecular and Cellular Biology

Guelph, Ontario, Canada

ABSTRACT

EXPLORING THE POTENTIAL OF ATENOD93 AND *ARABIDOPSIS THALIANA* AS A MODEL SYSTEM TO STUDY ENOD93-LIKE PROTEINS

Nathaniel Secord **Advisor:**

University of Guelph, 2017 **Professor S. J. Rothstein**

The human population has increased substantially in recent years and is expected to increase further. To feed this population, it is important to develop an agricultural system that is efficient in terms of resource use. Nitrogen is a prominent nutrient for crop productivity and application of nitrogenous fertilizers has allowed for the current population expansion at the expense of damage to the environment. An avenue for developing efficient agriculture may be through the study and generation of crops that utilize nitrogen efficiently, reducing the need for fertilizers. The *OsENOD93-1* gene in rice was found to improve yields under nitrogen-limiting conditions, but other ENOD93 genes exist and warrant further study. In this study, the phenotype of a mutant with the expression of the only Arabidopsis ENOD93 gene knocked-out was characterized and this gene was implicated in the control of the GABA shunt's activity.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisory committee, Dr. Steven Rothstein and Dr. Tariq Akhtar, for providing me invaluable feedback during the experimentation process and while writing this thesis. Secondly, I want to extend a very big thanks to the past and current members of the Rothstein Lab who all played some role in making this thesis possible. Mei, thank you for always being there to go through possible experiments. Bin, thank you for keeping everything organized and functioning in the lab and managing the ordering of reagents so efficiently - it really helped. Jose, Kashif, Kosala and Zhen Hua, thank you for being there to bounce ideas off of and providing suggests. Gabriella, you were a tremendous help in the initial stages of the experiments. Ping, you worked so very hard to help the last few experiments come together. I'm sorry we had to go through those long days for so many months, but know that it is very much appreciated.

I'd also like to thank all of those behind the scenes who provide funding opportunities for students. I was fortunate to receive funding from the Natural Sciences and Engineering Research Council of Canada and the Ontario Graduate Scholarship program and it was a huge help in dealing with the ever-rising costs of living and tuition.

Lastly, thank you to my amazing wife, Elaine. It hasn't been easy spending our first years of marriage both as graduate students, but your support and understanding made it drastically easier than it could have been. I've been so blessed to have you walk with me through this journey and look forward to countless more.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ADP	Adenosine Diphosphate
Ala	Alanine
ATP	Adenosine Triphosphate
cDNA	Complementary DNA
Cit	Citrate
DAS	Days After Sowing
ENOD93	Early-Nodulin 93
ETC	Electron Transport Chain
GABA	γ-aminobutyric acid
GABA-P	GABA Permease
GABA-T	GABA Transaminase
GAD	Glutamate Decarboxylase
GAT1	GABA Transporter 1
GFP	Green Fluorescent Protein
Gln	Glutamine
Glu	Glutamate
GOGAT	Glutamine Oxoglutarate Aminotransferase
GS	Glutamine Synthetase
Isocit	Isocitrate
MS	Murashige and Skoog Basal Salts
NADH/NAD+	Nicotinamide Adenine Dinucleotide

NUE	Nitrogen Use Efficiency
PCR	Polymerase Chain Reaction
Pyr	Pyruvate
RT-PCR	Real Time-Polymerase Chain Reaction
SALK	Salk Institute for Biological Studies
SE	Standard Error
SSA	Succinate Semialdehyde
SSADH	SSA Dehydrogenase
Succ	Succinate Semialdehyde
TAIR	The Arabidopsis Information Resource
TCA	Tricarboxylic Acid Cycle
T-DNA	Transfer DNA
YFP	Yellow Fluorescent Protein
α-KG	α-Ketoglutarate

CHAPTER 1. INTRODUCTION

According to the United Nations, Department of Economic and Social Affairs, Population Division (2013), the population of human beings on Earth is predicted to increase to 9.6 billion by the year 2050 and then 10.9 billion by 2100. Whether these predictions come to fruition is dependent on a number of factors, including policies related to child birth, the economics of the world and culturally related family size preferences, but they do underscore the need to prepare our infrastructure to cope with population increases. One extremely important aspect of global infrastructure that is often overlooked is that of agriculture. Simply put, if the world is to sustain the predicted population growth while maintaining or even enhancing an individual's quality of life, it will likely be necessary to increase the yield of crops in a limited quantity of arable land (Bi et al., 2009). This concept is supported by the prediction that yields of crops worldwide will have to double in the next 50 years to sustain the expected population, while arable land per person is expected to decrease, despite an increase in total arable land (Rothstein, 2007; Alexandratos and Bruinsma, 2012). Such a feat will require the improvement of multiple aspects of crop production, including enhancements to agronomic practices and crop genetics, all of which will require considerable research efforts.

While there are a number of aspects of plant growth that can be examined to improve yields, one that may prove to be of great benefit to, at least in part, address the above challenges is the improvement in crop nitrogen use efficiency (NUE) (Fischer et al., 2013). Although NUE can be defined in various ways, Good et al. (2004), it is generally considered to be a measure of a plant's ability to uptake, utilize and remobilize nitrogen that is in its environment (Mcallister et al., 2012). Efficient uptake, use and remobilization of all nutrients are important to minimize the

waste of inputted fertilizers. However, nitrogen has the unique feature of being the main factor controlling plant growth and productivity, with only water deficiency having a larger effect (Morot-Gaudy and Lea, 1997). Indeed, insufficient nitrogen leads to a number of symptoms that ultimately impair crop yields, including diminished biomass production, increased chlorosis, wasting resources to facilitate increase root exploration and issues with reproduction, such as seed development (Fischer et al., 2013). Altering the use of particular crops and the practices to grow them to support a growing population is hardly a new concept. In fact, with the onset of the Green Revolution in the 1960s, large amounts of chemical nitrogenous fertilizers started to be applied to crops, which ultimately produced gains in yield (Mulvaney et al., 2009). Such a practice has often been poorly managed, with excessively applied fertilizers leading to both economic waste and, more importantly, serious environmental damage due to nitrogenous compounds polluting both air and water (Cassman et al., 1993).

Nitrogen can be taken up by plants from the soil in three main forms: nitrate (NO_3^-), ammonia (NH_3) or urea (CH_4N_2O) (Fischer et al., 2013). However, the majority of the chemical fertilizer is derived from the Haber-Bosch process (shown below), which uses nitrogen gas, hydrogen gas, high temperature and high pressure to form ammonia (Marschner, 1995).

$$N_2 + 3\ H_2 + \text{Heat} + \text{Pressure} \rightarrow 2\ NH_3$$

Due to the requirements of high pressure and temperature, this process consumes about 1% of the world's energy supply (Smith, 2002). This fact is problematic, considering that ~87% of energy produced is non-renewable and ~82% is derived through the burning of fossil fuels (World Energy Council, 2013). Furthermore, natural gas is a major component of the process, raising concerns of increased fertilizer demand spurring the harvesting of natural gas through

environmentally damaging means, such as fracking (Engelder et al., 2011; Agriculture and Agri-foods Canada, 2013) Currently, natural gas accounts for 70-90% of ammonia production costs (Agriculture and Agri-foods Canada, 2013). With chemical nitrogen fertilizers constituting ~8% of Canadian farmers operating expenses, a dependence of the farming industry on these fertilizers is linked to economic difficulties for farmers and consumers today and in the future, especially should prices of energy and natural gas increase (Agriculture and Agri-foods Canada, 2013). These concerns are exacerbated by the fact that many farmers apply chemical nitrogen fertilizers in a prescribed fashion to maximize yields without considering current soil nitrogen content and time of year (Glass, 2003). The result can be excessive nitrogen content in the soil as uptake by plants is fairly inefficient, with cereals only showing ~33% of the applied nitrogen being taken up into the plants (Raun and Johnson, 1999). Moreover, at least in rice and maize, efficiency of nitrogen uptake decreases with addition of nitrogen fertilizers past a certain point (Cassman et al., 2002).

The application of chemical nitrogen fertilizers to crops also poses a serious environmental threat. Due to the aforementioned inefficiencies in plant nitrogen uptake, significant amounts of these fertilizers remain in the soil (Kant et al., 2011). This remaining nitrogen has several fates. Approximately 19% is lost via denitrification and uptake by soil microorganisms (Good and Beatty, 2011). Roughly 20% is processed by soil microbes to generate nitrous oxide, a known greenhouse gas that has been linked to climate change, and ozone depletion (Ravishankara et al., 2009; Wuebbles, 2009; Ivens et al., 2011). As well, about 20% of the remaining nitrogen can leach into water systems (Good and Beatty, 2011). Firstly, this provides another source for nitrous oxide production as microbes within marine environments have been shown to produce this compound like their terrestrial cousins (Cole and

Caraco, 2001). Secondly, the surplus nitrogen in water systems can lead to the excessive growth of phytoplankton and algae, which can produce an array of toxins, increase turbidity, and cause oxygen depletion, ultimately killing certain marine wildlife (Rabalais et al., 2002). Despite these consequences, world nitrogenous fertilizer consumption has increased over time and is expected to increase further (Tilman, 1999; Heffer and Prud'homme, 2014). Therefore, by discovering new ways of improving the NUE of crops, it may be possible to mitigate some or all of the above issues as yields could support the population's needs while reducing dependency on chemical nitrogen fertilizers.

One way to achieve such an improvement in NUE is to understand the underlying molecular features that are attributed to plant nitrogen uptake, assimilation and remobilization. A number of these features have already been identified and in fact have been altered in attempts to improve NUE (Fischer et al., 2013). However, focusing these discoveries on pathways that are present in agriculturally important crops, such as rice, may prove useful in staving off interim food shortages (Food and Agriculture Organization of the United Nations, 2014). One such molecular target in rice which appears to mediate NUE belongs to the class of proteins referred to as Early-Nodulin 93 and is the subject of this study (Bi et al., 2009).

In the pages that follow, an examination of the underlying principles of nitrogen uptake, assimilation and remobilization will be discussed. Second, a background on the discovery of genes encoding Early-Nodulin 93 proteins and the current state of knowledge of these genes will be presented. Finally, the results of a project aimed to study these genes in more detail will be described.

1.1 NITROGEN FIXATION, UPTAKE AND ASSIMILATION

Molecular nitrogen gas is quite abundant in the Earth's atmosphere, comprising 78% of the total, with a mass of 4×10^{18} kg (Sanhueza, 1982). Despite this abundant store of nitrogen, plants are unable to utilize it directly (Kraiser et al., 2011). First, it must be fixed to ammonia through processes collectively referred to as nitrogen fixation (Postgate, 1998). A chemical process involving lightning is one such process, in which energy from the lightning converts atmospheric nitrogen gas to nitrogen oxides and eventually nitrate (Hill et al., 1980). The nitrate can then be transported to the soil by rain droplets (Sanhueza, 1982). The second process, which constitutes the largest contribution to the biosphere, is carried out by soil prokaryotes, collectively called diazotrophic organisms (Lovell et al., 2000; Lodwig and Poole, 2003). All of these organisms utilize a family of enzymes called nitrogenases to fix atmospheric nitrogen to ammonia, with each member of the family containing different metal cofactors and having different structures (Clarke et al., 2014). In all cases, though, a nitrogenase will catalyze the following general, ATP-dependent, chemical reaction (Igarashi and Seefeldt, 2003):

$$N_2 + 8e^- + 16\ ATP + 8H^+ \rightarrow 2NH_3 + 16\ ADP + 16\ Pi + H_2$$

Plants can utilize the fixed nitrate and ammonia present in the soil by first taking them up into root cells via specific transporters. In the case of nitrate, transporters belonging to the family of NRT transporters are utilized (Masclaux-Daubresse et al., 2010). Ammonia, on the other hand, is taken up by ammonia transporters (Masclaux-Daubresse et al., 2010; Wang et al., 2012). Once in root cells, nitrate can be reduced to nitrite in the cytoplasm by nitrate reductase and then nitrate to ammonia in plastids by nitrite reductase (Suzuki and Knaff, 2005). Whichever way ammonia is generated or taken into the plant, it is eventually assimilated into glutamine and then other amino acids in a series of enzymatic reactions that take place

throughout the plant cell, generating and using organic acids in the process (Suzuki and Knaff, 2005; Mcallister et al., 2012). Of these amino acids, some are remobilized from one part of the plant to another during processes that signal amino acid or nitrogen transport, such as away from leaves during their senescence and towards seeds during their development (Pate, 1973; Rolletschek et al., 2005; Masclaux-Daubresse et al., 2010).

1.2 γ-AMINOBUTYRIC ACID AND CARBON-NITROGEN BALANCING

During normal plant growth and response to stressors it is vital that the ratio between carbon and nitrogen (C:N ratio) be maintained (Weigelt et al., 2008; Zheng, 2009). At the center of the maintenance of this ratio are the amino acids generated after the assimilation of nitrogen into glutamine, one of which is γ-aminobutyric acid (GABA) (Weigelt et al., 2008). This compound's interaction with C:N ratio balancing was supported by the fact that high levels of ammonium and amino acids forced a redistribution of it in various tissues, including in developing seeds (Fait et al., 2008; Weigelt et al., 2008; Ariz et al., 2013; Batushansky et al., 2014; Li et al., 2016). Furthermore, the GAD mutant constitutively produced GABA from glutamate, an amino acid that is a precursor for many other amino acids, suggesting that GABA production can alter amino acid homeostasis (Baum et al., 1996). Also, diurnal shifts in metabolite concentrations have revealed a link between GABA levels and glutamate levels, supporting the notion that GABA can act as a transient storage form of carbon and nitrogen (Masclaux-Daubresse et al., 2002). A series of glutamate receptors have been discovered in Arabidopsis (Lacombe et al., 2011). Inhibiting the expression of one of these, AtGLR1.1, provided evidence for its role in C:N ratio regulation as the transcripts levels for a series of enzymes relating to the C:N ratio were altered in response (Kang and Turano, 2003). Thus, alterations in glutamate levels caused by GABA metabolism may signal C:N maintenance

pathways. Additionally, in both *A. thaliana* seedlings and *Brassica napus* L., exogeneous GABA induced increased nitrate uptake, with the expression of a nitrate transporter being up-regulated in the latter (Beuve et al., 2004; Barbosa et al., 2010). These results imply that GABA is not only important in maintaining C:N but has a role in nitrogen uptake regulation as well.

Since many organic acids derived from amino acid break-down are also synthesized in the TCA cycle and glutamate can be generated from the cycle, GABA's interaction with the cycle in this regard is particularly important (Gauthier et al., 2010). The way by which GABA interacts with the TCA cycle is called the GABA shunt, which is demonstrated in Figure 1. The levels of GABA in a cell can be regulated through three pathways. Firstly, cytosolic glutamate, generated in plastids by the assimilation of nitrogen from the environment, can be used by one of five glutamate-decarboxylase enzymes (GAD1-5) to form GABA in a non-reversible reaction (Shelp et al., 2012). GAD activity is controlled by the Ca^{2+}/CaM binding domain in proportion to the concentration of Ca^{2+} ions in the cytosol (Baum et al., 1996). The entire pathway is still unclear, but evidence from Yu et al. (2006) suggests that there are GABA binding sites on the plasma membrane which can activate an influx of Ca^{2+} ions into the cytosol. Secondly, the *A. thaliana* GAT1 (GABA transporter 1) transporter was shown to transport extracellular GABA into the cytosol when constitutively expressed in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes void of their native GABA transporters (Meyer et al., 2006). What's more, it was discovered that the transporter localized to the plasma membrane when expressed in tobacco protoplasts, suggesting that plant cells are capable of transporting GABA from the apoplast to their cytoplasm (Meyer et al., 2006). Lastly, AtALDH10A8 and AtALDH10A9, a set of enzymes belonging to the Arabidopsis aldehyde dehydrogenase 10 family of proteins, was shown to affect levels of GABA and plants' abilities to respond to salt stress (Zarei et al., 2016).

The first protein is localized to plastids and is involved in the pathway responsible for generating GABA from arginine (Zarei et al., 2016). The latter is involved in a similar pathway but is found in peroxisomes and uses spermine as a substrate (Zarei et al., 2016).

GABA produced within the cell or transported into the cell can enter the mitochondria through a recently discovered GABA-permease (GABA-P), where it is utilized by GABA-transaminase (GABA-T) to form succinate-semialdehyde (SSA) and alanine, while transferring an amino group to pyruvate (Renault et al., 2010; Michaeli et al., 2011; Renault et al., 2011). Subsequently, the SSA is used to form succinate by succinate-semialdehyde-dehydrogenase (SSADH), generating NADH from NAD^+ in the process (Bouché et al., 2003). Succinate is an intermediate compound in the TCA cycle (Ludewig et al., 2008). Thus, the GABA shunt allows the TCA cycle to continue in situations where intermediate compounds are low, such as when photosynthesis is impaired due to low light conditions (Michaeli et al., 2011). Additionally, the NADH generated in the final step can be used by the electron transport chain to generate a proton gradient across the mitochondrial inner-membrane and eventually ATP (Bouché et al., 2003). At the same time, the alanine generated by the shunt can be used as an amino-donor to regenerate glutamate from α-ketoglutarate (Gauthier et al., 2010). However, the α-ketoglutarate generated from the TCA cycle may also be transported to plastids, where it can be used to facilitate the assimilation of nitrogen from the environment to glutamate. The importance of this process, especially under stressful conditions, is underscored in numerous studies that modify the activity or presence of GAD, GABA-P or GABA-T. The unregulated over-active GAD mutant mentioned earlier continued to pool GABA in the cytosol, as the only way of metabolizing GABA is through the shunt (Baum et al., 1996). This lead to stunted growth as glutamate was being redirected from use elsewhere (Baum et al., 1996). As well, GABA-P mutants were not

able to cope with stresses affecting the TCA cycle, such as low light and the absence of added sucrose, and thereby exhibit reduced growth (Michaeli et al., 2011). Also, mutations in GABA-T resulted in plants with shorter roots and reduced chlorophyll content compared to wild-type plants when subjected to exogenous GABA (Renault et al., 2011). Lastly, the *ssadh* mutant exhibited dwarfed growth, indicating that the final step of the shunt, where GABA "meets" the TCA cycle is vital to the shunt's function (Ludewig et al., 2008).

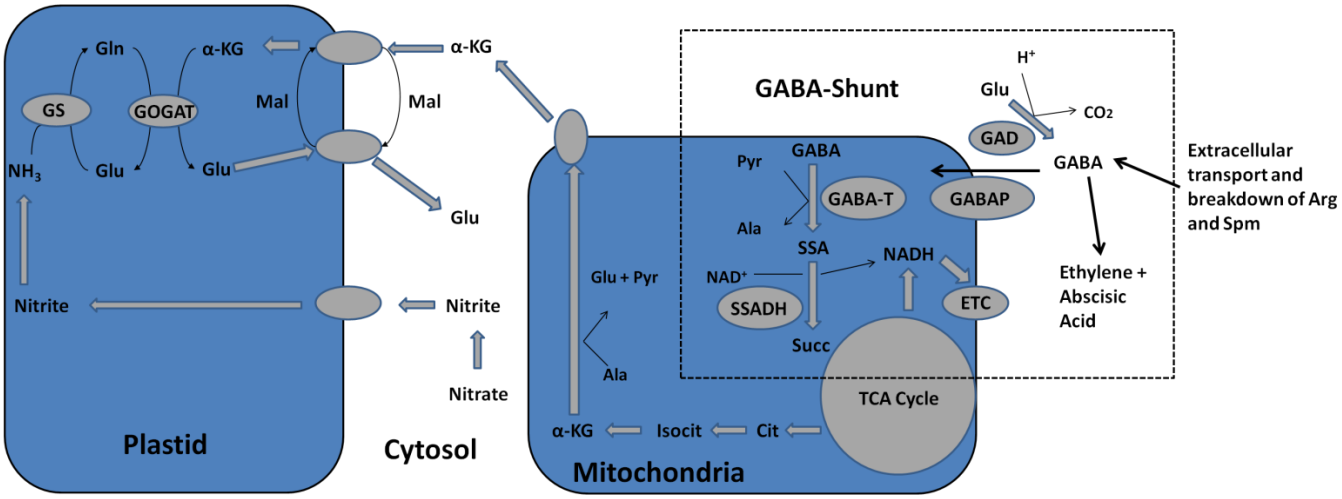


Figure 1. The GABA shunt and its interactions with some key processes in mitochondria and plastids.

GABA also appears to cause the alteration of expression of genes within plants, with some suggesting it acts as a signaling molecule in the truest sense, while others believe expression changes are caused by resulting metabolic changes (Batushansky et al., 2014; Ramesh et al., 2015). Regardless of how GABA exerts changes in the transcriptome, production of GABA or exogenous GABA has been shown to alter expression of genes involved in nitrogen uptake (increased), cell wall expansion (decreased) and the synthesis of the phytohormones abscisic acid and ethylene (increased) (Shi et al., 2010; Renault et al., 2011). The latter is

important as abscisic acid and ethylene can subsequently alter the expression of amino acid, ammonium and nitrate transporter genes, thereby linking GABA to nitrogen availability signaling (Kiba et al., 2011).

1.3 ROOT NODULATION

Some diazotrophs freely live in the soil, without ever entering the roots they surround, while others can colonize plant tissue and fix nitrogen within the plant (Perin et al., 2006; Coelho et al., 2009). Others still, may live freely in the soil initially, but can infect plant roots and form a symbiotic relationship within plant-derived structures called nodules, providing the plant with fixed nitrogen and receiving organic acids in exchange (Pawlowski and Bisseling, 1996; Lodwig and Poole, 2003). One group of these symbiotic diazotrophs is that of *Rhizobium*, which typically infect plants of the *Leguminosae* family (legumes) (Lodwig and Poole, 2003). Only one case of a non-legume plant forming a nodule with *Rhizobium* has been recorded, and that is in the *Parasponia* genus (Appleby et al., 1983; Op den Camp et al., 2012). Another group of bacteria that can form nodules is that of the genus *Frankia* which infects a variety of non-legume plants jointly call actinorhizal plants (Benson and Silvester, 1993). During the nodule formation event, plant genes are induced after receiving chemical signals from the bacteria (Verma et al., 1992). These induced genes are classified based on their temporal expression, being classified as early-nodulins when expressed during infection and nodule organogenesis and late-nodulins when expressed during or after the release of bacteria from the infection thread but before nitrogen fixation occurs (Verma et al., 1992). It is in the nodule structures of soybeans that the particularly interesting early-nodulin gene, *GmENOD93*, was discovered (Kouchi and Hata, 1993).

1.4 EARLY NODULIN-93-LIKE PROTEINS

Kouchi and Hata (1993) began their experiments with the goal of discovering which genes were differentially expressed in nodule root cells versus their non-nodule counterparts. They did so through subtractive cDNA hybridization screening of libraries and were able to identify 20 nodulin genes specifically expressed in nodule cells. Out of the 20, 4 were newly discovered and *GmENOD93* was one of these (Kouchi and Hata, 1993). The cDNA did not hybridize to the *Bradyrhizobium japonicum* genomic DNA, confirming that the source of the gene was indeed the plant cell and not of the infecting bacteria. A Northern blot analysis did not yield signal for uninfected leaves, stems, and roots or infected root segments 4 days after sowing and inoculation with *B. japonicum* (Kouchi and Hata, 1993). However, the same analysis showed expression in nodules 8, 10, 13 and 17 days after sowing and inoculation and they designated the gene as an early-nodulin gene (Kouchi and Hata, 1993). The cDNA of *GmENOD93* was generated and it's sequence analyzed to determine the amino acid sequence of the protein, which was noted as being 105 amino acids long and having a high percentage of alanine and serine residues - 22.9 and 10.5%, respectively (Kouchi and Hata, 1993). An *in situ* hybridization was used to determine the localization of transcripts in the nodule at various time points (Kouchi and Hata, 1993). Firstly, 5 days after sowing and inoculation, a weak signal was apparent at the site of nodule primordia, near the point of infection, indicating a role in nodule initiation. Signals from nodules 8 days after inoculation were concentrated in the nodule meristem, suggesting the gene has a part in nodule growth. After 14 days, the signal was only present in infected cells. Interestingly, the authors noted that nitrogen fixation had begun at this point, while meristematic activity was decreased markedly in the nodule, and they postulated that the signals in the primordia and meristem were signs of early bacterial infection rather than

relating to nodule development (Kouchi et al., 1990; Hata et al., 1991; Kouchi and Hata, 1993). However, due to the prevalence of genes induced within uninfected plant cells by bacterial infection of the root, it seems entirely plausible that *GmENOD93* could be induced during nodule development without directly interacting with the bacteria (Carvalho et al., 2013).

Later, Reddy et al. (1998) used documented rice expressed sequence tags that were similar to *GmENOD93* to screen a rice genomic library and isolate homologues of the soybean gene. In doing so, two genes were found and designated *OsENOD93a* and *OsENOD93b* (Reddy et al., 1998). Sequencing of the cDNA showed that the open reading frame for *OsENOD93a* and *OsENOD93b* had 69.4% and 49.5% similarity to *GmENOD93*, respectively (Reddy et al., 1998). Subsequent amino acid predictions based on these sequences, determined the amino acid sequences of the two had 77.4% and 52.4% similarity to the soybean gene, respectively, and that there was a highly conserved region in the three proteins (Reddy et al., 1998). Unlike *GmENOD93*, which was only expressed in developing soybean root nodules, a Northern blot revealed that *OsENOD93a* was expressed at low levels in roots and slightly more in etiolated leaves, but not at all in green leaves, whereas *OsENOD93b* was expressed strongly in roots and less in etiolated and green leaves (Reddy et al., 1998). As well, the blot indicated expression of both genes in rice suspension culture, signifying some function in undifferentiated and/or dividing tissues in rice and providing evidence for the notion that *GmENOD93* expression in the meristem could be related to this tissue's activity (Reddy et al., 1998).

Shortly after this study, it was discerned that an actinorhizal plant, *Datisca glomerata*, contains a homologue of *GmENOD93*, referred to as *Dg93*, through screening of a *D. glomerata* nodule cDNA library (Okubara et al., 2000). Using database searches, the nucleotide sequence of *Dg93* was shown to be 83% identical to *GmENOD93* and the protein products 74% identical

(Okubara et al., 2000). While the predicted polypeptide of *Dg93* was less similar to *OsENOD93a* and *OsENOD93b* (44 and 40% identity, respectively), it did share a central conserved region with the rice and soybean proteins (Okubara et al., 2000). Unlike the rice genes, but similar to the soybean gene, *Dg93* expression was only present in nodules as early as 4 days after inoculation with *Frankia* and not in other plant tissues (Okubara et al., 2000). *In situ* hybridization was performed on developing nodules to measure temporal and spatial expression of *Dg93*. Conversely to soybeans, meristematic activity persisted in actinorhizal nodules and there was strong signal in the meristem of nodules 6 months after inoculation (Pawlowski and Bisseling, 1996; Okubara et al., 2000). These results support the idea that *ENOD93*-like genes can have functions in undifferentiated cells, as outlined above. During the same time point, signal appeared in the pericycle, the phloem and xylem vessels, meaning the gene may have a role in nutrient transport, vascular tissue development and/or long-distance signaling (Okubara et al., 2000). As well, signal was produced in the periderm and within mature infected cells (Okubara et al., 2000). Alternatively, nodules 1 month after inoculation showed signal only in a the newly infected cells, but not the mature infected cells, and in inner layer of periderm cells only (Okubara et al., 2000). However, at both time points signal was detected in the meristem and vascular bundles (Okubara et al., 2000). Okubara et al. (2000) noted that the predicted polypeptide of *Dg93* shared sequence identity with a number of proteins from non-legume/actinorhizal plants other than rice, including a maize protein which was 73% identical.

Indeed, the massive amount of genomic, transcriptomic and proteomic data collected and inputted into databases has shown that ENOD93-like proteins are common in the plant kingdom. The basis for classification as an ENOD93-like protein is centered on the conserved regions noted in the genes mentioned above. This conserved region has been designated the ENOD93

domain and analysis of the Pfam online protein database currently yields 99 sequences in 28 species that contain the domain (Finn et al., 2014). However, only 6 of these sequences are part of a group of identified genes that are situated very close to one another on chromosome 6 of rice (*Oryza sativa japonica*, specifically), as detailed in Table 1, and that have similar sequences (Schwacke et al., 2003). In this table, *OsENOD93a* corresponds to *Os06g4990*. *OsENOD93b*, on the other hand, corresponds to a gene on chromosome 2, classified as *Os02g0227200*. An alignment of their cDNA sequences shows prominent similarities in their structures (Figure 2). This fact raises concerns when considering the previously mentioned spatial and temporal expression patterns as it is uncertain if the probes used are reading only the transcript of interest, or a number of very similar transcripts. Moreover, the sequence similarities complicate any micro-array data produced as it is uncertain if the probes are truly gene-specific. It is very important that all micro-array discussed be considered in light of this. With that said, it is still clear that *ENOD93*-like genes are expressed and likely serve some function in plants. As well, the alignment of the predicted proteins for each transcript, in Figure 3, emphasizes the large similarities between these genes.

Table 1. Structural properties of ENOD93-like rice genes and proteins

Locus Name	CDS Coordinates on Chromosome 6[c]	Predicted Protein Length[a]	Number of Transmembrane Domains Predicted[b]	AramLocCon Chloroplast Value[d]	AramLocCon Mitochondria Value[d]	AramLocCon Secretory Pathway Value[d]
Os06g04940.1	2176926 - 2175882	139	2	7.8	5.2	9.2
Os06g04940.2 (alternate splice)	2176926 - 2175882	116	2	5.0	13.5	1.7
Os06g04950	2181116 - 2180198	115	2	3.4	14.7	2.0
Os06g04990	2200544 - 2199242	115	2	4.2	15.3	1.9
Os06g05000	2206534 - 2205577	115	2	3.3	14.2	1.7
Os06g05010.1	2209562 - 2208673	116	2	3.9	13.7	1.5
Os06g05010.2 (alternate splice)	2209562 - 2208673	100	2	1.9	7.2	13.0
Os06g05020.1	2213563 - 2212543	115	2	4.2	15.4	1.7
Os06g05020.2 (alternate splice)	2213563 - 2212543	110	2	4.7	18.4	1.6

[a](Schwacke et al., 2003)

[b](Arai et al., 2004)

[c] (Ouyang et al., 2007)

[d] (Schwacke et al., 2007)

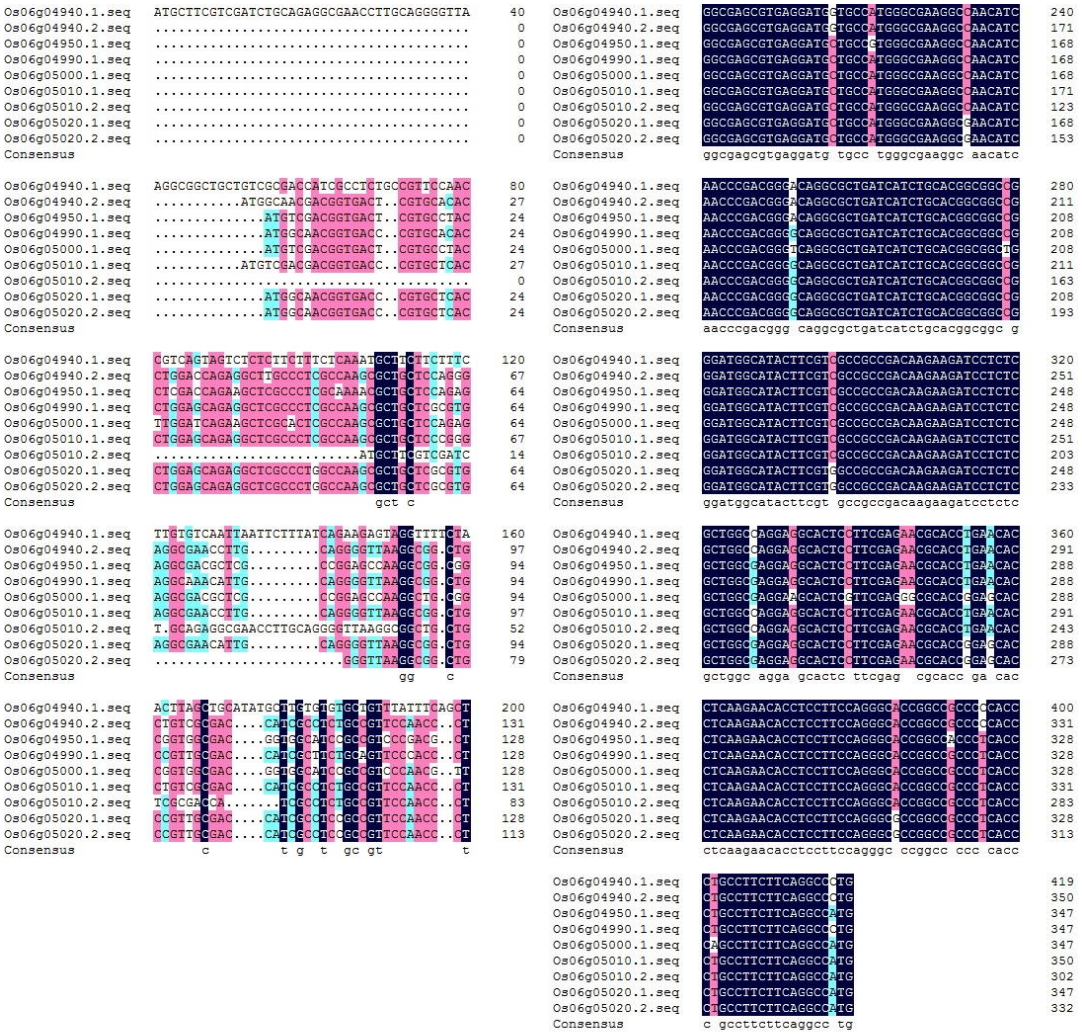


Figure 2. Alignment of transcripts generated from the OsENOD93 genes using DNAMan software. Dark blue indicates high level of consensus, pink less consensus, light blue even less consensus and white very low to no consensus. Sequence goes from to top left to bottom right, moving to the top left of each subsequent section. The labels refer to the last 6 characters of the locus names from Table 1.

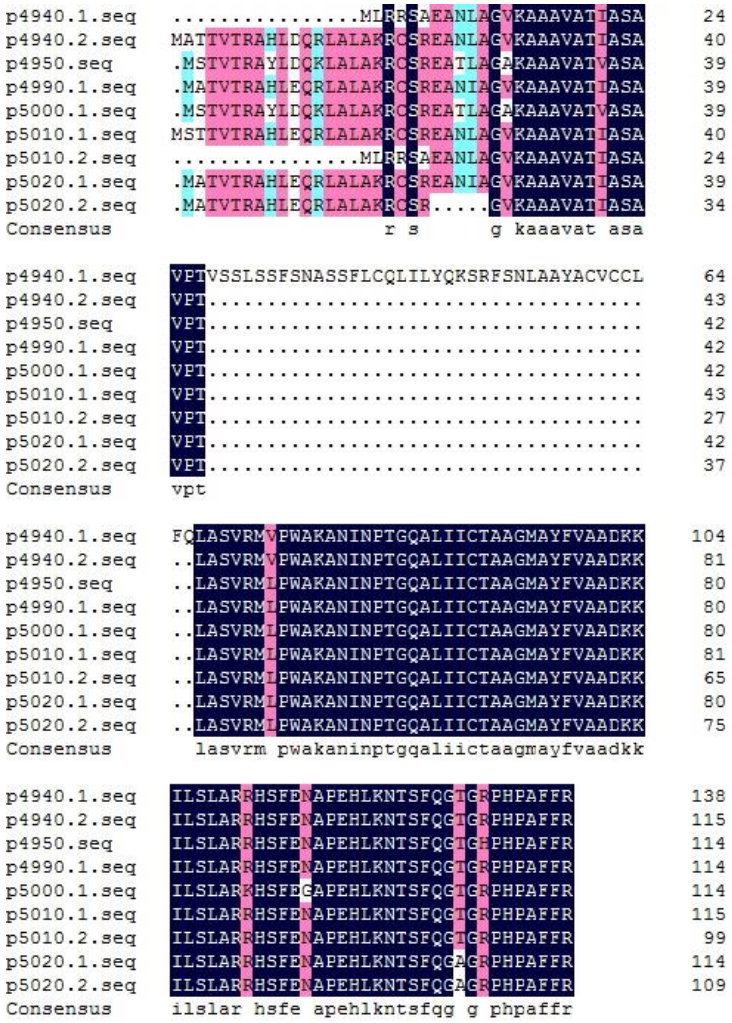


Figure 3. Alignment of the amino acid sequence of the proteins predicted to be generated from *OsENOD93* gene using DNAMan software. Dark blue indicates high level of consensus, pink less consensus, light blue even less consensus and white very low to no consensus. The labels refer to the last 6 characters of the locus names from Table 1.

One of the six genes, *Os06g05010* (designated *OsENOD93-1*), was studied in some depth by Bi et al. (2009). A micro-array transcriptomic analysis of rice experiencing nitrogen limitation stress revealed drastic increases in expression of the gene when moved from a high nitrogen to low nitrogen environment (reduction) and from a low nitrogen to high nitrogen environment (induction) (Bi et al., 2009). It was noted that other genes found to respond significantly to reduction and induction were those known to take part in "...nitrogen and carbon metabolism; transportation of nitrate, ammonium, amino acid and growth hormones; signal transduction, and transcriptional regulation" (Bi et al., 2009). These findings supported the notion that this gene, and possibly the other *OsENOD93*'s, is important in the plant response to fluctuations in nitrogen levels. What is more, the fact that the expression increase was so great under induction *and* reduction may provide evidence that this gene is involved in maintaining some form of homeostasis in rice, such as the C:N ratio. Data from a transcript expression profile of genes at various developmental stages showed a large increase in expression of the gene in the root during panicle emergence and lesser expression increases in the root during tillering, panicles during booting, mature stems, inflorescences and in seeds after anthesis (Zhu et al., 2003). These data may substantiate the concept that the gene is involved in a response to nitrogen needs as the reproductive stage is quite nitrogen intensive (Hirel et al., 2007). When provided with 3 mM of nitrate, a value shown to limit growth, lines over-expressing *OsENOD93-1* had greater biomass, higher number of spikes and spikelets, and greater seed yield by weight than the wild-type plants (Bi et al., 2009). As well, the number of spikes and spikelets, and seed yield by weight were greater than wild-type plants grown in the presence of 10 mM nitrate, an amount shown to maximize growth (Bi et al., 2009). Evidence for how this may come about is found in the fact that total amino acid levels per fresh weight in roots were

higher in over-expression lines than the wild-type 60 days after sowing (DAS) and grown at 3 and 10 mM nitrate (Bi et al., 2009). At the same time points and conditions, the percentage of nitrogen per dry weight remained steady (Bi et al., 2009). Thus, it seems as though *OsENOD93-1* is involved in the generation of amino acids, but in a coordinated fashion so as to balance the nitrogen levels with plant growth. As mentioned earlier, the GABA pathway and the TCA cycle are heavily involved in the balancing of C:N and it may be that *OsENOD93-1* interacts with these to regulate the ratio. Indeed, a protein localization experiment performed by Bi et al. (2009) showed that OsENOD93-1 fused with YFP was situated at the membrane of or within mitochondria of onion epithelial cells, implying a role for this protein in the aforementioned process. Through an *in situ* hybridization experiment in roots, it was found that transcription of the gene occurred in the epidermis, endodermis and vascular bundle of roots (Bi et al., 2009). This observation along with the fact that amino acid concentrations in xylem sap was higher in over-expression lines grown at 1 and 3 mM nitrate led to the theory that this protein is involved in the transport of amino acids to vascular tissue (Bi et al., 2009). On the basis of the sub-cellular localization data collected by Bi et al. (2009), the involvement would not be one of direct transport across the plasma membrane, but could be in the regulation of other signals to increase amino acid transport. As well, it could simply be that the gene increases amino acid levels in roots, which could inherently lead to greater xylem transport, especially in response to low-nitrogen conditions. Regardless of how this protein product is involved in cell metabolism, the improvements in growth and yield certainly make *OsENOD93-1* a candidate gene for use in improving NUE.

The other five *OsENOD93* genes may prove useful candidates as well. Expression analysis of these six genes, based on transcriptomic data available through Rice eFP Browser,

indicate that they were generally found in the same tissues at the same time points and conditions, although not always to the same degree (Winter et al., 2007). Firstly, data from (Jain et al. (2007) outlined that expression of all six genes occurred during numerous stages of inflorescences development, with expression dropping drastically when pollen vacuolates. While not having nearly the levels as in inflorescences, expression of the genes occurred in seeds throughout development as well (Jain et al., 2007). Also, a very low level of expression of the six genes occurs in the shoot apical meristem, in seedling roots and mature leaves, but almost none was present in young leaves (Jain et al., 2007). Data from Li et al. (2007) showed that expression of all the genes occurs in reproductive tissues as well, with notable expression in embryos and the endosperm. These expression data indicate that the genes may be involved in seeds development and, keeping in mind the improved yields witnessed by Bi et al. (2009), regulating how nitrogen integrates into this stage of plant development. As well, expression for all genes occurred in suspension culture, confirming and expanding on the data from Reddy et al. (1998), which showed the same for two *OsENOD93* genes (Li et al., 2007). However, the role of these genes appears to extend beyond dealing with nitrogen-limitation stress. That is to say, data from Lasanthi-Kudahettige et al. (2007) and Jain et al. (2007) demonstrated that all of the genes were expressed at greater levels compared to the controls during anoxic, drought and salinity stresses. This may mean that the genes are involved in management of the C:N ratio under these stress conditions, as was theorized for nitrogen stress earlier. It was discussed above that phytohormones have a part in signaling nitrogen availability, and these processes may be a link between how the *OsENOD93* genes are activated during nitrogen stress. Transcriptomic data obtained by Hirose et al. (2007) showed that all of the genes are up-regulated in leaves and roots 30 minutes and 120 minutes after exposure with zeatin, a cytokinin, with the exception of

Os06g04940, which was only up-regulated in the roots. Likewise, these genes are potential candidates to study for NUE improvements based on the predicted sub-cellular localizations in Table 1, which outlines that the five genes other than *OsENOD93-1* also have at least one protein product that is likely to localize to mitochondria. The two protein products that aren't predicted to localize to the mitochondria (from *Os06g04940.1* and *Os06g05010.2*) are much different in size from the others and have greater possibilities of entering the secretory pathway, with an unknown fate. In addition, the sequence similarities of the genes and their generated proteins, outlined in Figures 2 and 3, highlight their shared candidacy as genes to study to improve NUE.

1.5 ARABIDOPSIS EARLY-NODULIN 93

While the analysis of the *OsENOD93* genes holds promise to improve the NUE of rice, the number of genes and their similarities pose challenges to understand their biochemical function. Firstly, it becomes impossible to develop primers that will only target individual transcripts when assessing the genes' responses to environmental conditions. Secondly, any reduction in expression of specific active OsENOD93 proteins through mutations or transgenic event would likely be masked by the genetic redundancy of the other OsENOD93s. What's more, growing rice requires a fair amount of space, takes a long time to mature and requires specialized and time-consuming techniques to transform. These challenges are not restricted to rice, however. In fact, as shown in Table 2, other important crops that take time and space to grow and are difficult to genetically manipulate have multiple ENOD93-like genes.

Table 2. Agriculturally important species of plants that produce proteins containing the ENOD93 domain

Species	Number of Proteins
Tomato (*Solanum lycoperscium)*	4
Banana (*Musa acuminata*)	5
Corn (*Zea mays*)	4
Grape (*Vitis vinifera*)	2
Rice (*Oryza sativa*)	7
Thale Cress (*Arabidopsis thaliana*)	1

(Schwacke et al., 2003)

Notable from Table 2 is the fact that *Arabidopsis thaliana* only produces one ENOD93-like protein from locus *At5g25940* (henceforth referred to as AtENOD93). Upon examining the sequence of the AtENOD93 protein, it was found that both it and OsENOD93-1 share much of the same central sequence, only varying to any extent at the C- and N-terminals (shown in Figure 4). As well, as demonstrated in Figure 5, both proteins have two similarly positioned hydrophobic regions, predicted to be transmembrane domains. What's more, AramLocCon predicts AtENOD93 localizes to the mitochondria as it did for OsENOD93-1 and a series of proteomics data place the former in mitochondrial membranes (Schwacke et al., 2003; Brugière et al., 2004; Heazlewood et al., 2004; Nikolovski et al., 2012).

```
p5010.1.seq     ..............MSTTVTRAHLEQRLALAKRCSREANLA      27
pa25940.seq     MENRSEMGNRRQQNLFVIASPDEIAKIHRAETSKQAGAIA      40
Consensus                                       a         a

p5010.1.seq     GVKAAAVATIASAVPTLASVRMLPWAKANINPTGQALIIC      67
pa25940.seq     GAKAAAVAAVASAIPTVAAVRMFPWAKANLNYTAQALIIS      80
Consensus       g kaaava  asa pt a vrm pwakan n t qalii

p5010.1.seq     TAAGMAYEVAADKKIISLARRHSFENAPEHLKNTSFQGTG     107
pa25940.seq     SASIAAFEITADKTIIQGARRNTEAQLKKVQQDSK.....     115
Consensus        a   a f  adk il  arr

p5010.1.seq     RPHPAFFR                                     115
pa25940.seq     ........                                     115
Consensus
```

Figure 4. Alignment of OsENOD93-1 (top sequence) and AtENOD93 (bottom sequence) amino acid sequence.

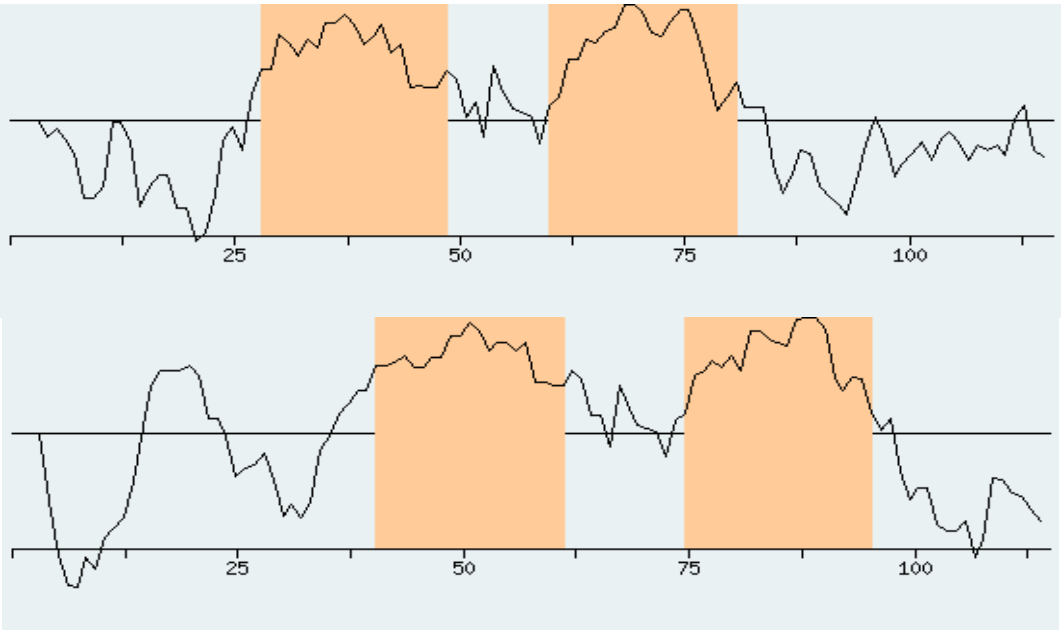


Figure 5. Alignment of OsENOD93-1 (top) and AtENOD93 (bottom) hydrophobic regions predicted to be transmembrane domains (Arai et al., 2004).

Readily available transcriptomic data sets of *A. thaliana* developmental stages and under stress conditions also yielded similarities between *AtENOD93* and the *OsENOD93* genes. According to data collected by Schmid et al. (2005), expression of *AtENOD93* occurred in all tissues during development, but, as with *OsENOD93s*, was up-regulated in floral tissues and in seeds. Furthermore, *AtENOD93-1* showed the same high expression during embryo development (Casson et al., 2005). While detailed transcriptomic data of roots at various developmental stages as was shown in Bi et al. (2009) is not available, *AtENOD93* appears to be expressed in the roots, with the highest levels in the procambium (Brady et al., 2007). Furthermore, as with the *OsENOD93s*, *AtENOD93* was shown to be up-regulated during hypoxia stress (Branco-Price et al., 2005). Application of the hormone zeatin, a cytokinin, to wild-type plants resulted in increased expression of *AtENOD93*, as with the *OsENOD93s* (Kiba, 2004). Additionally, an even greater increase was found when a line over-expressing ARR22, a stress response regulator involved in signal transduction, was treated with zeatin, providing evidence for *AtENOD93'*s involvement in modulating stress response (Kiba, 2004).

1.6 OBJECTIVES AND HYPOTHESIS

The similarities in structure and possible overlap in localization and expression patterns between the *OsENOD93s* and the single *AtENOD93* gene suggest a conservation of function. The presence of only one ENOD93-like gene, together with the vast array of molecular and genetic resources that are available, could make *A. thaliana* an ideal species to examine the function of the *OsENOD93s* genes. The aim of this project was to develop a series of parameters to define the effects of a knock-out mutation in *AtENOD93* on phenotype and its metabolic function, so that this information might be used in the future to examine the *OsENOD93s* genes as well as other ENOD93-like genes in important crops that could have an impact on agricultural

yields. If enough similarities are found, it could be possible to use *AtENOD93* to more easily test hypotheses and then extend those that show promise to individual *OsENOD93*s.

As discussed earlier, GABA plays a central role in modulating the C:N balance of plants, during normal growth, under stressful conditions and during seed development, a function that is directly linked to the mitochondria and the effectiveness of the GABA shunt (Shelp et al., 1999; Weigelt et al., 2008). Also discussed was the possible function of *OsENOD93-1* being involved with this C:N maintenance. Moreover, as mentioned, the higher expression of the *OsENOD93* genes and *AtENOD93* follows situations where GABA would be important, such as during stress and seed development. Therefore, it was hypothesized that AtENOD93 functions by modulating the effectiveness of GABA use in the GABA shunt at the mitochondria, whereby a decrease in AtENOD93 would result in decreased GABA shunt activity.

CHAPTER 2. MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds were obtained from the Arabidopsis Biological Resource Center. The T-DNA line was designated SALK_204202 and was in the Columbia-0 background (Alonso et al., 2003). Wild-type seeds were received from TAIR as well. When planted on soil, seeds were stratified in distilled water for 48 hours and then spread on LB2 nutrient-free soil (SunGro Horticulture Canada Ltd., BC, Canada) in standard 20" x 10" trays and then thinned to contain 12 plants each. Plants were grown in a growth chamber maintained under a 16 hour photoperiod (150 µmol/m^2 of light using a combination of cool white fluorescent and incandescent lamps) at 21°C during the day and 18°C during the dark, with a consistent relative humidity of 60%. Following a recipe based on that used by Peng et al. (2007), after 1 week, trays were given 2 L of nutrient

solution containing 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 50 μM Fe-EDTA, 25 μM H_3BO_4, 6 μM $MnSO_4$, 0.5 μM $ZnCl_2$, 0.5 μM $CuSO_4$, 0.1 μM Na_2MoO_4. For minimal nitrate, 0.5 mM KNO_3 was added and for maximal nitrate, 5 mM KNO_3 was added. Another 2 L of nutrient solution was added weekly thereafter, only providing reverse-osmosis water when trays were dry and always adding the same volume to each tray when necessary.

When planted in hydroponics, a method based on Tocquin et al. (2003) was employed. In this, 1.5 mL centrifuge tubes were cut in half and filled with 0.65% agar. The tubes were then placed in holes cut out in the lids of 15 L plastic bins covered in thick black duct tape (24 per bin), with a piece of plastic screen on the bottom to prevent the agar from sliding out. The bins were filled to the top with MiliQ water to ensure the agar remained in contact and was moist. Sterile seeds were applied to each agar tube using a sterile toothpick. The chamber was then set to 4°C and 24 hours of dark at 70% relative humidity for 48 hours, after which it was changed to the settings used in soil-grown plants, but relative humidity was kept at 70% to help the agar remain moist. Plants were thinned to 1 per agar tube after a week and nutrient solution added. Stock nutrient solutions were used to yield final concentrations of 10 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 μM H_3BO_4, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4. For minimal nitrate, 0.3 mM KNO_3 was used and for maximal nitrate, 3 mM KNO_3 was used, based on concentrations used in Bi et al. (2007). The nutrient solution was removed and renewed every 2 weeks after the first week. Seeds were sterilized by shaking for 10 mins in 70% ethanol and 0.05% Triton-X. The solution was removed and seeds were allowed to sit for 5 mins in 100% ethanol and then placed in fresh ethanol for an additional 3 mins. Seeds were allowed to dry on sterile filter paper in a flow hood and kept in sterile microfuge tubes. Only the same sterilized seed stocks were used for any given experiment.

For all experiments other than those that specify they were without additional nitrogen sources or sucrose, plants grown on agar plates were done so using 1% agar supplemented with 1/2 the standard concentration of Murashige & Skoog basal salts (Phytotechnologies, KS, USA) and 1% sucrose. For γ-aminobutryic acid experiments where no nitrogen was added, a modified Murashige & Skoog basal salt mixture was used which had no nitrate- or ammonia- containing salts (Phytotechnologies, KS, USA). 1-Aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA) and γ-aminobutryic acid (GABA) were filter sterilized and added after the base media had been autoclaved and cooled to 55°C. Seeds were sterilized using the same procedure mentioned for the hydroponics. They were placed at equal intervals on plates, sealed with parafilm and allowed to stratify at 4°C in the dark for 48 hours. Plates were unopened for the duration of the experiment. Unless stated otherwise, plates were grown under a 16 hour photoperiod as described above. Plates with ACC added were kept covered with aluminum foil during stratification and once in the chamber.

Leaf area measurements and root length measurements were conducted by taking pictures and then using ImageJ (Image J; National Institutes of Health; http://rsb.info.nih.gov/ij).

Genotyping, Quantitative-PCR and Sequencing to Verify T-DNA Insertion Site

Wild-type and *atenod93* lines were genotyped using primers designed via SALK's iSect Primer program (atenod93-LP: ATCGGTCAGAAATGGGTAACC, atenod93-RP: CGAAGAAGACTGCAACAGAGG and LBb1.3: ATTTTGCCGATTTCGGAAC). PCR cycling condition were: 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min 20 sec; and then 1 cycle at 72°C for 10 min. DNA was extracted using the TPE method outlined in Kim et al. (2016).

RNA from wild-type and *atenod93* plants was extracted using Novagen's Total Plant/Fungal RNA Isolation Kit (Norgen BioTek, ON, Canada) and cDNA generated using the qScript cDNA Supermix kit (Quanta Bio, MA, USA). Quantitative-PCR was performed using Perfecta SYBR Green Supermix (Quanta Bio, MA, USA), with Actin-7 used as the endogenous control (Actin-L: TGCACCGCCAGAGAGAAAAT and Actin-R: TGAGGGATGCAAGGATTGATC). The primers atenod93-Rq (AAGAACGCAGCGATAGAAGC) and atenod93-Lq (CACAGTAGCTGCCGTTCGTA) were used to amplify *AtENOD93* cDNA and measure quantities. Relative quantification was calculated using Applied Biosystem's included software, which used the $2^{-\Delta\Delta CT}$ method.

To sequence the left border of the T-DNA insert and the adjacent genomic sequence, the region was first amplified using the primers TDNA Seq LB-R (CGAGCCGAAACTTCCAAACA) and LBb1.3: (ATTTTGCCGATTTCGGAAC). Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and cycling conditions were: 1x cycle at 98°C for 30 sec; 30x cycle at 98°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec; and then 1x cycle at 72°C for 10 min. The resulting amplicon was sequenced using the same primers used for the amplification at the Advanced Analysis Center-Genomics Facility (University of Guelph).

Chlorophyll Measurements

Chlorophyll was extracted from frozen and ground samples that consisted of a pool of the rosette leaves from individual plants. ~100 mg of each sample was transferred to a centrifuge tube and 80% acetone added according to Arnon (1949). The samples were shaken for 3 hours at RT, after which time the supernatant was transferred to a 15 mL tube. New acetone was added

and left to shake over-night, followed by another session for 3 hours. The supernatants from these sessions were pooled in the appropriate 15 mL tube, generating a total of 3 mL of acetone-chlorophyll extract. Samples were kept in the dark as much as possible to prevent degradation of chlorophyll. Soon after the third wash, aliquots of the pooled supernatants were transferred to a microplate and absorbance values at 645 nm and 663 nm were measured using a Fisher Scientific accuSkan GO UV/Vis Microplate Spectrophotometer. The total chlorophyll per mg was calculated using the equation devised by Arnon (1949).

Sub-cellular Localization

RNA from wild-type plants was extracted using Novagen's Total Plant/Fungal RNA Isolation Kit (Norgen BioTek, ON, Canada) and cDNA generated using qScript cDNA Supermix (Quanta Bio, MA, USA). The coding sequence of AtENOD93 was then amplified using PCR, adding a NcoI restriction site to the 5' end (atenod93-cds-L: AAACCATGGATGGAAAATCGGTCAGAAATG) and a BamHI site to the 3' end (atenod93-cds-R: AAAGGATCCTTTAGAGTCTTGCTGAACTTTC). Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and cycling conditions were: 1x cycle at 98°C for 30 sec; 30x cycle at 98°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec; and then 1x cycle at 72°C for 10 min. This sequence was cloned into the pUC18-GFP construct which was kindlyprovided by Dr. Robert Mullen (University of Guelph), placing the coding sequence under the control of a CaMV 35S promoter and in-frame with GFP so that a fusion protein would be expressed with GFP on the C-terminus of AtENOD93. A pRTL2-based construct that produces TraB, a previously reported mitochondrial protein, with mCherry fused to its C-terminus was also provided by Dr. Mullen as a marker for mitochondrial localization (Marty et al., 2014). Protoplasts from wild-

type Columbia-0 plants were prepared according to Wu et al. (2009) and the constructs used to transform them were extracted using a PureLink HiPure Plasmid Midiprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). After 16 hours at 26°C and 100 µmol/m^2 light, the protoplasts were viewed using an upright Leica DM 6000B microscope connected to a Leica TCS SP5 system in the Advance Analysis Center (University of Guelph). GFP was viewed using an excitation laser of 488 nm and detected using an emission range of 500-540 nm, mCherry was viewed using an excitation laser of 543 nm and detected using an emission range of 600-640 nm and chloroplast autofluorescence was viewed using an excitation laser of 470 nm and detected using an emission range of 680-700 nm.

CHAPTER 3. RESULTS

Seeds stocks for the T-DNA insertion mutants in almost all genes in *A. thaliana* were generated by SALK and kept at the Arabidopsis Biological Resource Center (Alonso et al., 2003). Insertion mutant information was obtained from the SIGnAL website at http://signal.salk.edu and it was found that one of these insertions was putatively in the *AtENOD93* gene and that the seed stock was already homozygous for the T-DNA insert. However, it was important to confirm the homozygosity status of the seed stock before beginning any experiments that relied on a homogenous seed stock for accurate comparisons. This was especially true as this was the only insertion mutant line available. To begin, plants were grown from the wild-type and *atenod93* seed stocks, DNA was extracted and genomic PCR performed for genotypic analysis. The premise of this PCR is outlined in Figure 6 and uses the insertion site of the T-DNA sequence provided by SALK. The amplification time of the PCR was set so that atenod93-LP and -RP can amplify the region between them in wild-type DNA but

not in DNA with the T-DNA insertion. A second PCR using LBb1.3 and atenod93-RP was performed, where amplification of a product occurred when the T-DNA insert was present (as LBb1.3 binds to the T-DNA sequence) and not in wild-type. In doing this, it was possible to confirm those lines that are truly wild-type and those that are homozygous for the T-DNA insert insertion in *AtENOD93* (Figure 7). Seeds from individual plants that were found to meet these criteria were harvested and 3 plants were grown from these stocks. Quantitative-PCR was performed to prove that the insertion led to the expected reduction in expression of *AtENOD93* mRNA. As shown in Figure 7, this was the case for all homozygous *atenod93* plants grown. *atenod93* and wild-type seeds from the genotyped stocks were used in further experiments on this basis.

As a measure of precaution, the region around the left border of the T-DNA insert was sequenced. A portion of the left border and the adjacent genomic sequence were amplified using a primer that bound to the left border and one that bound to the genomic sequence. The resulting amplicon was then sequenced using the same primers at the Advance Analysis Center (University of Guelph). As shown in Figure 7, the T-DNA insert is indeed located in *AtENOD93.*

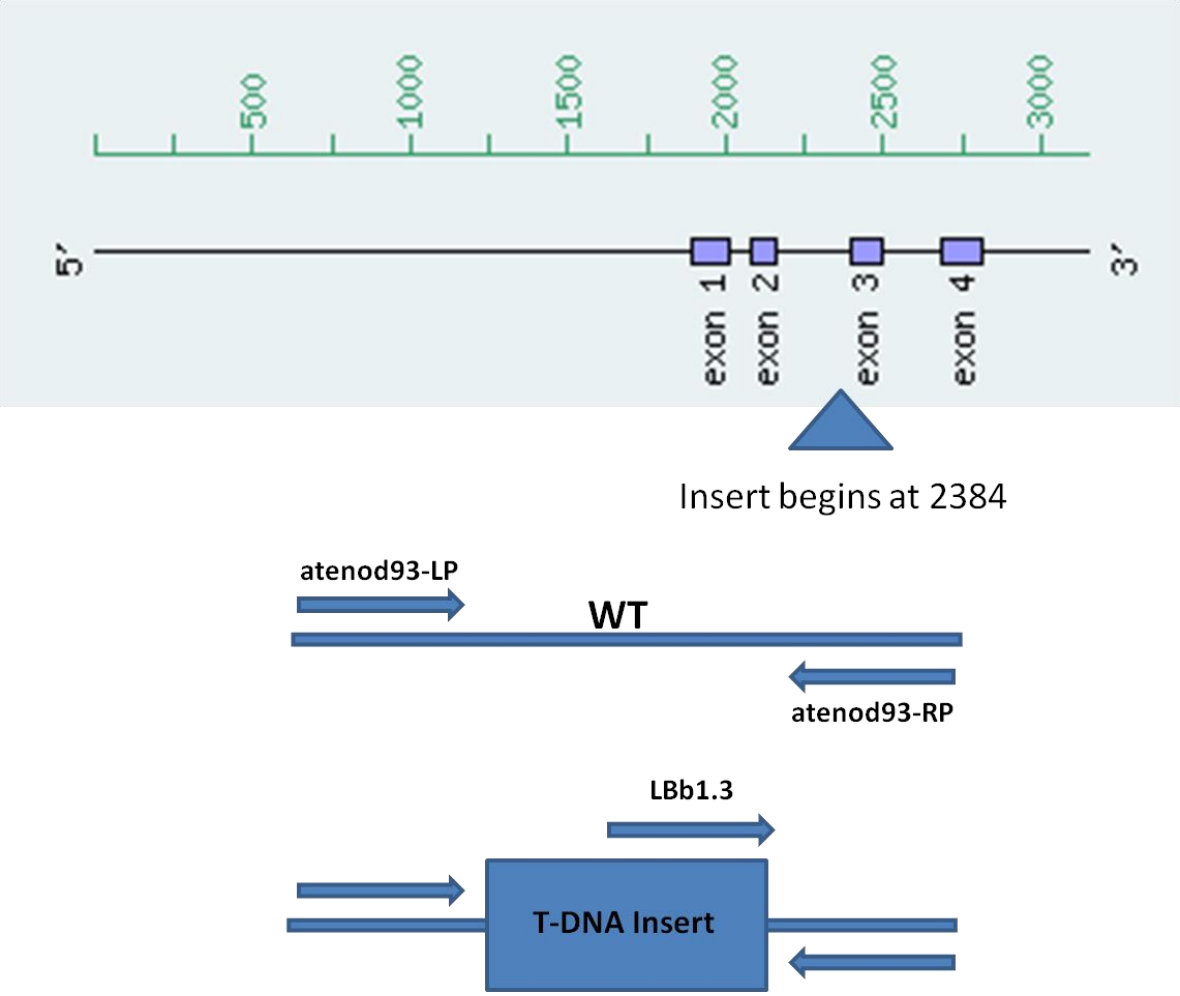


Figure 6. Representation of the *AtENOD93* gene and where the T-DNA insert is located. The top is a representation of the entire genomic sequence of *AtENOD93*, with the numbers representing the nucleotide number starting at the promoter. The representation was obtained from Aramemnon, a plant membrane protein database and the insert from information provided by SALK (Alonso et al., 2003; Schwacke et al., 2003). The bottom section also shows how the primer sets are designed to allow for genotyping of plants to determine if they are homozygous.

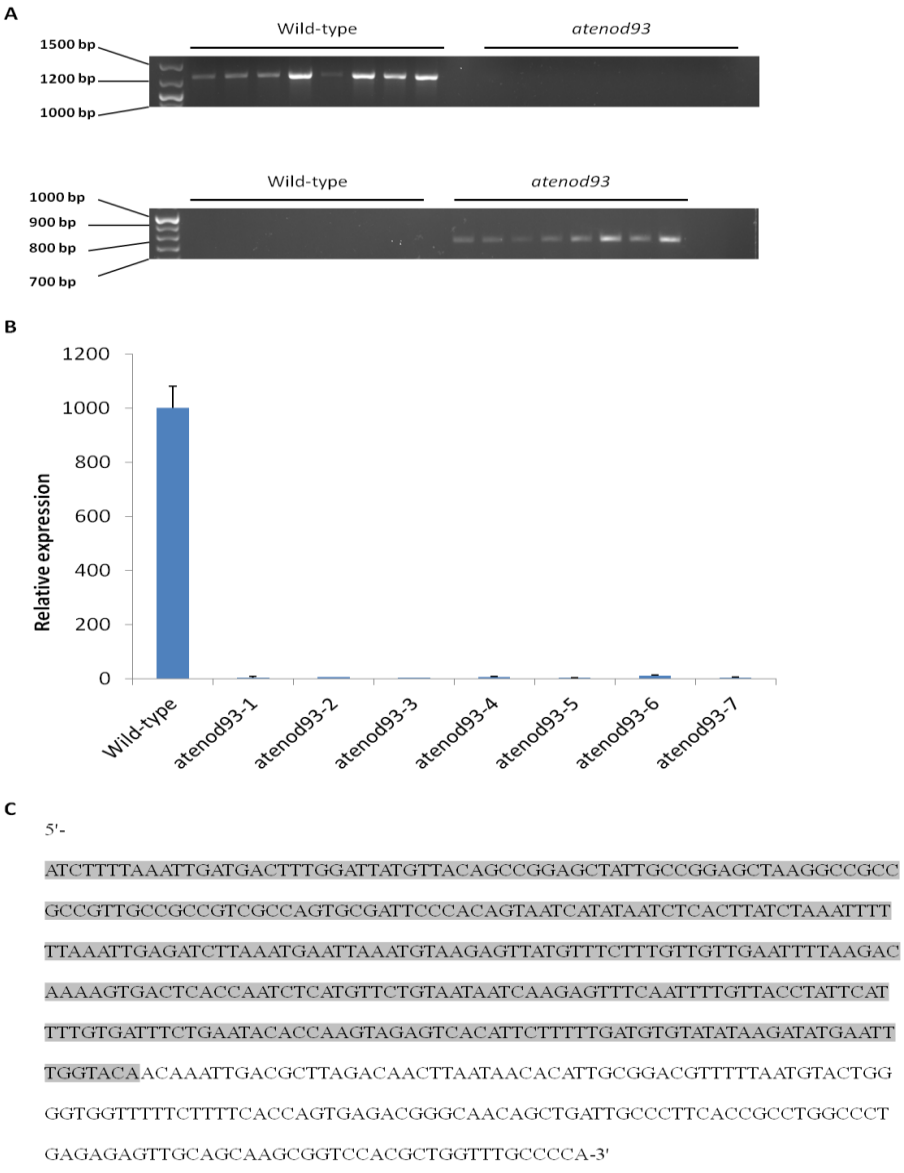


Figure 7. Genetic characteristics of the wild-type and *atenod93* lines. (A) Genotype analysis results to determine if plants are homozygous for the T-DNA insert. The top section shows results of using atenod93-LP and -RP primers and the bottom shows results of using LBb1.3 and atenod93-RP primers. (B) Relative expression of *AtENOD93* in wild-type and homozygous *atenod93* plants (error bars are SE, n=3) (C) Sequence at T-DNA insertion's left border - *AtENOD93* sequence is shaded grey, T-DNA left border is not shaded.

Upon confirming the T-DNA insertion site, it became important to establish a profile of the phenotype generated by the mutation. The first noticeable difference was in the flowering times. As shown in Figure 8, the *atenod93* line flowered an average of 2.4 days sooner than the wild-type under both high and low nitrate conditions.

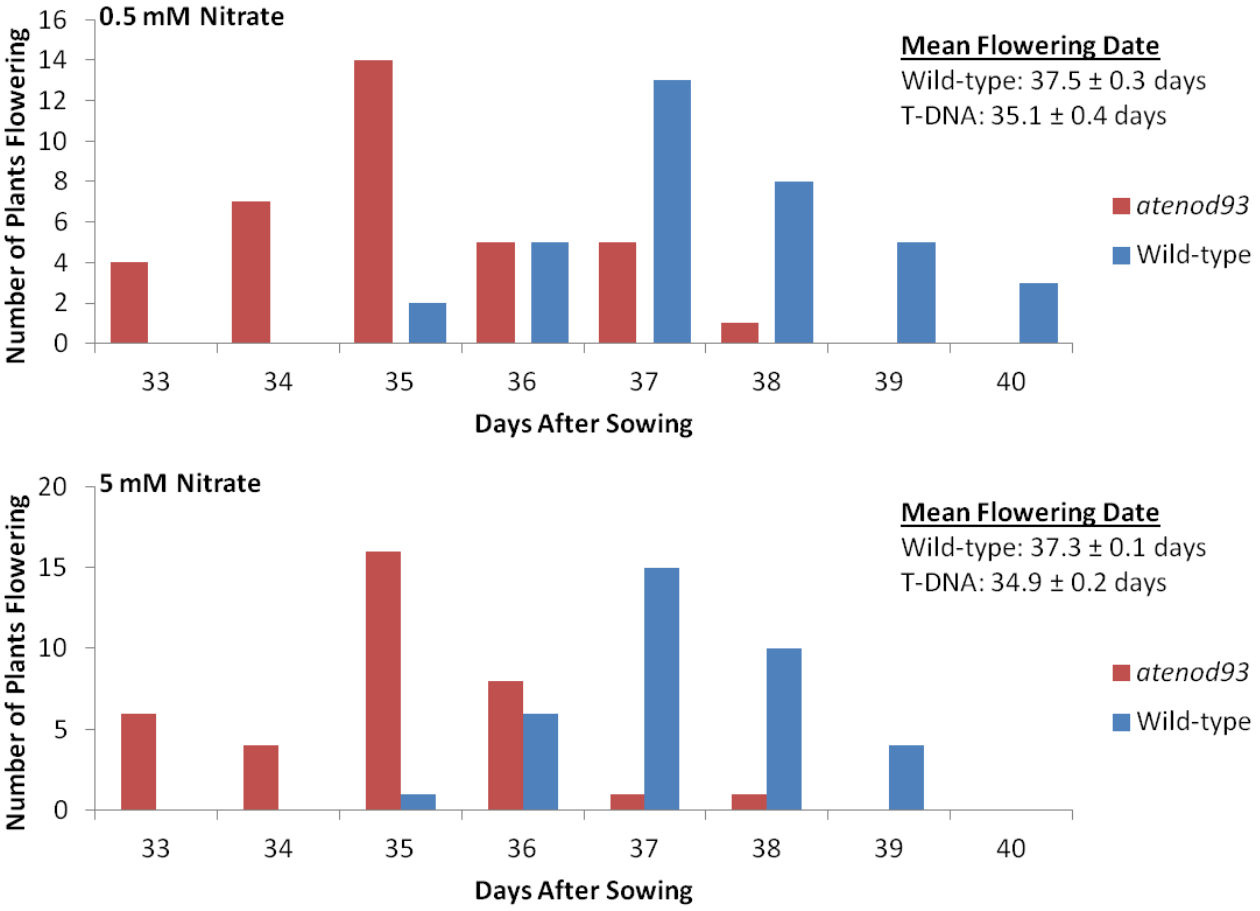


Figure 8. Flowering time distribution of wild-type and *atenod93* plants grown in soil at minimal and maximal nitrate levels. Mean flowering dates were proven significantly different using an unpaired t-test (n=36, representing 12 plants from 3 technical replicates, $p<0.05$, SE shown)

The next phenotypic differences were only witnessed after the first 4 weeks of growth. For the first 4 weeks, plants grew similarly, with no noticeable differences in size or number of the rosette leaves or their colouration (Figure 9). However, as the plants entered the reproductive phase and began to bolt, a clear divide between wild-type and *atenod93* could be visualized. After 6 weeks, when plants were well into the flowering stage, wild-type had more rosette leaves, slightly larger rosette leaves and were a darker green colour versus the light green-yellow colour of *atenod93* plants.

These observations were quantified in Figure 10 and Figure 11. As described above, the only differences between wild-type and *atenod93* were at 6 weeks. Total leaf area of *atenod93* was 67% that of WT at 0.5 mM nitrate, while the difference was reduced to 59% at 5 mM nitrate. Similarly, the number of rosette leaves on *atenod93* plants was 77% and 64% that of wild-type, under 0.5 mM nitrate and 5 mM nitrate, respectively. When the ratio of leaf area:leaf number was calculated, *atenod93* plants had significantly lower ratios compared to wild-type at both 0.5 mM nitrate and 5 mM nitrate, indicating individual *atenod93* leaves were on average smaller than wild-type.

To quantify the difference in colour observed, total chlorophyll was extracted from flash frozen rosette leaves and the results displayed in Figure 11. As with the leaf areas and counts, chlorophyll content between wild-type and *atenod93* was only different at 6 weeks. At this point, *atenod93* plants had 84% and 85% of total chlorophyll compared to wild-type at 0.5 mM nitrate and 5 mM nitrate, respectively.

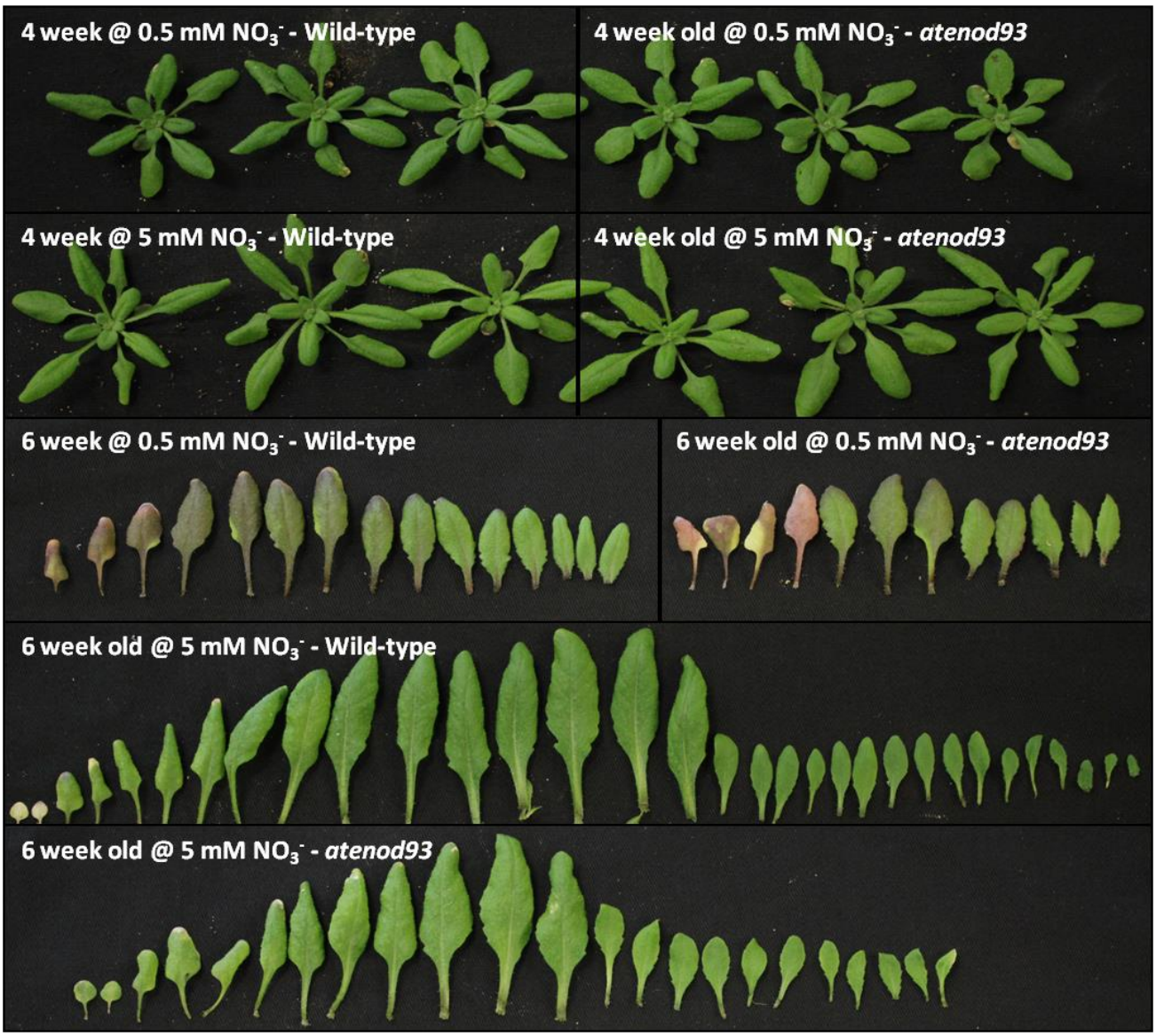


Figure 9. Rosette leaves from *atenod93* and wild-type plants at 4 and 6 weeks, grown in soil under 0.5 and 5 mM nitrate. 4 week old plants are those slightly before flowering while 6 week old plants have already reached flowering.

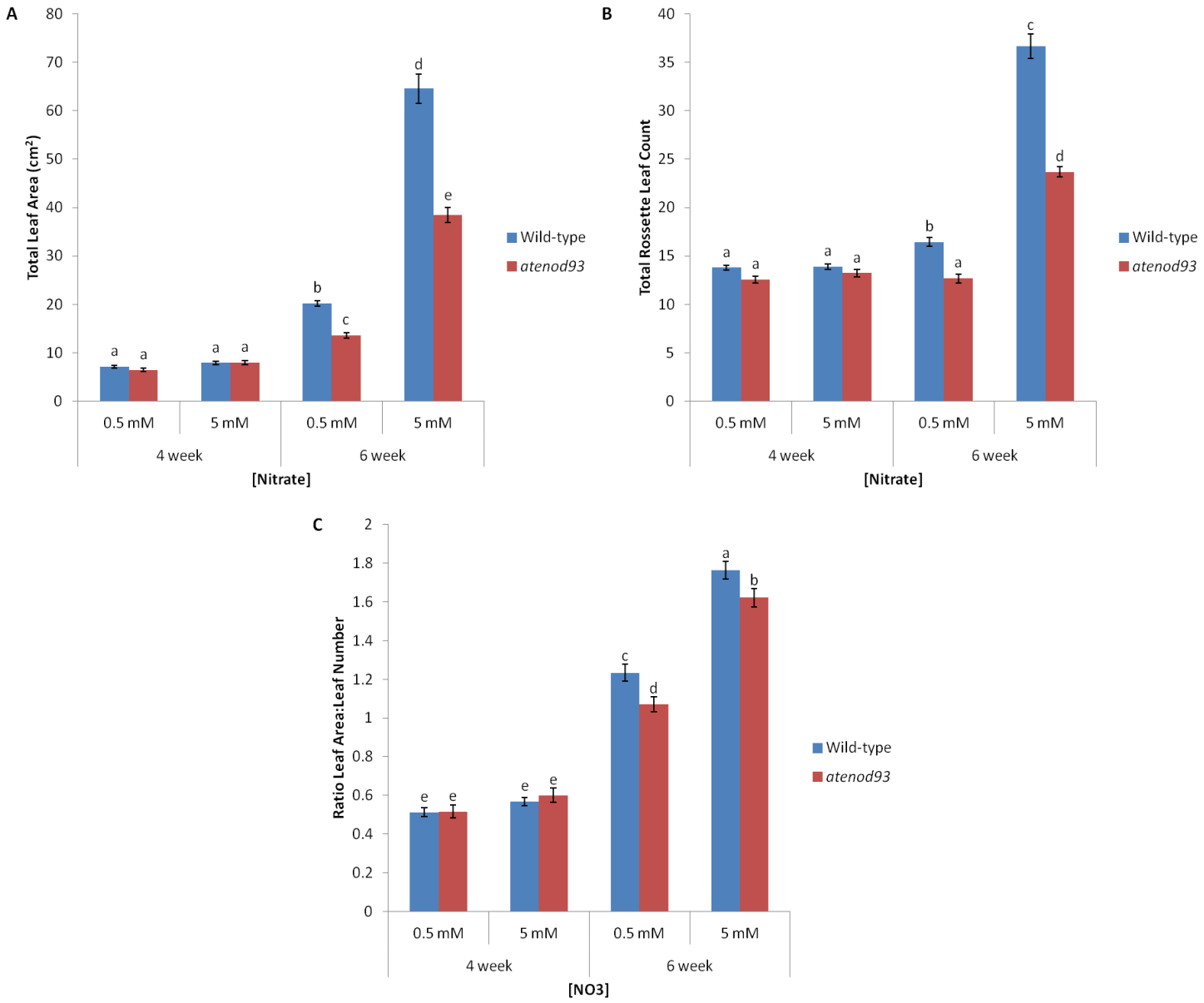


Figure 10. Rosette leaf characteristics of *atenod93* and wild-type plants at 4 and 6 weeks, grown in soil under 0.5 and 5 mM nitrate. (A) The average total leaf areas. (B) The average total number of rosette leaves on plants. (C) The average ratios of the total leaf area:total leaf count of individual plants. In all cases n=9, representing 3 plants from 3 technical replicates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

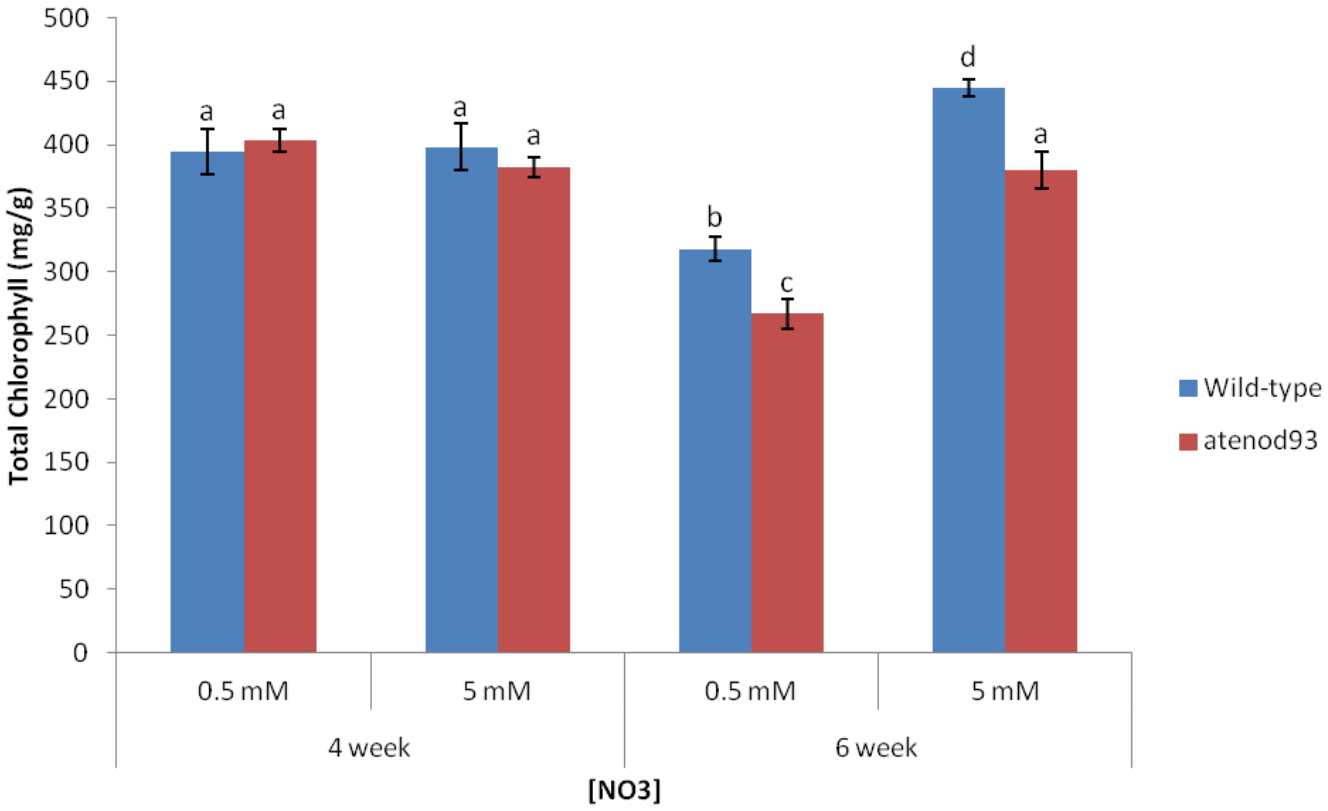


Figure 11. Average total chlorophyll of *atenod93* and wild-type plants at 4 and 6 weeks, grown in soil under 0.5 and 5 mM nitrate. Rosette leaves were pooled from individual plants from individual plants. n=9, representing 3 plants from 3 technical replicates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

At 7 weeks, close to the end of the plants' life cycles, the differences in their overall health were still observable (Figure 12). To quantify and compare the phenotype of these mature plants to those of the *OsENOD93-1* over-expression lines created by Bi et al. (2009), similar attributes were tested. As a representation of overall growth, the shoot fresh weights, root dry weights, root lengths and number of branches were measured. As a measure of reproductive capacity, the number of siliques were counted. Table 3 summarizes these measurements. At both minimal and maximum nitrate concentrations, the shoot weights, root weights and number of siliques were significantly greater in wild-type plants as compared to *atenod93* plants. The root lengths, however, showed no difference. Furthermore, the number of branches was limited to 1 at minimal nitrate for both lines, while at maximum nitrate, the wild-type had slightly more branching.

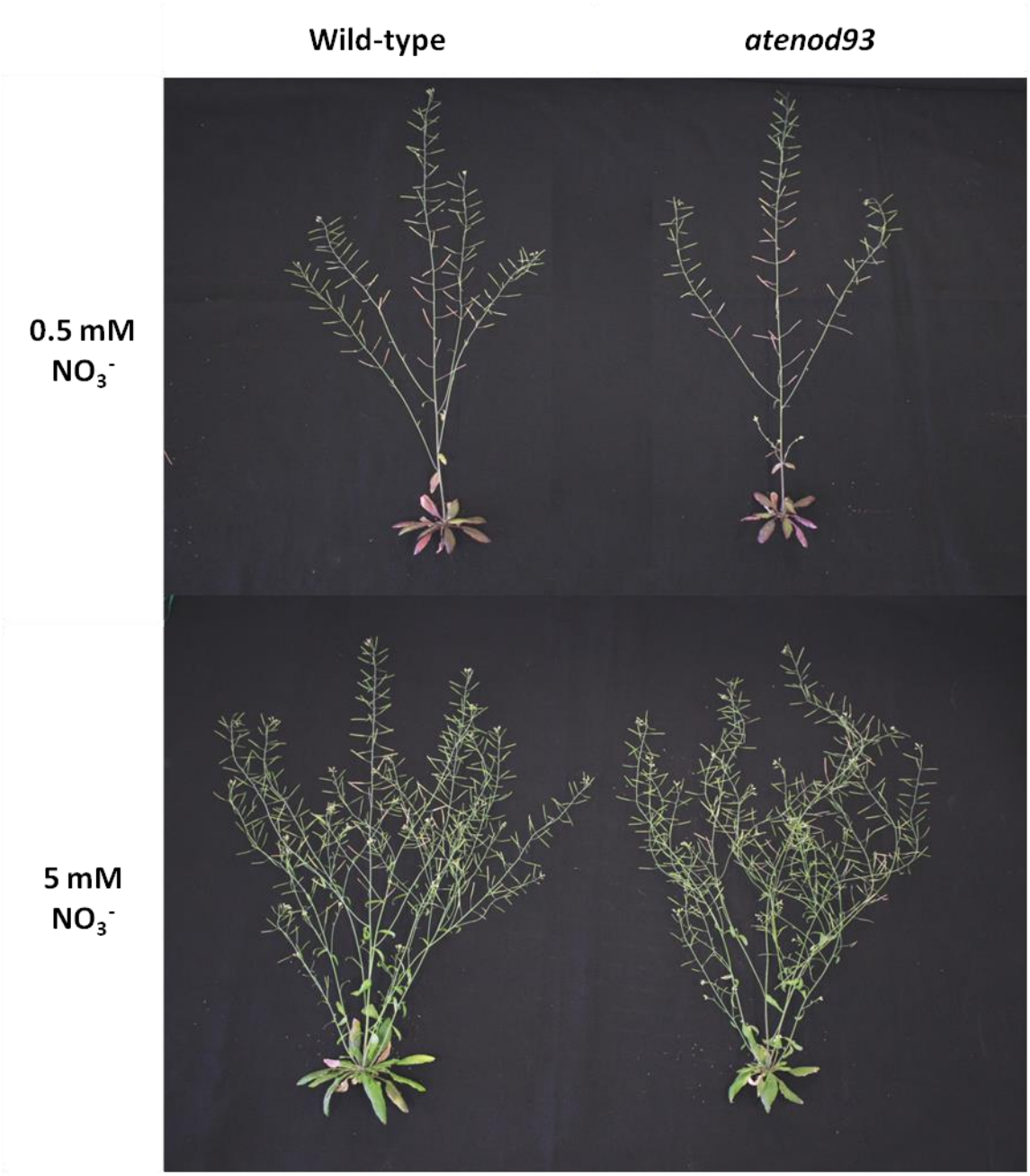


Figure 12. Shoot phenotype of wild-type and *atenod93* plants at 7 weeks, grown in soil under 0.5 and 5 mM nitrate.

Table 3. Quantified phenotype of wild-type and *atenod93* plants at 7 weeks, grown in soil under 0.5 and 5 mM nitrate.

	Shoot FW (g)	Root DW (mg)	Root Length (cm)	Number of Branches	Number of Siliques
Minimal Nitrate					
Wild-type	1.31 ± 0.05 *	119.9 ± 5.4 *	20.8 ± 0.8	1 ± 0	131 ± 3 *
atenod93	1.05 ± 0.04 *	97.4 ± 2.5 *	20.0 ± 0.9	1 ± 0	111 ± 3 *
Maximum Nitrate					
Wild-type	8.79 ± 0.30 *	171.5 ± 11.8 *	19.3 ± 1.2	6.2 ± 0.2 *	635 ± 17 *
atenod93	7.02 ± 0.22 *	113.6 ± 4.7 *	18.5 ± 0.8	5.6 ± 0 .2*	561 ± 26 *

Shoot fresh weights, number of branches and numbers of siliques were measured from 7 week old soil grown plants (n=9, from 3 technical replicates), from while root dry weights and lengths were measured from 7 week old hydroponically grown plants (n=7-8, from 3 technical replicates). Significance was measured using an unpaired t-test ($p<0.05$), with * representing differences.

It would be useful for further studies to have a phenotype that can be studied in younger plants. In an attempt to see if the reduced growth of the *atenod93* line was visible during early stages of growth, despite the lack of difference in the shoots of *atenod93* and WT until after 4 weeks, plants were grown on 1/2 MS agar plates supplemented with 1% sucrose. Only 7 days after being placed in a chamber, there were signs of impaired root growth in the *atenod93* line. This impairment became very noticeable after 14 days, with primary root growth being stunted (Figure 13). Additionally, the *atenod93* seedlings appeared to have a greater density of root hairs.

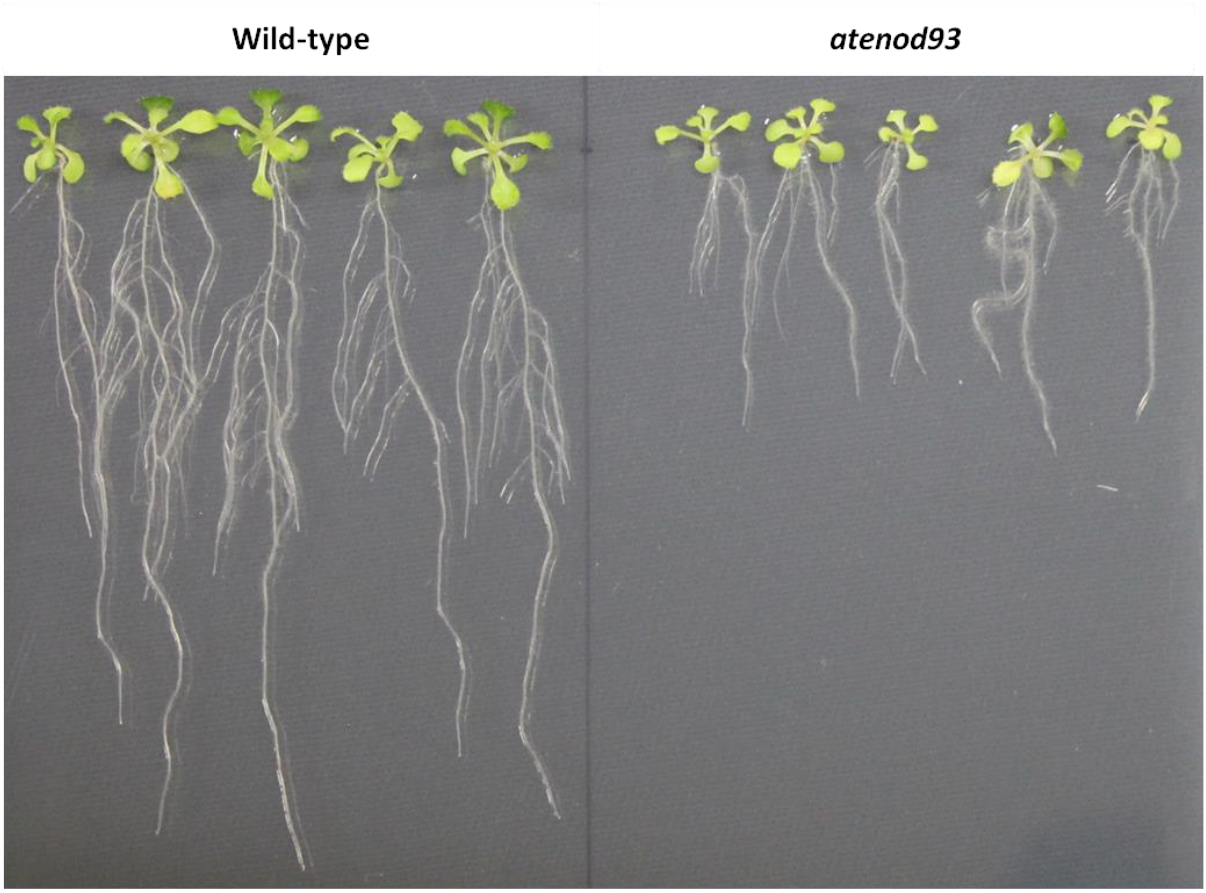


Figure 13. Wild-type and *atenod93* seedlings grown on 1/2 MS+1% Sucrose agar plates for 14 days after being moved from cold storage to a chamber.

The discovery of the seedling stunted root phenotype allowed for the rapid testing of a number of conditions regarding GABA utilization. Since GABA can be used to create succinate as an intermediate of the TCA cycle and NADH to feed the mitochondrial electron transport chain, addition of exogenous GABA can supplement TCA cycle intermediates that are decreased under stress conditions (Michaeli et al., 2011; Shelp et al., 2012). As well, the use of GABA to transfer an amino group to pyruvate, creating alanine, which can then be used to generate glutamate, means that exogenous GABA could be used as a nitrogen source in nitrogen-depleted environments (Van Cauwenberghe et al., 2002). As such, plants were grown on three groups of

plates - 1/2 MS with nitrogen sources and 1% sucrose; 1/2 MS with nitrogen sources but no sucrose; and 1/2 MS with no nitrogen sources and 1% sucrose. The intensity of light was also varied between high (150 μmol/m^2) and low light (50 μmol/m^2) to put further pressure on the plants as light is used to generate ATP for nitrogen assimilation and to form base carbon compounds that eventually enter the TCA cycle (Nelson and Ben-Shem, 2004; Foyer et al., 2011). Exogenous GABA was added at 0, 1, 5, and 10 mM concentrations. On each plate, half of the seedlings were wild-type and half were the mutant line, allowing for growth comparisons on each plate individually. As demonstrated in Figures 14, 15 and 16, under low light conditions, there were no obvious trends in root growth as concentrations of added GABA increased. This may be due to variations of light conditions between groups of plates caused by their positions in the chambers. To account for any differences among plates, each plate was analyzed separately. The percentage of *atenod93* primary root length to that of wild-type was calculated and this value was averaged with other plates from the same treatment. As shown in Figures 17 and 18, the proportional root growth improved with increasing concentrations of GABA under most conditions of stress (low light and no sucrose, low light and no nitrogen and high light and no sucrose). Therefore, under moderate stress conditions increasing the exogenous GABA enhanced the growth of the *atenod93* mutant line somewhat more than what occurred with wild-type. Clearly, this experiment would need to be done again to try to get a more homogeneous light condition. Despite the measurable changes in root length, no obvious trends in root hair growth were observed with varying GABA concentrations, but it was noted that all *atenod93* plants had a higher density of root hairs (Appendix I: Root Pictures).

Interestingly, mutant seedlings grown on plates with no added nitrogen and at high light grew close to 100% that of wild-type across all concentrations of GABA, rather than starting lower than wild-type and increasing, as was found under low light. This may be due to the fact that both wild-type and *atenod93* appeared to be under extreme stress at these conditions. The growth of both lines was actually impaired compared to low light grown plants (Figure 15) and the resulting plants appeared to be stunted and damaged by chlorosis (Appendix I: Root Pictures). Thus, it is possible that the high light in combination with metabolic deficiencies allowed photodamage that neither line could tolerate more than the other. That is to say, the metabolic stress could have resulted in the inefficient production of compounds involved in reactive oxygen species scavenging, such as α-tocopherol, ascorbic acid and carotenoids, thereby promoting photodamage to photosystem II (Bonfils et al., 2000; Takahashi and Badger, 2011).

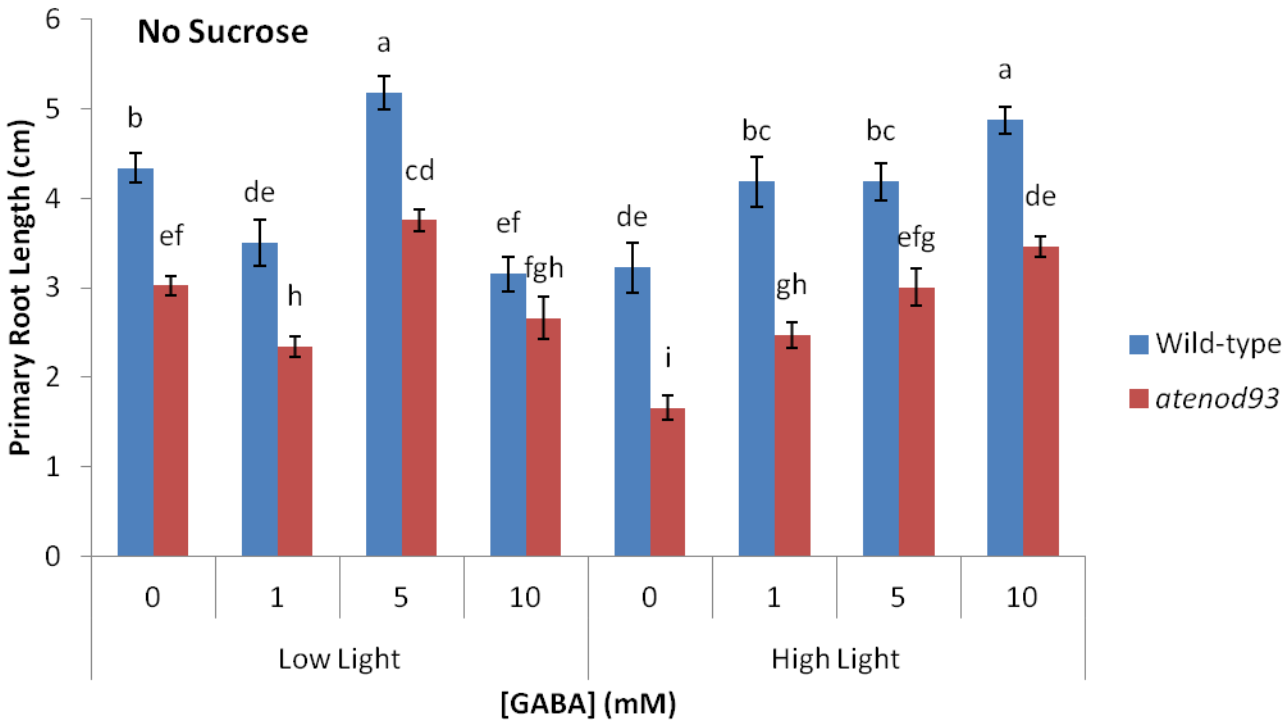


Figure 14. The effects of GABA treatments on primary root length under varying light conditions when grown with no added sucrose but with added nitrogen. Values are average primary root lengths of *atenod93* and wild-type seedlings after 14 days, when grown with no sucrose but with nitrogen at high (150 µmol/m^2) and low light (50 µmol/m^2) and with 0, 1, 5, 10 mM GABA. n=10-12 seedlings from 3 separate plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

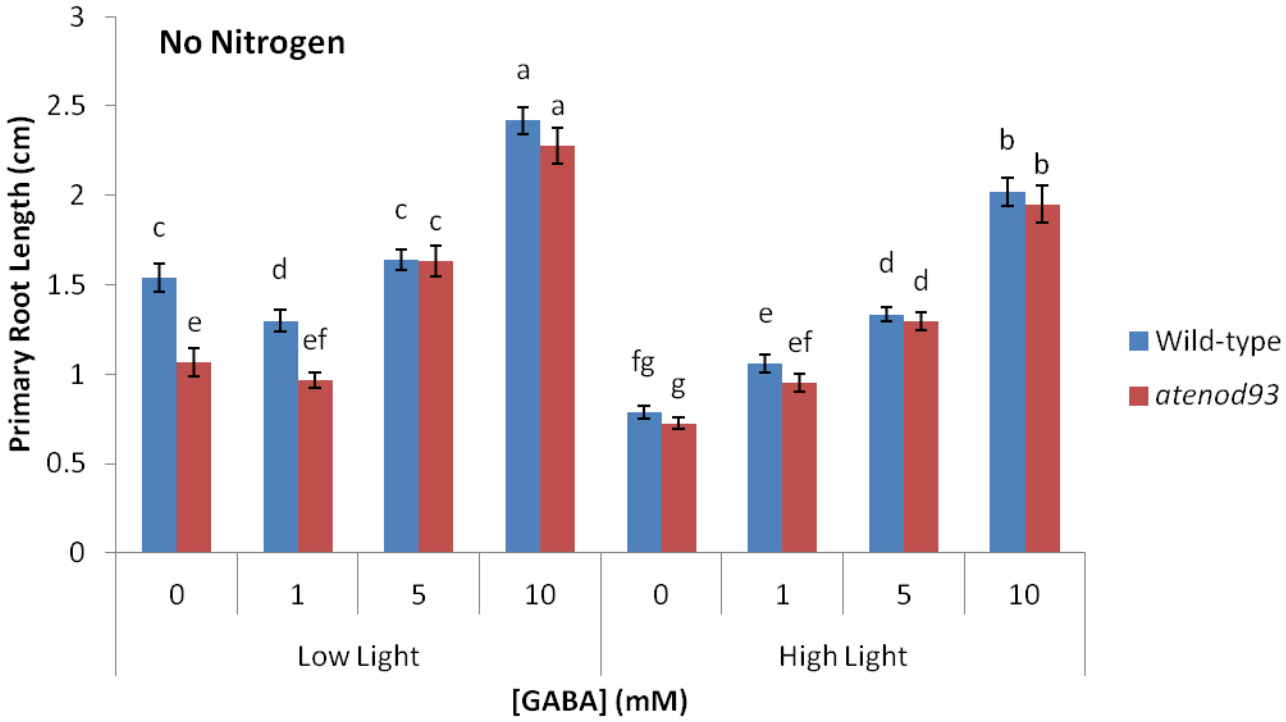


Figure 15. The effects of GABA treatments on primary root length under varying light conditions when grown with added sucrose but no added nitrogen. Values are average primary root lengths of *atenod93* and wild-type seedlings after 14 days, when grown with 1% sucrose but no nitrogen at high (150 µmol/m^2) and low light (50 µmol/m^2) and with 0, 1, 5, 10 mM GABA. n=10-12 seedlings from 3 separate plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

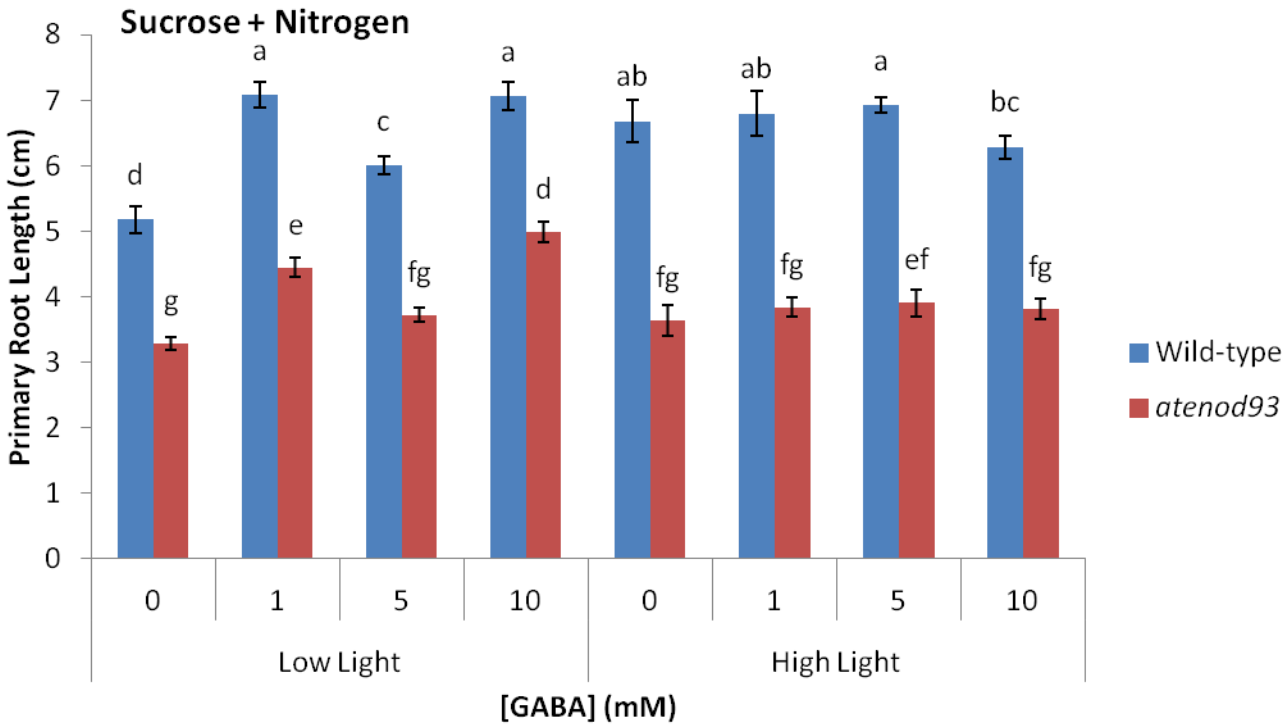


Figure 16. The effects of GABA treatments on primary root length under varying light conditions when grown with added sucrose and added nitrogen. Values are average primary root lengths of *atenod93* and wild-type seedlings after 14 days, when grown with nitrogen and 1% sucrose at high (150 µmol/m^2) and low light (50 µmol/m^2) and with 0, 1, 5, 10 mM GABA. n=10-12 seedlings from 3 separate plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

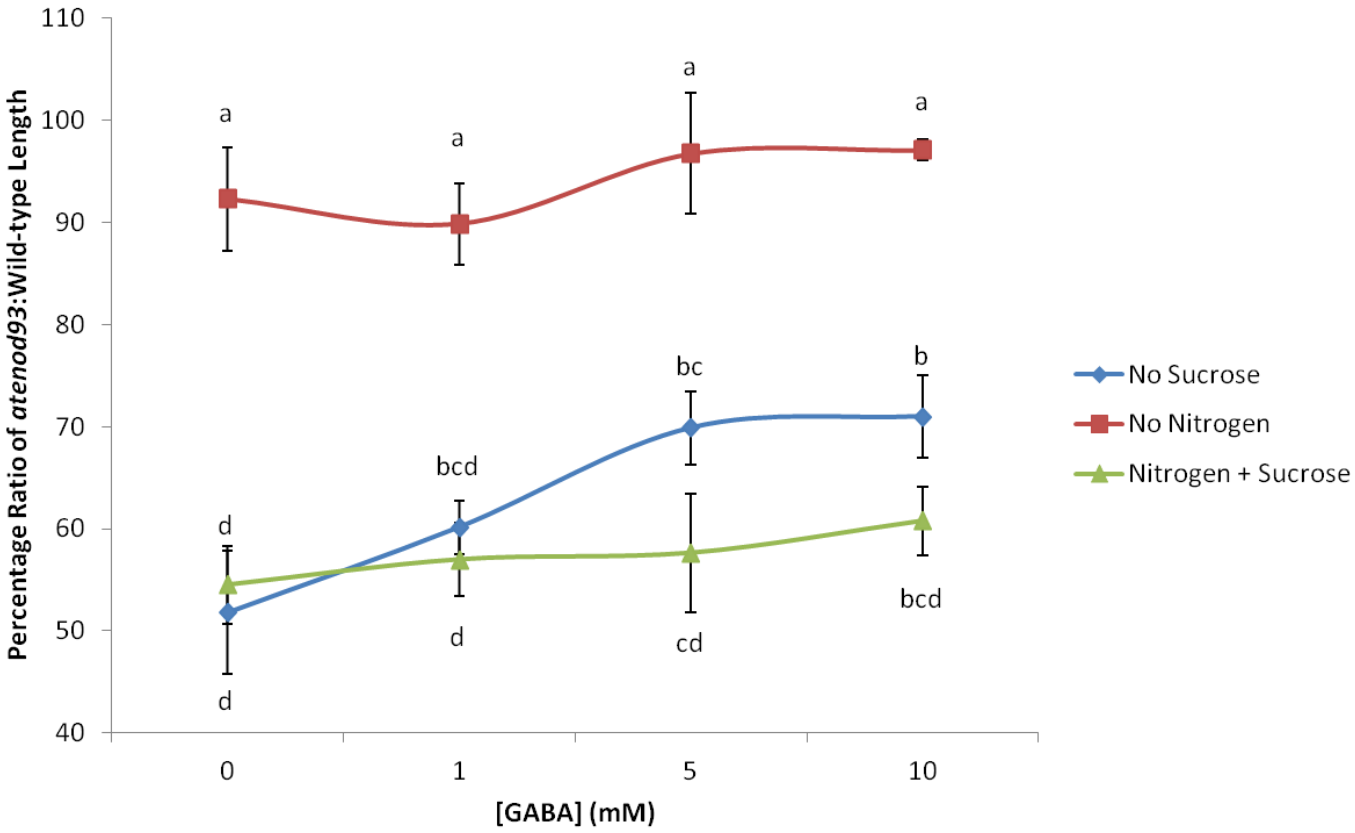


Figure 17. The effects of GABA treatments on relative primary root growth of *atenod93* plants as compared to wild-type plants under a high light condition. Values are the percentages of 14-day old *atenod93* primary root lengths relative to wild-type primary root lengths at 150 µmol/m^2 light, when grown on plates with combinations of varying conditions, including no sucrose, no nitrogen, nitrogen + sucrose and 0, 1, 5, and 10 mM GABA. Root lengths were measured for 3-4 plants of each line on 3 separate plates. The values were averaged for each plate and percentage generated for each plate. The values shown are the averages of the 3 plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

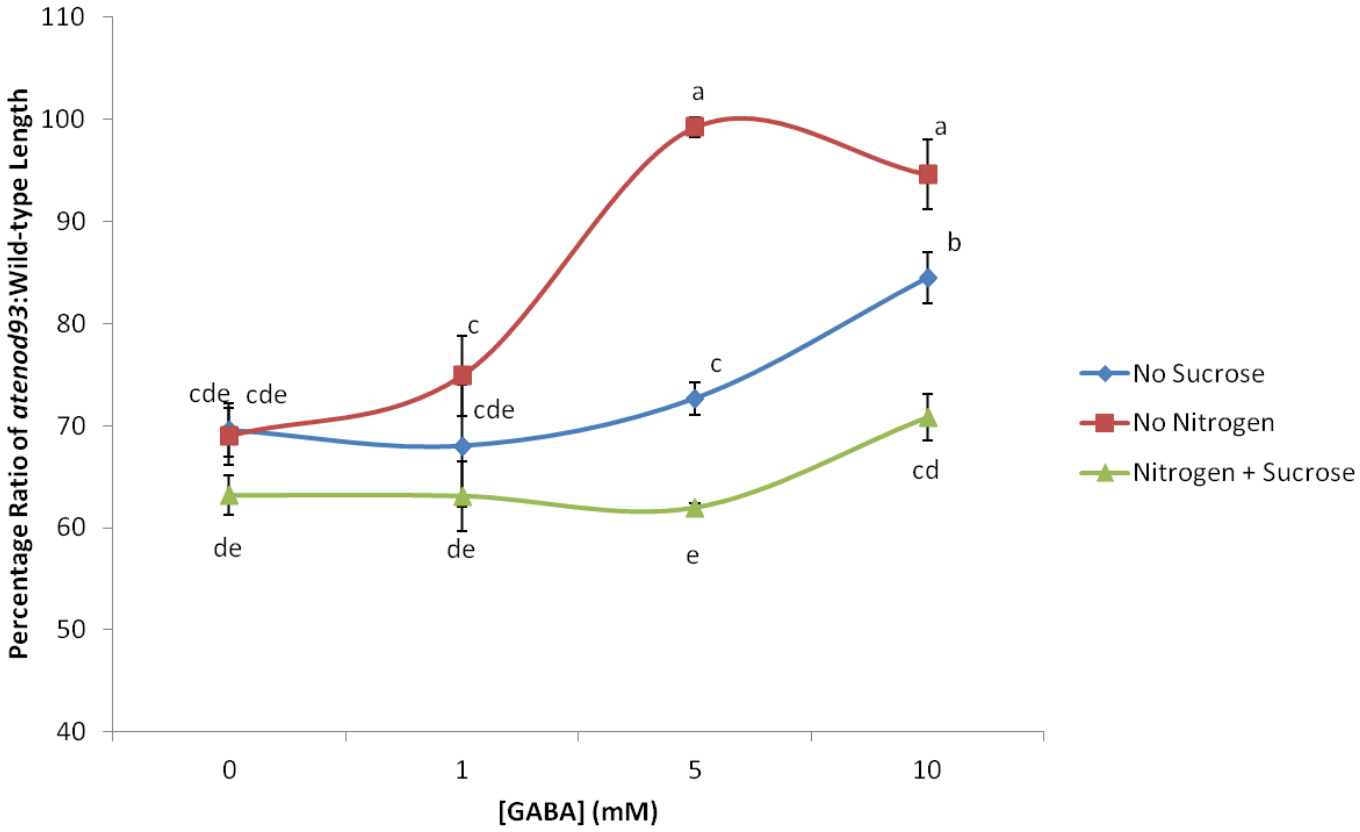


Figure 18. The effects of GABA treatments on relative primary root growth of *atenod93* plants as compared to wild-type plants under a low light condition. Values are the percentages of 14-day old *atenod93* primary root lengths relative to wild-type primary root lengths at 50 µmol/m^2 light, when grown on plates with combinations of varying conditions, including no sucrose, no nitrogen, nitrogen + sucrose and 0, 1, 5, and 10 mM GABA. Root lengths were measured for 3-4 plants of each line on 3 separate plates. The values were averaged for each plate and percentage generated for each plate. The values shown are the averages of the 3 plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

Since it has been shown that exposure to GABA up-regulates the expression of ethylene and abscisic acid (ABA) synthesis genes and results in plants actually producing more ethylene (Kathiresan, A et al., 1997; Shi et al., 2010), two assay were performed to test the sensitivity of *atenod93* plants to these hormones versus the wild-type. The first relies on the fact that 1-amino-cyclopropane-1-carboxylic acid (ACC) is a precursor for ethylene production and that increased ethylene production results in a triple response when seedlings are grown in the dark (Guzman and Ecker, 1990; Van de Poel and Van Der Straeten, 2014). In this response, hypocotyls swell, reduce in length and exhibit an apical hook (Van de Poel and Van Der Straeten, 2014). When *atenod93* and wild-type seeds were grown in this fashion, both lines showed a similar mild apical hook phenotype and mild swelling of the hypocotyls (Figure 19). Similarly, as seen in Figure 20, at 0 and 1 mM ACC, there was no significant difference in the hypocotyl length. However, at 0.2 and 0.5 mM ACC, the *atenod93* were slightly, but significantly shorter than the wild-type. These results imply that *atenod93* plants were hypersensitive to the increased ethylene production, but not as drastically as previously recorded ethylene hypersensitive mutants (Van de Poel and Van Der Straeten, 2014).

The assay for increased production of ABA was based on the fact that ABA inhibits seed germination in a proportional manner (Yang et al., 2014). Figure 21 outlines the results of this assay. The *atenod93* line showed a clearly reduced germination rate at all concentrations of ABA, though the difference was most pronounced at 1 mM ABA. At this concentration, even after 60 hours, a time point where 100% of wild-type seeds were germinated, some *atenod93* seeds remained dormant. This could not be due to low germination rates of the seed stock as at 0 mM ABA all of the *atenod93* seeds germinated by 60 hours. Therefore, *atenod93* seeds were hypersensitive to increases in exogenous ABA.

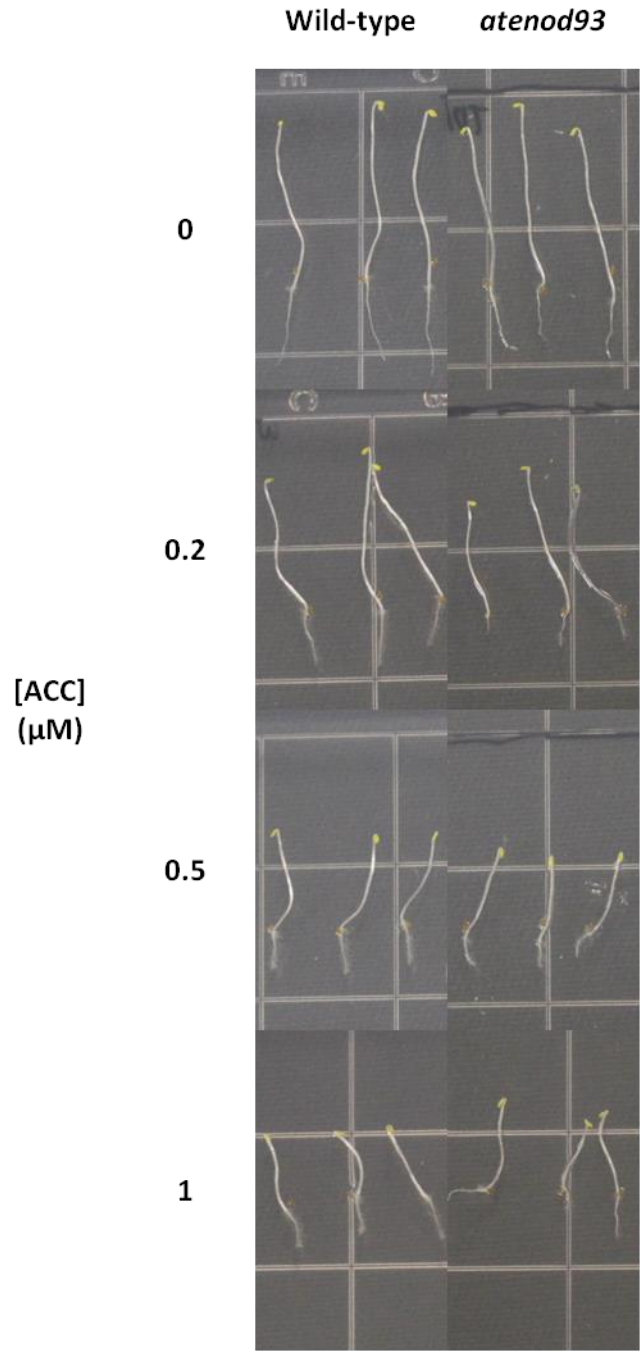


Figure 19. *atenod93* and wild-type seedlings grown in the dark for 4 days on 1/2 MS agar plates supplemented with various concentrations of ACC.

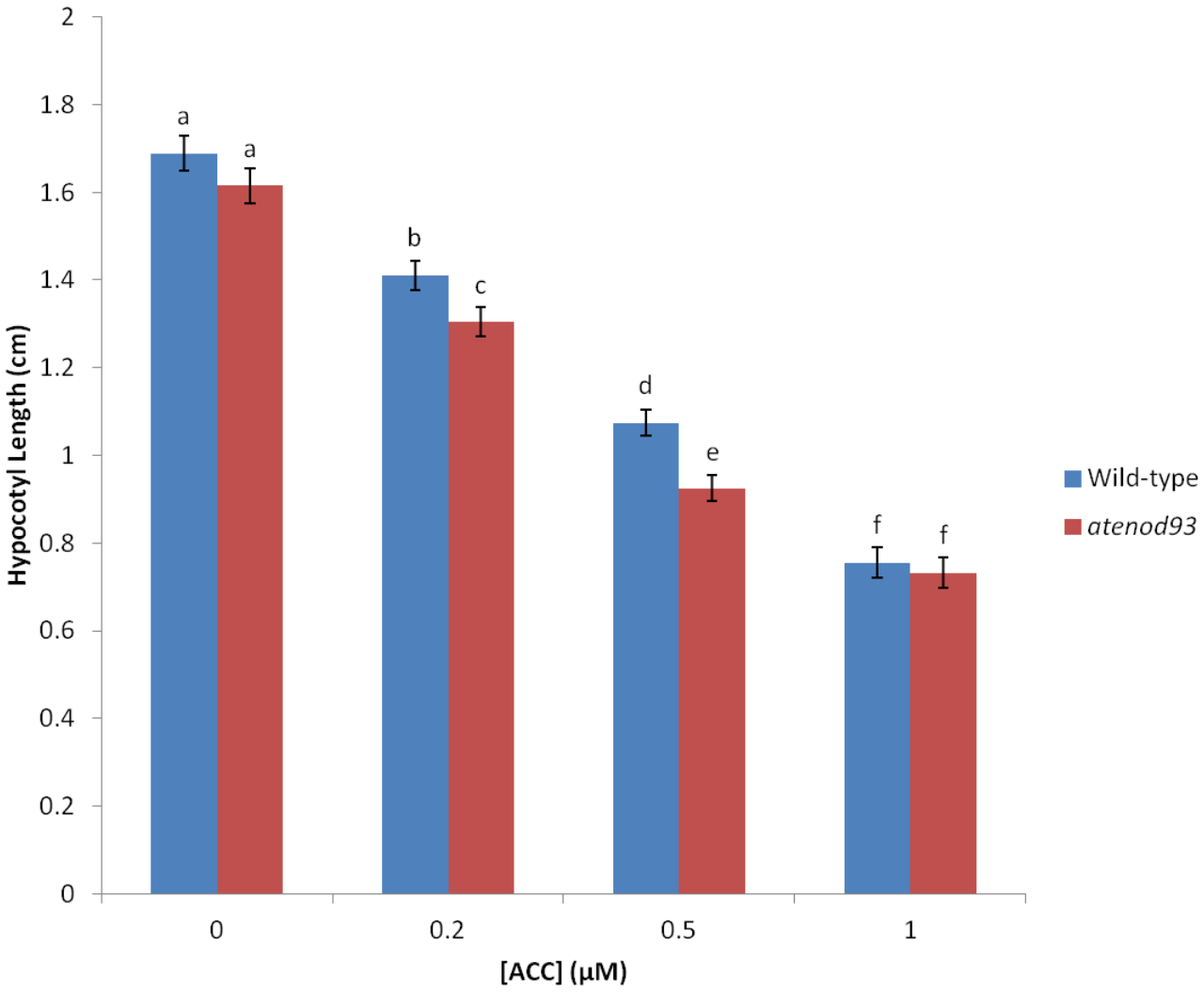


Figure 20. Average hypocotyl lengths of *atenod93* and wild-type seedlings grown for 4 days on 1/2 MS agar plates with additions of various concentrations of ACC. n=18 seedlings, representing 6 seedlings from 3 separate plates. Significantly different values are denoted by different letters as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE.

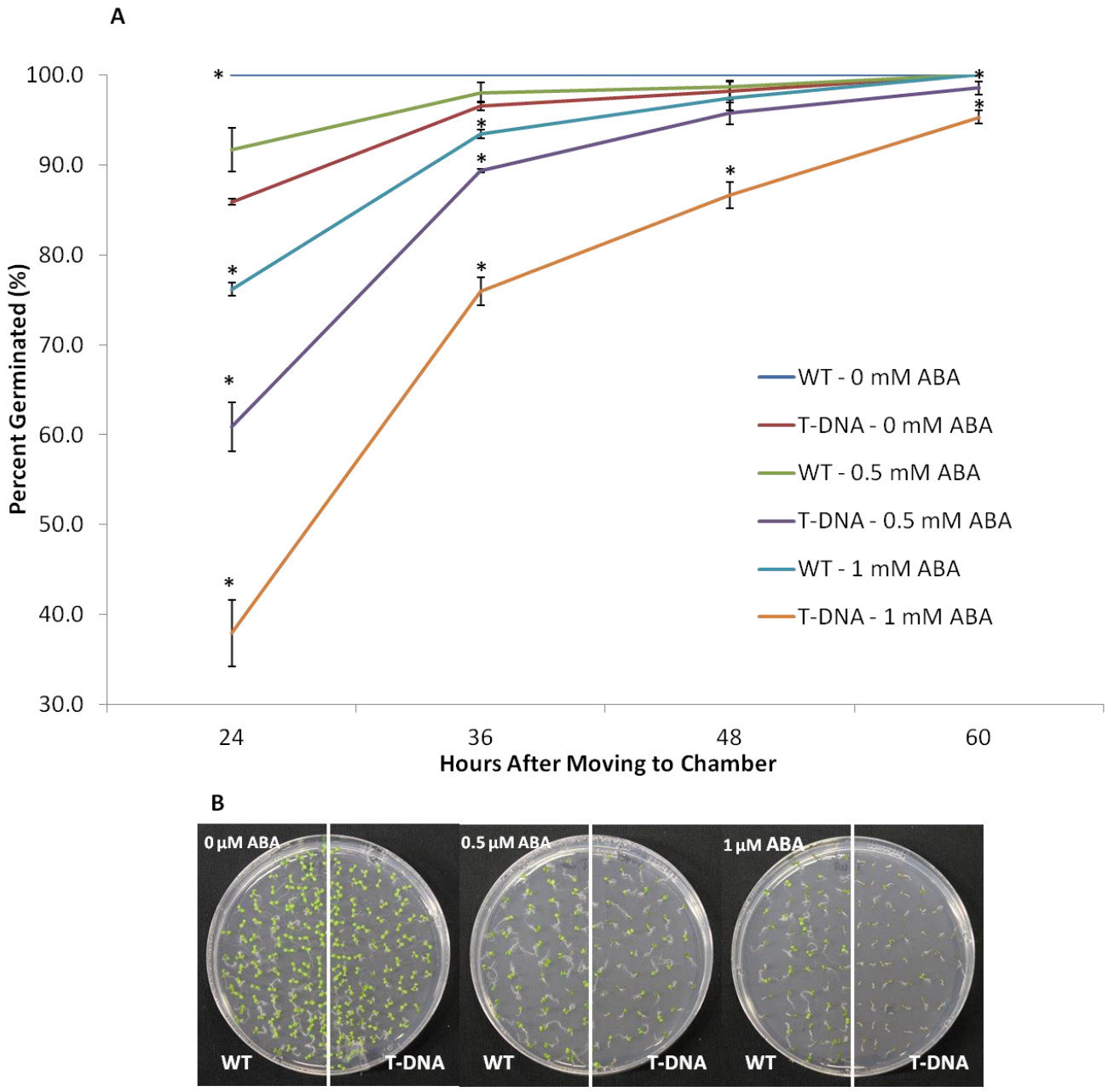


Figure 21. Effects of ABA on germination. (A) Germination rates of *atenod93* and wild-type seeds during 12 hour intervals starting after 24 hours after moving the plates to the chamber. n=140-170, representing seeds counted across 3 separate plates. Asterisks indicate unique values as determined using the Fischer's Least Significant Difference method ($p<0.05$). Bars are SE. (B) Example of the plates 7 days after moving to the chamber.

The sub-cellular localization of AtENOD93 was determined in order to better understand its metabolic roles. Figure 22 outlines the images of protoplasts transformed with various constructs. Three constructs were utilized for this experiment. The first was pUC18-GFP which constitutively expressed GFP. Using this, a new construct, pUC18-GFP-AtENOD93, was made by cloning the AtENOD93 coding sequence upstream of the N-terminal end GFP. Thus, this construct constitutively expressed a C-terminal GFP fusion protein. The last construct used was pRTL2-mCherry-TraB, which constitutively expressed TraB, a mitochondrial protein, with the red fluorescent protein, mCherry, fused to its C-terminus (Marty et al., 2014). Untransformed protoplasts served as a negative control and exhibited only chlorophyll auto-fluorescence. When the pUC18-GFP construct with no additional sequence was transfected into protoplasts, the GFP signal was very strong, but diffuse, indicating the produced GFP alone had no specific localization and remained in the cytosol. Protoplasts transformed with only pRTL2-mChr-TraB showed mostly concentrated signals of mCherry caused by localization to mitochondria, but no GFP signal, meaning any GFP signal localized along with TraB-mCherry was not from this protein. When pUC18-GFP-AtENOD93 was transformed in conjunction with pRTL2-mChr-TraB, concentrated signals of both mCherry and GFP were seen at the same location, suggesting AtENOD93 localizes to mitochondria.

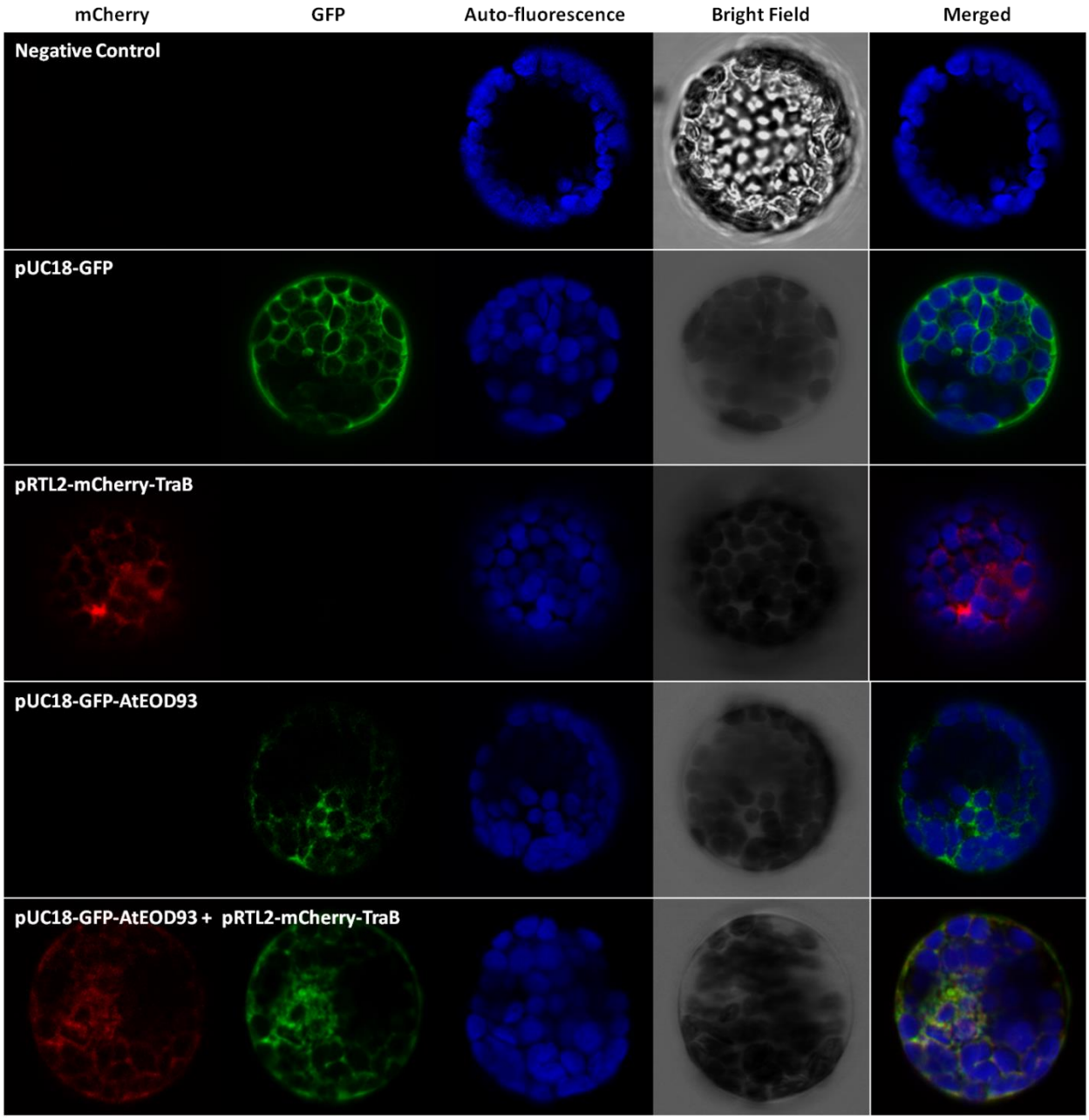


Figure 22. Images taken with a confocal microscope of protoplasts transformed with various constructs.

CHAPTER 4. DISCUSSION AND CONCLUSIONS

This project sought to characterize some of the phenotypic and metabolic effects of modifying the singular ENOD93-like gene in *A. thaliana*. The understanding of how this gene works in Arabidopsis could then hopefully be used to identify ways in which the ENOD93-like family of genes could be modified in important crop plants to improve NUE. The *atenod93* phenotypes are opposite to that seen when over-expressing *OsENOD93-1* in rice. For instance, the knock-out of *AtENOD93* reduced biomass accumulation and reproductive capacity in that silique numbers are reduced. On the other hand, the over-expression lines had higher biomass accumulation and improved reproductive capacity compared to wild-type plants as evident by increased seed yield and weight. Additionally, both mutant and over-expression lines exhibited their respective phenotypes under minimal and maximum nitrate conditions, meaning their functions are not simply in response to low nitrogen stresses, but are beneficial for normal growth as well. The AtENOD93 over-expression lines have been produced, although there was not time to generate data from these in this thesis. It will be of interest to see if these do mimic what was seen in rice.

Bi et al. (2009) placed OsENOD93-1 at the center of the complex process of C:N ratio balancing in cells and it may be that this extends to AtENOD93 as well. Chlorophyll content in leaves is closely linked to the available nitrogen content within a plant (Netto et al., 2005). This is due to the fact that glutamate is the initial compound used in the tetrapyrrole pathway that eventually synthesizes chlorophyll (Gomez-Silva et al., 1985). The *atenod93* line had a reduced capacity to generate chlorophyll after 6 weeks, when the plants were in the flowering stage, whether grown under minimal or maximum nitrate, while at 4 weeks, which was before flowering, no significant difference was found. This result is consistent with the idea that

AtENOD93 improves NUE as nitrogen requirements increase during flowering to cope with the rapid development of new tissues (Hirel et al., 2007).

The finding that the *atenod93* line had stunted primary root growth on 1/2 MS agar plates allowed for the study of the effects of GABA on its growth compared to wild-type. It has been shown previously that adding varied concentrations of exogenous GABA leads to a dose-dependent increased uptake of GABA, permitting the rapid testing of GABA's effects on the mutant's growth on plates under a variety of conditions (Michaeli et al., 2011). The results of these experiments showed that applying GABA at increasing concentrations was able to improve primary root growth of *atenod93* seedlings relative to wild-type seedlings under various moderate stress conditions involving withholding additional nitrogen sources or sucrose under low and high light. However, additions of GABA were unable to improve the root growth of *atenod93* seedlings compared to wild-type when sucrose and other nitrogen sources were provided. Thus, under more ideal growth conditions, there is no relative improvement of the mutant compared to the wild-type with the addition of GABA. The only relative improvement comes when stress limits the growth of wild-type. Under these conditions GABA does increase the relative growth of the mutant compared to wild-type. In fact, under low light and no nitrogen, the growth of the mutant and wild-type in the presence of high levels of GABA were identical.

In order to develop hypotheses on the function of AtENOD93, it was important to establish experimentally the sub-cellular localization. The co-transformation of protoplasts with constructs that expressed AtENOD93 fused to GFP and a mitochondrial marker protein, TraB, fused to mCherry, generated GFP and mCherry signals at the same locations. This indicates that AtENOD93 is localized to the mitochondria, and, in conjunction with the similar structure,

localization and expression pattern discussed earlier, suggests that OsENOD93-1 and AtENOD93 could share the same or similar functions in mitochondria.

The series of previously studied mutants with reduced activity of mitochondrial GABA shunt enzymes show similarities to the results here and imply that the phenotypes seen here are related to GABA shunt deficiencies at the mitochondrial level. Firstly, a mutant lacking the one discovered GABA-P transporter was shown to have stunted primary root growth under low light conditions and when sucrose was withheld from plates (Michaeli et al., 2011). However, under high light conditions and those where sucrose was provided, the root growth was the same between wild-type and mutant. Thus, a reduction in root length, as found with the *atenod93* mutant, can be a result of GABA's inability to supplement the TCA cycle. The authors found that GABA transport into isolated mutant mitochondria was only reduced by ~50% to that of wild-type, revealing the likely presence of multiple transporters. It may be that AtENOD93 is somehow involved in regulating the quantity and/or activity of both transporters rather than just one, causing the severe reduction in growth of the *atenod93* line even under ideal conditions. Additionally, the GABA transporter(s) are attractive potential targets for AtENOD93's modulation of the GABA shunt as AtENOD93 is theoretically localized to the mitochondrial membrane (Brugière et al., 2004).

A mutation in the only discovered GABA-transaminase in plants showed no phenotypic difference compared to wild-type until exogenous GABA was applied (Renault et al., 2011). Upon this addition, the mutant's primary root growth was stunted to a greater extent than wild-type and endogenous concentrations of GABA were larger than those in wild-type. This proves two points: 1) when a plant is impaired in its use of GABA, it pools GABA and 2) the pooled GABA signals reductions in primary root growth. In fact, the study attributed this reduction in

growth to a shift in expression of cell wall synthesis genes and an overall reduction in cell elongation. The results from plates with sucrose and nitrogen and GABA do not match the results by Renault et al. (2011), though, showing no decrease in root growth either in the absolute measurements or proportional measurements. This difference may point to the possibility that the method used by Renault et al. (2011) evokes a greater GABA-mediated response than the one used in the current study. The difference may lie in the fact that Renault et al. (2011) grew seedlings on plates with no GABA first and then transferred them to GABA-containing plates, whereas the current study grew seedlings on GABA plates from germination. The former might cause a spike in GABA when plants are moved to the new plates as the entire root will come into contact with GABA. By growing the seedlings continuously on GABA plates, the root would have time to take up and metabolize GABA surrounding it as it grows. In spite of this deviation, the studies combined show that it is possible for GABA shunt deficiencies to reduce primary root length by not supplementing the TCA cycle and through queuing gene expression changes that result in reduced growth.

In a similar fashion, previously characterized mutations affecting the GABA shunt can be used to explain the phenotypes discovered when growing the plants on soil and hydroponics. For instance, previous studies have shown that disruption of the other enzyme of the GABA shunt, SSA-dehydrogenase, can have a drastic effect on shoot growth (Bouché et al., 2003; Ludewig et al., 2008). Bouché et al. (2003) noted that mutants produced greater quantities of reactive oxygen species (ROS), which likely contributed to the observed reduced biomass, reduced leaf area and reduced chlorophyll content compared to wild-type plants. Moreover, Li et al. (2016) found that exogenous GABA had a positive effect on the growth of plants subjected to heat stress and that this was, at least in part, due to a reduction in ROS. Likewise, exogenous GABA

applied to sunflower up-regulated genes involved in processing ROS and reduced the accumulation of ROS under salt stress (Shi et al., 2010). A buildup of ROS might be why the GABA-transaminase mutant mentioned above also had reduced chlorophyll compared to wild-type and continued to lose chlorophyll in a manner proportional to supplied GABA (Renault et al., 2011). As such, these results support the idea that the *atenod93* mutant is deficient in its GABA shunt ability as it too had reduced leaf area, biomass and chlorophyll content and it may be that these phenotypes are caused by a buildup of ROS.

Ethylene concentrations modulated through pooled GABA may also have played a role in developing the phenotypes witnessed. Exogenously applied GABA was shown to up-regulate the ethylene synthesis genes for ACC oxidase, the enzyme that creates ethylene from ACC, and produced a measurable increase in ethylene (Kathiresan, A et al., 1997; Shi et al., 2010). Tholen et al. (2004) demonstrated that ethylene sensitivity resulted in reduced rosette leaf growth, shortened seedling primary roots and reduced chlorophyll content compared to ethylene insensitive plants. Moreover, application of exogenous ethylene produced longer root hairs, which may explain this observation during the GABA plate experiments (Ruzicka et al., 2007). Ogawara et al. (2003) compared mutants with varying responses to ethylene and found an increased flowering time and decreased rosette leaf number with increasing sensitivity, a result very much like the *atenod93* line compared to wild-type.

ABA production may also have a part in generating the phenotype as ABA2, a reductase involved in ABA synthesis, was up-regulated when sunflowers were exposed to exogenous GABA (Shi et al., 2010). Mutants that were hypersensitive to ABA had reduced seed germination and primary root growth when exposed to exogenous ABA (Yang et al., 2014). As well, exposing wild-type plants to as little as 0.1 μM ABA increased the density of root hairs (Xu

et al., 2013). Thus, it is possible that GABA was pooling in the *atenod93* line due to a deficiency in GABA shunt activity and caused increases in ABA and ethylene production. This would explain the differences in hypocotyl lengths discovered when grown with 0.2 and 0.5 µM ACC and the reduced seed germination of *atenod93* plants grown with ABA. It also accounts for the reduced primary root growth and the lower rosette leaf area, number and size of *atenod93* plants compared to wild-type. The responses do not need to be limited to one of the two hormones, though, as a recent study showed ABA and ethylene have compounding effects on primary root elongation and seed germination (Thole et al., 2014).

Although the results obtained during this study imply that GABA shunt activity is impaired in the *atenod93* mutant, they are not complete enough to say with certainty that this is the mode of action of AtENOD93. The GABA plate experiments point to a lack of GABA shunt activity in the *atenod93* line and all of the phenotypic traits can be linked to a reduction in this activity, but due to the complex nature of the mitochondria, many other possibilities remain. For instance, when the main regulatory component of the TCA cycle, mitochondrial enzyme phophoenolpyruvate carboxylase, was exposed to nitrate, its activity increased, indicating PEPC regulation is directly linked to the maintenance of the C:N as well (Le Van Quy et al., 1991; Foyer et al., 2003). Despite this, it was proven the AtENOD93 is localized to the mitochondria and disruption of its expression produced the expected phenotype based on the OsENOD93-1 over-expression lines in rice. The similarities open the door for expedited experiments using *A. thaliana* and AtENOD93 as models in the hopes of unlocking the mysteries of the ENOD93-like protein family and their potential for improving crop yield.

CHAPTER 5. RECOMMENDATIONS

Firstly, even though the *atenod93* mutant was confirmed to have the insert in *AtENOD93*, other insertions cannot be ruled out. Lines over-expressing AtENOD93 in the wild-type background and in the *atenod93* background were produced but still need to be examined. The wild-type-over-expression lines will prove if AtENOD93 has the ability to improve nitrogen use, as OsENOD93-1 did. The T-DNA-over-expression lines will clarify if the phenotype of *atenod93* is wholly due to the absence of AtENOD93 as a reversion to the wild-type phenotype will confirm complementation.

Constructs with GUS under the control of the *AtENOD93* promoter were also generated. The promoter-GUS sequence was stably inserted into wild-type plants using an *Agribacterium*-mediated transformation to that can be used to visualize the location of *AtENOD93* expression in various tissues at different developmental stages and under different conditions. The visualization still needs to be completed. In addition, constructs to express AtENOD93 with a C- or N-terminal Myc tag were generated and the sequences were stably inserted into wild-type plants using an *Agribacterium*-mediated transformation. In the future, these lines could be used to perform a co-immunoprecipitation experiment to determine which proteins associate with AtENOD93 and provide new insight into the role of AtENOD93.

The GABA plate experiments did not produce results that completely matched those of previous studies and the trends in growth with increasing concentration of GABA were not consistent amongst low light plates. These should be repeated in a larger chamber to control the light hitting each tray. Also, to better compare results with those published before, seedlings could be moved from optimal trays to trays with the experimental conditions sometime after germination. If the lines once again show a response to GABA, a transcriptional analysis could

be performed. Namely, it would be valuable to map the expression of genes relating to the GABA shunt, such as those that encode the enzymes involved (GAD, GABA-P, etc.). Further evidence for a reduction in GABA shunt activity being a result of the *atenod93* mutation could be generated by performing plate experiments with the addition of metabolites that circumvent the GABA shunt but have the same function, such as SSA, succinate, alanine and glutamate. Additionally, to prove a direct metabolic connection between AtENOD93 and the GABA shunt, GABA content could be measured within plants. As well, to show the metabolic impairment of the TCA cycle, the functions of the mitochondria can be monitored, like ATP production, and could provide a picture of mitochondrial health.

Ultimately, the goal of working with ENOD93-like proteins is to discern how they function in agriculturally important plants and how they can be used to improve agricultural productivity. As such, once a clearer picture of the function of AtENOD93 is formed, it would be beneficial to begin focusing on the OsENOD93s. Should the *atenod93* mutant phenotype be confirmed to occur as a result from the single insertion in *AtENOD93,* over-expression lines producing any one of the OsENOD93s could be created. Complementation of the *atenod93* phenotype could be used as a simple screen for OsENOD93s with the same function as AtENOD93. From there, expression of various combinations of OsENOD93s in *A. thaliana* could be attempted to see if traits could be improved over the over-expression of just one gene.

REFERENCES

Agriculture and Agri-foods Canada (2013) Market outlook report: canadian farm fuel and fertilizers.

Alexandratos N, Bruinsma J (2012) World agriculture towards 2030/2050.

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Gadrinab C, et al (2003) Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana. Science (80-) **301**: 1849

Appleby CR, Tjepkema JD, Trinick MJ (1983) Nitrogen fixation. Science (80-) **220**: 951–953

Arai M, Mitsuke H, Ikeda M, Xia JX, Kikuchi T, Satake M, Shimizu T (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res **32**: 390–393

Ariz I, Asensio AC, Zamarreño AM, García-Mina JM, Aparicio-Tejo PM, Moran JF (2013) Changes in the C/N balance caused by increasing external ammonium concentrations are driven by carbon and energy availabilities during ammonium nutrition in pea plants: The key roles of asparagine synthetase and anaplerotic enzymes. Physiol Plant **148**: 522–537

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24**: 1–15

Barbosa JM, Singh NK, Cherry JH, Locy RD (2010) Nitrate uptake and utilization is modulated by exogenous γ-aminobutyric acid in Arabidopsis thaliana seedlings. Plant Physiol Biochem **48**: 443–450

Batushansky A, Kirma M, Grillich N, Toubiana D, Anh Pham P, Balbo I, Fromm H, Galili G, Fernie AR, Fait A (2014) Combined transcriptomics and metabolomics of arabidopsis thaliana seedlings exposed to exogenous gaba suggest its role in plants is predominantly metabolic. Mol Plant **7**: 1065–1068

Baum G, Lev-Yadun S, Fridmann Y, Arazi T, Katsnelson H, Zik M, Fromm H (1996) Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. EMBO J **15**: 2988–2996

Benson DR, Silvester WB (1993) Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev **57**: 293–319

Beuve N, Rispail N, Laine P, Cliquet J-B, Ourry A, Le Deunff E (2004) Putative role of g-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in Brassica napus L . Plant Cell Environ **27**: 1035–1046

Bi YM, Kant S, Clark J, Gidda S, Ming F, Xu J, Rochon A, Shelp BJ, Hao L, Zhao R, et al (2009) Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling. Plant, Cell

Environ **32**: 1749–1760

Bi Y-M, Wang R-L, Zhu T, Rothstein SJ (2007) Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. BMC Genomics **8**: 1–17

Bonfils MHJ, Lu C, Niyogi KK (2000) Photodamage of the Photosynthetic Apparatus and Its Dependence on the Leaf Developmental Stage in the. Society **124**: 273–284

Bouché N, Fait A, Bouchez D, Møller SG, Fromm H (2003) Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. Proc Natl Acad Sci **100**: 6843–6848

Brady SM, Orlando DA, Lee J-Y, Wang JY, Koch JR, José D, Daniel M, Ohler U, Benfey PN (2007) A High-Resolution Root Spatiotemporal Map Reveals Dominant Expression Patterns. Cell 801–806

Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J (2005) Genome-wide analysis of transcript abundance and translation in arabidopsis seedlings subjected to oxygen deprivation. Ann Bot **96**: 647–660

Brugière S, Kowalski S, Ferro M, Seigneurin-Berny D, Miras S, Salvi D, Ravanel S, D'Hérin P, Garin J, Bourguignon J, et al (2004) The hydrophobic proteome of mitochondrial membranes from Arabidopsis cell suspensions. Phytochemistry **65**: 1693–1707

Carvalho GAB De, Batista JSS, Marcelino-Guimarães FC, Nascimento LC Do, Hungria M (2013) Transcriptional analysis of genes involved in nodulation in soybean roots inoculated with Bradyrhizobium japonicum strain CPAC 15. BMC Genomics **14**: 153

Cassman KG, Dobermann A, Walters DT (2002) Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio **31**: 132–140

Cassman KG, Kropff MJ, Gaunt J, Peng S (1993) Nitrogen use efficiency of rice reconsidered: what are the key constraints? Plant Soil **155**–**156**: 359–362

Casson S, Spencer M, Walker K, Lindsey K (2005) Laser capture microdissection for the analysis of gene expression during embryogenesis of Arabidopsis. Plant J **42**: 111–123

Van Cauwenberghe OR, Makhmoudova A, McLean MD, Clark SM, Shelp BJ (2002) Plant pyruvate-dependent gamma-aminobutyrate transaminase: identification of an Arabidopsis cDNA and its expression in Escherichia coli. Can J Bot **80**: 933–941

Clarke VC, Loughlin PC, Day DA, Smith PMC (2014) Transport processes of the legume symbiosome membrane. Front Plant Sci **5**: 1–9

Coelho MRR, Marriel IE, Jenkins SN, Lanyon C V., Seldin L, O'Donnell AG (2009) Molecular detection and quantification of nifH gene sequences in the rhizosphere of sorghum (Sorghum bicolor) sown with two levels of nitrogen fertilizer. Appl Soil Ecol **42**: 48–53

Cole JJ, Caraco NF (2001) Emissions of nitrous oxide (N2O) from a tidal, freshwater river, the Hudson River, New York. Environ Sci Technol **35**: 991–996

Engelder T, Howarth R, Ingraffea a (2011) Should fracking stop? Nature **477**: 271–275

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2008) Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci **13**: 14–19

Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, et al (2014) Pfam: the protein families database. Nucleic Acids Res **42**: 222–230

Fischer JJ, Beatty PH, Good AG, Muench DG (2013) Manipulation of microRNA expression to improve nitrogen use efficiency. Plant Sci **210**: 70–81

Food and Agriculture Organization of the United Nations (2014) Food outlook. doi: 10.1044/leader.PPL.19102014.18

Foyer CH, Noctor G, Hodges M (2011) Respiration and nitrogen assimilation: Targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. J Exp Bot **62**: 1467–1482

Foyer CH, Parry M, Noctor G (2003) Markers and signals associated with nitrogen assimilation in higher plants. J Exp Bot **54**: 585–593

Gauthier PPG, Bligny R, Gout E, Mahé A, Nogués S, Hodges M, Tcherkez GGB (2010) In folio isotopic tracing demonstrates that nitrogen assimilation into glutamate is mostly independent from current CO2 assimilation in illuminated leaves of Brassica napus. New Phytol **185**: 988–999

Glass A (2003) Nitrogen Use Efficiency of Crop Plants: Physiological Constraints upon Nitrogen Absorption. CRC Crit Rev Plant Sci **22**: 453–470

Gomez-Silva B, Timko MP, Schiff JA (1985) Chlorophyll biosynthesis from glutamate or 5-aminolevulinate in intact Euglena chloroplasts. Planta **165**: 12–22

Good AG, Beatty PH (2011) Fertilizing nature: A tragedy of excess in the commons. PLoS Biol **9**: 1–9

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci **9**: 597–605

Guzman P, Ecker JR (1990) Exploiting the Triple Response of Arabídopsís To ldentify Ethylene-Related Mutants. Plant Cell **2**: 513–523

Hata S, Kouchi H, Suzuka I, Ishii T (1991) Isolation and characterization of cDNA clones for plant cyclins. EMBO J **10**: 2681–2688

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Harvey Millar A (2004) Experimental Analysis of the Arabidopsis Mitochondrial Proteome Highlights Signaling

and Regulatory Components, Provides Assessment of Targeting Prediction Programs, and Indicates Plant-Specific Mitochondrial Proteins. Plant Cell **16**: 241–256

Heffer P, Prud'homme M (2014) Fertilizer Outlook 2014-2018. Paris

Hill R, Rinker G, Wilson H (1980) Atmospheric Nitrogen Fixation by Lightning. J Atmos Sci **37**: 179–192

Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot **58**: 2369–2387

Hirose N, Makita N, Kojima M, Kamada-Nobusada T, Sakakibara H (2007) Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. Plant Cell Physiol **48**: 523–539

Igarashi RY, Seefeldt LC (2003) Nitrogen fixation: the mechanism of the Mo-dependent nitrogenase. Crit Rev Biochem Mol Biol **38**: 351–384

Ivens WPMF, Tysmans DJJ, Kroeze C, Löhr AJ, van Wijnen J (2011) Modeling global N2O emissions from aquatic systems. Curr Opin Environ Sustain **3**: 350–358

Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, Kapoor S, Tyagi AK, Khurana JP (2007) F-box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiol **143**: 1467–1483

Kang J, Turano FJ (2003) The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100**: 6872–6877

Kant S, Bi Y-M, Rothstein SJ (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J Exp Bot **62**: 1499–1509

Kathiresan, A, Tung P, Chinnappa C, Reid D (1997) gamma-Aminobutyric acid stimulates ethylene biosynthesis in sunflower. Plant Physiol 129–135

Kiba T (2004) Arabidopsis Response Regulator, ARR22, Ectopic Expression of Which Results in Phenotypes Similar to the wol Cytokinin-Receptor Mutant. Plant Cell Physiol **45**: 1063–1077

Kiba T, Kudo T, Kojima M, Sakakibara H (2011) Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. J Exp Bot **62**: 1399–1409

Kim S, Yang J, An G, Jena KK (2016) A Simple DNA Preparation Method for High Quality Polymerase Chain Reaction in Rice. **2016**: 99–106

Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet **238**: 106–119

Kouchi H, Tsukamoto M, Tajima S (1990) Differential expression of nodule-specific (nodulin) genes in the infected, uninfected cortical cells of soybean (Glycine max) root nodules. J Plant Physiol **135**: 608–617

Kraiser T, Gras DE, Gutiérrez AG, González B, Gutiérrez R a. (2011) A holistic view of nitrogen acquisition in plants. J Exp Bot **62**: 1455–1466

Lacombe B, Becker D, Hendrich R, DeSalle R, Hollmann M, Kwak JM, Schroeder JI, Novere N, Nam HG, Spalding EP, et al (2011) The identity of plant glutamate receptors. **292**: 1486–1487

Lasanthi-Kudahettige R, Magneschi L, Loreti E, Gonzali S, Licausi F, Novi G, Beretta O, Vitulli F, Alpi A, Perata P (2007) Transcript profiling of the anoxic rice coleoptile. Plant Physiol **144**: 218–231

Li M, Xu W, Yang W, Kong Z, Xue Y (2007) Genome-wide gene expression profiling reveals conserved and novel molecular functions of the stigma in rice. Plant Physiol **144**: 1797–1812

Li Z, Yu J, Peng Y, Huang B (2016) Metabolic pathways regulated by γ -aminobutyric acid (GABA) contributing to heat tolerance in creeping bentgrass (Agrostis stolonifera). Nat Publ Gr 1–16

Lodwig E, Poole P (2003) Metabolism of Rhizobium bacteroids. CRC Crit Rev Plant Sci **22**: 37–78

Lovell CR, Piceno YM, Quattro JM, Bagwell CE (2000) Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, Spartina alterniflora. Appl Environ Microbiol **66**: 3814–3822

Ludewig F, Hüser A, Fromm H, Beauclair L, Bouché N (2008) Mutants of GABA transaminase (POP2) suppress the severe phenotype of succinic semialdehyde dehydrogenase (ssadh) mutants in arabidopsis. PLoS One. doi: 10.1371/journal.pone.0003383

Marschner H (1995) Nitrogen fixation. Miner. Nutr. High. Plants, 2nd ed. Academic Press, London, p 201

Marty NJ, Teresinski HJ, Hwang YT, Clendening E a., Gidda SK, Sliwinska E, Zhang D, Miernyk J a., Brito GC, Andrews DW, et al (2014) New insights into the targeting of a subset of tail-anchored proteins to the outer mitochondrial membrane. Front Plant Sci **5**: 426

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot **105**: 1141–1157

Masclaux-Daubresse C, Valadier MH, Carrayol E, Reisdorf-Cren M, Hirel B (2002) Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. Plant, Cell Environ **25**: 1451–1462

Mcallister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J **10**: 1011–1025

Meyer A, Sepehr E, Grallath S, Doris R (2006) AtGAT1, a High Affinity Transporter for γ-Aminobutyric Acid in Arabidopsis thaliana. J Biol Chem **281**: 7197–7204

Michaeli S, Fait A, Lagor K, Nunes-Nesi A, Grillich N, Yellin A, Bar D, Khan M, Fernie AR, Turano FJ, et al (2011) A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. Plant J **67**: 485–498

Morot-Gaudy J-F, Lea PJ (1997) Plant Nitrogen. *In* J-F Morot-Gaudy, PJ Lea, eds, Plant Nitrogen. Springer-Verlag, Berlin, p vII

Mulvaney RL, Khan S a, Ellsworth TR (2009) Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. J Environ Qual **38**: 2295–2314

Nelson N, Ben-Shem A (2004) The complex architecture of oxygenic photosynthesis. Nat Rev Mol Cell Biol **5**: 971–982

Netto AT, Campostrini E, De Oliveira JG, Bressan-Smith RE (2005) Photosynthetic pigments, nitrogen, chlorophyll a fluorescence and SPAD-502 readings in coffee leaves. Sci Hortic (Amsterdam) **104**: 199–209

Nikolovski N, Rubtsov D, Segura MP, Miles GP, Stevens TJ, Dunkley TPJ, Munro S, Lilley KS, Dupree P (2012) Putative Glycosyltransferases and Other Plant Golgi Apparatus Proteins Are Revealed by LOPIT Proteomics. Plant Physiol **160**: 1037–1051

Ogawara T, Higashi K, Kamada H, Ezura H (2003) Ethylene advances the transition from vegetative growth to flowering in Arabidopsis thaliana. J Plant Physiol **160**: 1335–40

Okubara PA, Fujishige NA, Hirsch AM, Berry AM (2000) Dg93, a nodule-abundant mRNA of Datisca glomerata with homology to a soybean early nodulin gene. Plant Physiol **122**: 1073–1079

Op den Camp RHM, Polone E, Fedorova E, Roelofsen W, Squartini A, Op den Camp HJM, Bisseling T, Geurts R (2012) Deploys a broad Rhizobium host range strategy resulting in largely variable symbiotic effectiveness. Mol Plant-Microbe Interact **25**: 954–963

Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, Childs K, Thibaud-Nissen F, Malek RL, Lee Y, Zheng L, et al (2007) The TIGR rice genome annotation resource: improvements and new features. Nucleic Acids Res **35**: 8–11

Pate JS (1973) Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol Biochem **5**: 109–119

Pawlowski K, Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? Plant Cell **8**: 1899–1913

Peng M, Bi YM, Zhu T, Rothstein SJ (2007) Genome-wide analysis of Arabidopsis responsive

transcriptome to nitrogen limitation and its regulation by the ubiquitin ligase gene NLA. Plant Mol Biol **65**: 775–797

Perin L, Martínez-Aguilar L, Paredes-Valdez G, Baldani JI, Estrada-de los Santos P, Reis VM, Caballero-Mellado J (2006) Burkholderia silvatlantica sp. nov., a diazotrophic bacterium associated with sugar cane and maize. Int J Syst Evol Microbiol **56**: 1931–1937

Van de Poel B, Van Der Straeten D (2014) 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: more than just the precursor of ethylene! Front Plant Sci **5**: 640

Postgate J (1998) Nitrogen Fixation. Nitrogen Fixat., 3rd ed. Cambridge University Press, Cambridge, pp 1–3

Rabalais NN, Turner RE, Scavia D (2002) Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River. Bioscience **52**: 129–142

Ramesh SA, Tyerman SD, Xu B, Bose J, Kaur S, Conn V, Domingos P, Ullah S, Wege S, Shabala S, et al (2015) GABA signalling modulates plant growth by directly regulating the activity of plant-specific anion transporters. Nat Commun **6**: 7879

Raun WR, Johnson GV (1999) Improving nitrogen use efficiency for cereal production. Agron J **91**: 357–363

Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide(N2O): the dominant ozone-depleting substance emitted in the 21st century. Science (80-) **326**: 123–125

Reddy PM, Kouchi H, Ladha JK (1998) Isolation, analysis and expression of homologues of the soybean early nodulin gene GmENOD93 (GmN93) from rice. Biochim Biophys Acta **1443**: 386–392

Renault H, El Amrani A, Palanivelu R, Updegraff EP, Yu A, Renou JP, Preuss D, Bouchereau A, Deleu C (2011) GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in Arabidopsis thaliana. Plant Cell Physiol **52**: 894–908

Renault H, Roussel V, El Amrani A, Arzel M, Renault D, Bouchereau A, Deleu C (2010) The Arabidopsis pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. BMC Plant Biol **10**: 20

Rolletschek H, Hosein F, Miranda M, Heim U, Götz K-P, Schlereth A, Borisjuk L, Saalbach I, Wobus U, Weber H (2005) Ectopic expression of an amino acid transporter (VfAAP1) in seeds of Vicia narbonensis and pea increases storage proteins. Plant Physiol **137**: 1236–1249

Rothstein SJ (2007) Returning to our roots: making plant biology research relevant to future challenges in agriculture. Plant Cell **19**: 2695–2699

Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E (2007) Ethylene Regulates Root Growth through Effects on Auxin Biosynthesis and Transport-Dependent Auxin Distribution. Plant Cell Online **19**: 2197–2212

Sanhueza E (1982) The role of the atmosphere in nitrogen cycling. Plant Soil **67**: 61–71

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of Arabidopsis thaliana development. Nat Genet **37**: 501–6

Schwacke R, Fischer K, Ketelsen B, Krupinska K, Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice. Mol Genet Genomics **277**: 631–646

Schwacke R, Schneider A, van der Graaff E, Fischer K, Catoni E, Desimone M, Frommer WB, Flügge U-I, Kunze R (2003) ARAMEMNON, a novel database for Arabidopsis integral membrane proteins. Plant Physiol **131**: 16–26

Shelp BJ, Bown AW, McLean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci **4**: 446–452

Shelp BJ, Mullen RT, Waller JC (2012) Compartmentation of GABA metabolism raises intriguing questions. Trends Plant Sci **17**: 57–59

Shi SQ, Shi Z, Jiang ZP, Qi LW, Sun XM, Li CX, Liu JF, Xiao WF, Zhang SG (2010) Effects of exogenous GABA on gene expression of Caragana intermedia roots under NaCl stress: Regulatory roles for H2O2 and ethylene production. Plant, Cell Environ **33**: 149–162

Smith BE (2002) Nitrogenase reveals its inner secrets. Science (80-) **297**: 1654–1655

Suzuki A, Knaff DB (2005) Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. Photosynth Res **83**: 191–217

Takahashi S, Badger MR (2011) Photoprotection in plants: A new light on photosystem II damage. Trends Plant Sci **16**: 53–60

Thole JM, Beisner ER, Liu J, Venkova S V, Strader LC (2014) Abscisic acid regulates root elongation through the activities of auxin and ethylene in Arabidopsis thaliana. G3 (Bethesda) **4**: 1259–74

Tholen D, Voesenek LACJ, Poorter H (2004) Ethylene insensitivity does not increase leaf area or relative growth rate in Arabidopsis, Nicotiana tabacum, and Petunia x hybrida. Plant Physiol **134**: 1803–12

Tilman D (1999) Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc Natl Acad Sci U S A **96**: 5995–6000

Tocquin P, Corbesier L, Havelange A, Pieltain A, Kurtem E, Bernier G, Périlleux C (2003) A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of Arabidopsis thaliana. BMC Plant Biol **3**: 2

United Nations: Department of Social and Economic Affairs (2013) World population prospects.

Le Van Quy, Foyer C, Champigny ML (1991) Effect of light and NO(3) on wheat leaf

phosphoenolpyruvate carboxylase activity: evidence for covalent modulation of the C(3) enzyme. Plant Physiol **97**: 1476–1482

Verma D, Hu C, Zhang M (1992) Root nodule development: origin, function and regulation of nodulin genes. Physiol Plant **85**: 253–265

Wang WH, Köhler B, Cao FQ, Liu GW, Gong YY, Sheng S, Song QC, Cheng XY, Garnett T, Okamoto M, et al (2012) Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis. New Phytol **193**: 432–444

Weigelt K, Küster H, Radchuk R, Müller M, Weichert H, Fait A, Fernie AR, Saalbach I, Weber H (2008) Increasing amino acid supply in pea embryos reveals specific interactions of N and C metabolism, and highlights the importance of mitochondrial metabolism. Plant J **55**: 909–926

Winter D, Vinegar B, Nahal H, Ammar R, Wilson G V., Provart NJ (2007) An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One **2**: 1–12

World Energy Council (2013) World energy resources.

Wu F-H, Shen S-C, Lee L-Y, Lee S-H, Chan M-T, Lin C-S (2009) Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. Plant Methods **5**: 16

Wuebbles DJ (2009) Nitrous oxide: no laughing matter. Science (80-) **326**: 56–57

Xu W, Jia L, Shi W, Liang J, Zhou F, Li Q, Zhang J (2013) Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. New Phytol **197**: 139–150

Yang L, Zhang J, He J, Qin Y, Hua D, Duan Y, Chen Z, Gong Z (2014) ABA-Mediated ROS in Mitochondria Regulate Root Meristem Activity by Controlling PLETHORA Expression in Arabidopsis. PLoS Genet. doi: 10.1371/journal.pgen.1004791

Yu G, Liang J, He Z, Sun M (2006) Quantum Dot-Mediated Detection of γ-Aminobutyric Acid Binding Sites on the Surface of Living Pollen Protoplasts in Tobacco. Chem Biol **13**: 723–731

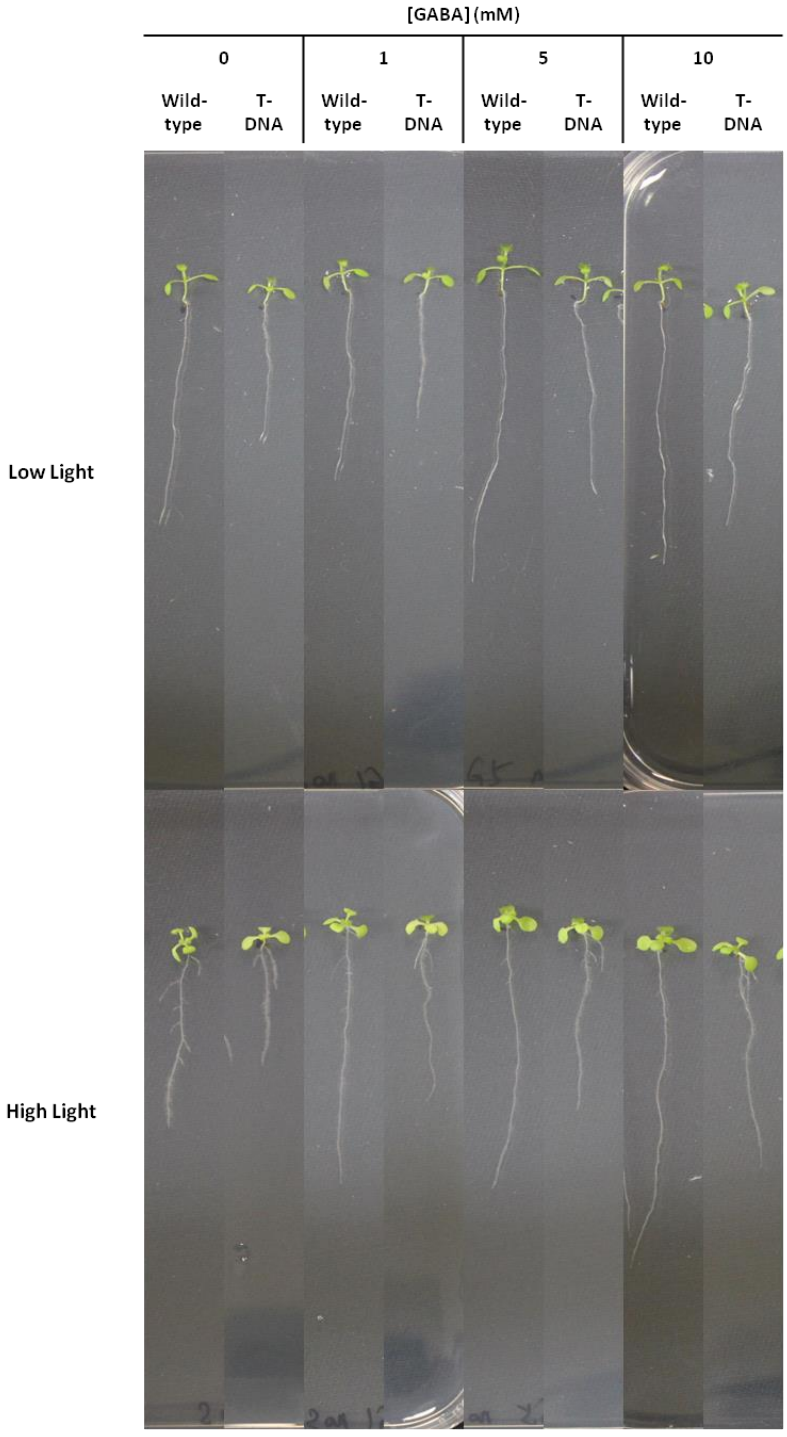
Zarei A, Trobacher CP, Shelp BJ (2016) Arabidopsis aldehyde dehydrogenase 10 family members confer salt tolerance through putrescine-derived 4-aminobutyrate (GABA) production. Sci Rep **6**: 35115

Zheng Z-L (2009) Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav **4**: 584–591

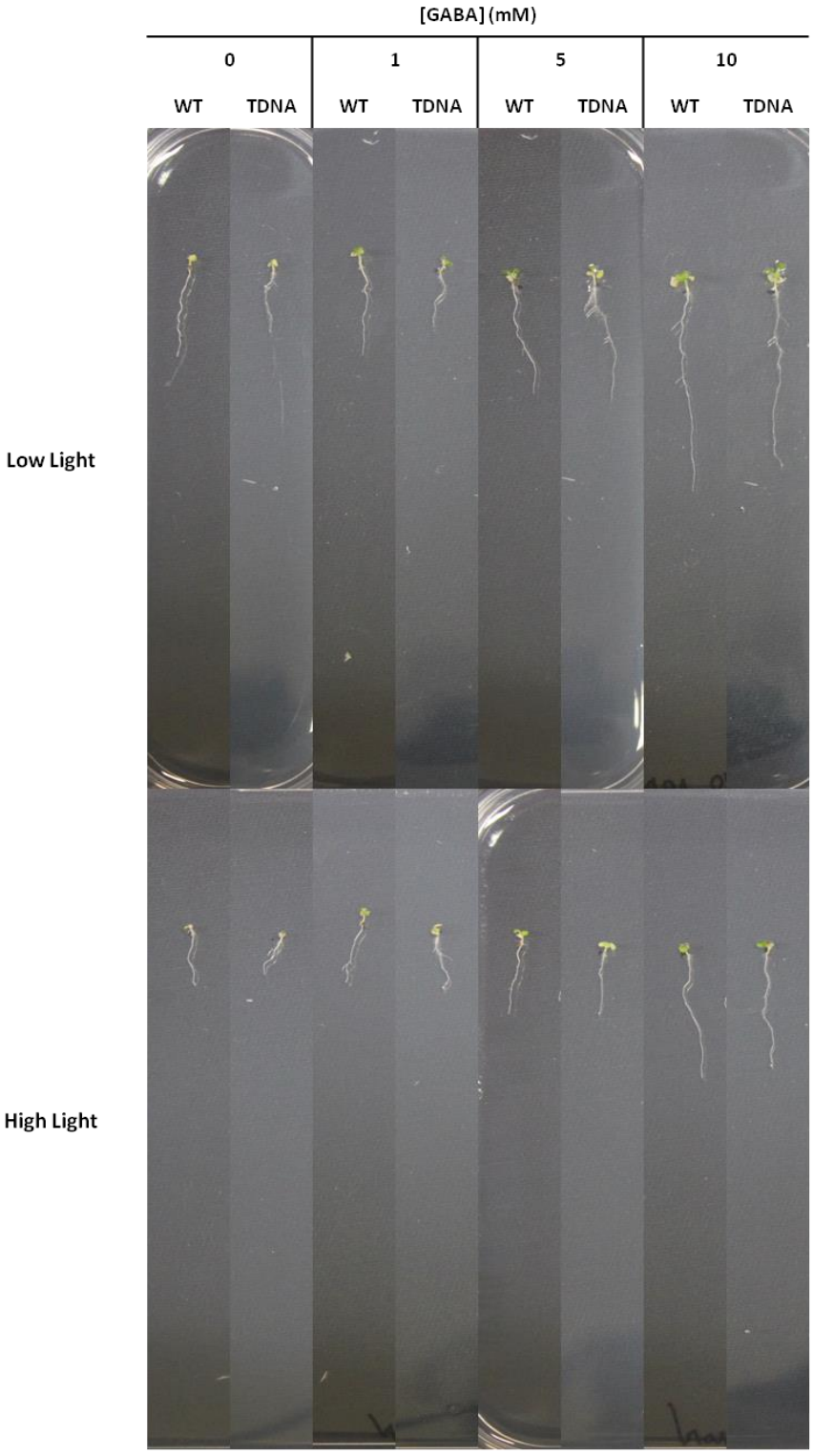
Zhou Li , Jingjin Yu , Yan Peng BH (2016) Metabolic pathways regulated by abscisic acid, salicylic acid and γ-aminobutyric acid in association with improved drought tolerance in creeping bentgrass (Agrostis stolonifera). Physiol Plant doi:10.1111/ppl.12483

Zhu T, Budworth P, Chen W, Provart N, Chang H-S, Guimil S, Su W, Estes B, Zou G, Wang X (2003) Transcriptional control of nutrient partitioning during rice grain filling. Plant Biotechnol J **1**: 59–70

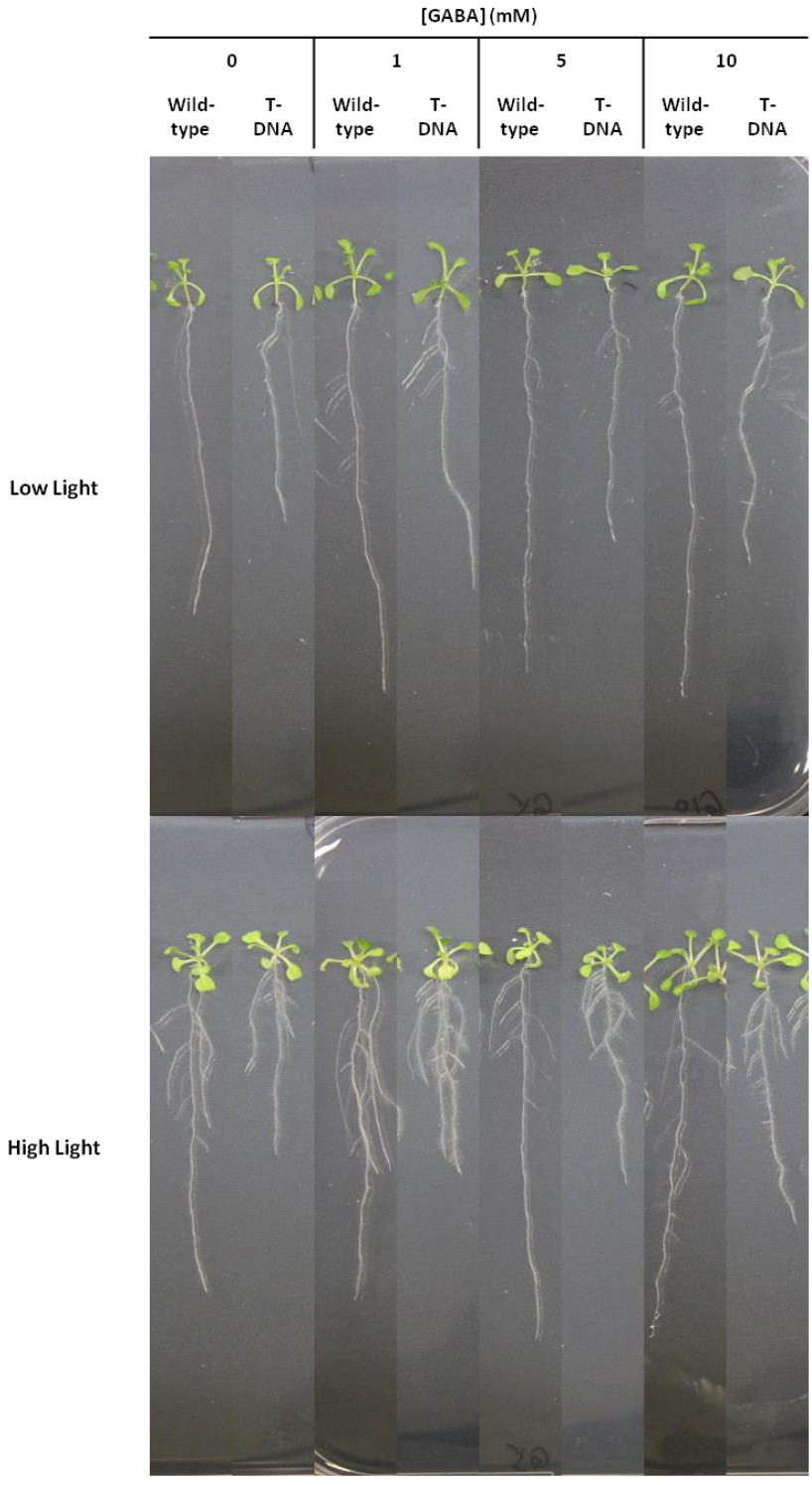
APPENDIX I: ROOT PICTURES



Appendix I - Figure 1. Seedlings grown for 14 days on 1/2 MS agar plates with no sucrose at low and high light conditions without added sucrose.



Appendix I - Figure 2. Seedlings grown for 14 days on 1/2 MS agar plates with no nitrogen at low and high light conditions without added nitrogen.



Appendix I - Figure 3. Seedlings grown for 14 days on 1/2 MS agar plates with sucrose at low and high light conditions with added sucrose and nitrogen.