Abstract

A Functional Analysis of microRNAs in *Nicotiana tabacum*

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microRNAs (miRNAs) are a newly discovered class of endogenous posttranscriptional gene regulators that are typically 20-22 nucleotides in length that do not code for proteins. miRNAs regulate gene expression by either inhibiting protein translation, or by targeting messenger RNAs (mRNAs) for cleavage. miRNAs are highly evolutionarily conserved and have been found in many plants such as corn, soybeans, rice, and *Arabidopsis*. *Nicotiana tabacum*, or cultivated tobacco, is an important economic, agricultural, and research crop that provided approximately $4.4 billion dollars to the United States economy in 2007. Although much genetic research has already been dedicated to tobacco, hardly any research regarding the role of miRNAs has been performed. In this project, the primary objective was to provide a functional analysis of miRNAs in *Nicotiana tabacum*. First, the expression profiles of miRNAs and their targets were generated for different organs through the use of quantitative real time PCR (qRT-PCR). It was found that all the miRNAs and targets that were tested were differentially expressed throughout different tissue types in tobacco. In particular, miR159 was found to be expressed the highest in all tissue types tested. Secondly, very few reference gene analyses have been performed in tobacco, therefore a reference gene analysis in tobacco was conducted. In this

analysis, 12 housekeeping genes were tested for their effectiveness in serving as reference genes for gene expression analyses in tobacco. qRT-PCR was used to quantify the amount of expression of each candidate reference gene, and a new comprehensive reference gene analysis tool, RefFinder, was used to rank the candidate reference genes based on the stability of their expression. The housekeeping genes that show the least amount of variability in expression were deemed appropriate reference genes for use. Through the use of RefFinder, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and PP2A (protein phosphatase 2A) were identified as being the most relaible reference genes for use in tobacco gene expression studies. Finally, microRNAs have been identified that mediate stress responses in plants to abiotic factors, but there have been few studies conducted on the effects nanoparticles may have on tobacco growth, development, and microRNA expression. To study this, tobacco seedlings were exposed to varying concentrations of aluminum oxide nanoparticles and tested to see which miRNAs were significantly changed in expression. It was found that the growth and development of tobacco seedlings was significantly adversely affected by increasing concentrations of aluminum oxide nanoparticles. In addition, several miRNAs were identified that may play a significant role in mediating plant responses to nanoparticles stress due to the magnitude of up regulation in expression. Overall, by providing a functional analysis of miRNAs in tobacco, these results will further help scientists to understand how plants react to their environment and will allow the further use of miRNA-mediated biotechnology to further improve crop yield and quality.

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A FUNCTIONAL ANALYSIS OF MICRORNAS IN NICOTIANA TABACUM

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ADH	Alcohol Dehydrogenase
AGO	ARGONAUTE
Al_2O_3	Aluminum Oxide
Al_3	Aluminum
ANOVA	Analysis of Variance
AP1	Apetala1
AP2	Apetala2
APX	Ascorbate Peroxidase
ARF	Auxin Response Factor
ATP	Adenosine Triphosphate
ATPS	ATP Sulfurylase
^oC	Celsius
CBC	Cap Binding Complex
cDNA	Complementary DNA
CSOD	Copper Chaperone for Superoxide Dismutase
C_T	Cycle Threshold

DDL	DAWDLE
EF1α	Elongation Factor 1α
FeSOD	Iron Superoxide Dismutase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GRF	Growth Regulating Factor
HEN1	HUA ENHANCER1
HrBP1	Hairpin Binding Protein1
HYL1	HYPONASTIC LEAVES1
L25	25S rRNA-binding Protein
L	Liter
LFY	LEAFY
mg	Milligram
miRNA	microRNA
mL	Milliliter
mm	Millimeter
mRNA	Messenger RNA
MYB	MYB Transcription Factor

nt	Nucleotide
PCR	Polymerase Chain Reaction
PE2U	Phosphate/E2 Ubiquitin Conjugating Protein
PP2A	Protein Phosphatase2
pri-miRNA	Primary microRNA
qRT-PCR	Quantitative Real Time PCR
rbcmtT	RUBISCO Large Subunit N-methyltransferase
RISC	RNA Induced Silencing Complex
Rn18S	18S Ribosomal RNA
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcription PCR
RUBISCO	Ribulose-1,5 bisphosphate carboxylase/oxygenase
SE	SERRATE
SOD	Copper Superoxide Dismutase
SPL	Squamosa-promoter Binding Protein-like
sRNA	Small RNA

TCP	TCP Transcription Factor
TUB	Tubulin
UB1	Ubiquitin
UV	Ultraviolet
Zn	Zinc
ZnO	Zinc Oxide

CHAPTER 1: INTRODUCTION

It is a common generalization among evolutionary biologists that animals and plants cope with environmental stressors in a different manner due to the nature of their lifestyles. Motile animals have an advantage over plants in evading environmental stressors due to their ability to simply migrate away from the stressor, whereas plants are sessile and must employ different mechanisms to evade stressors. Some scientists argue that plants can move away from environmental stressors by mechanisms involving seed dispersal or by simply growing away from stressors (i.e. vines) (*1*), but these mechanisms are not an immediate solution. Plants must evolve more complex internal mechanisms of evading or responding to environmental cues through phenotypic plasticity (*1*). Therefore in order to maintain phenotypic plasticity, plants must evolve complex mechanisms to alter the transcription and translation of genes into functional proteins. A newly discovered class of post transcriptional gene regulators called microRNAs (miRNAs) will be the focus of this thesis due to their primary mode of aiding plants in being "master" regulators through post transcriptional gene regulation.

Even though the first miRNA was discovered in 1993 in *Caenorhabditis elegans (2, 3)*, they were not recognized as a separate class of RNA until 2001 (*4-6*). The following year, the first plant miRNA, a Dicer homolog known as carpel factory, was discovered in *Arabidopsis (7)*. Since then, hundreds of miRNAs have been identified in both plants and animals (*8, 9*). miRNAs are a non-coding endogenous class of post transcriptional gene regulators that function to either target messenger RNAs (mRNAs) for cleavage, or through preventing translation (*10*). In plants, mature miRNAs tend to be 20-22 nucleotides in length and are many are highly evolutionarily conserved from mosses to monocots and eudicots which diverged approximately 125 million years ago (*11, 12*). Consequently, many microRNAs have a deep evolutionary

history that is shown through their major identified functions. Highly conserved plant microRNAs have been shown to largely regulate the expression of transcription factors, therefore microRNAs are considered to be master gene regulators within the cell. Some microRNAs are not highly conserved, and are thought to control more specific biological processes within the organism. microRNAs function to control many metabolic and biological processes, and include but are not limited to, organ maturation, cell proliferation, hormone signaling, phase change from vegetative to reproductive growth, and response to environmental stressors.

Most plant miRNAs are located in intergenic regions in the genome, and are transcribed largely by DNA-Dependent RNA Polymerase II (*13*). Once microRNA (MIR) genes are transcribed, the transcript is referred to as a primary miRNA (pri-miRNA) and they are processed like other transcripts with the addition a 5' cap as well as a 3' polyadenylated tail. Pri-miRNAs form an imperfect fold-back structure, which is stabilized through the RNA-binding protein, DAWDLE (DDL) which interacts with the protein, Dicer-like1 (*14*). Pri-miRNAs are processed into a stem-loop precursor (pre-miRNA) largely through the action of the enzyme, Dicer-like1. The function of the Dicer-like family is to produce sRNA strands with a specific size: between 18 and 24 nucleotides depending on the specific family member (*13*). In order to assist pri-miRNA to pre-miRNA conversion, the double-stranded RNA-binding protein, HYPONASTIC LEAVES1 (HYL1), the C2H2-zinc finger protein, SERRATE (SE), and the nuclear cap-binding complex (CBC) interact with Dicer-like1 in miRNA processing centers known as D-bodies (*15, 16*). The mature miRNA duplex (miRNA:miRNA*), excised by Dicer-like1, is then stabilized by the S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1) which acts to methylate the duplex prior to exportation from the nucleus, therefore preventing it from being degraded by small RNA degrading nucleases in the cytoplasm

(*13*). After methylation, the mature miRNA duplex is transported out of the nucleus through the plant homolog of exportin-5, HASTY (*17*).

The miRNA:miRNA* duplex consists of a sense, guide strand (miRNA), and a degraded strand (miRNA*). Once the miRNA* strand is degraded, the mature miRNA is incorporated into a RNA-Induced Silencing Complex (RISC), that contains one member of the ARGONAUTE (AGO) protein family. The RISC complex then scans the cell for either perfectly or near perfectly complementary mRNAs and binds to them. The RISC complex contains a sRNA-binding PAZ domain and a PIWI domain with catalytic residues that allows the complex to have endonucleolytic acitivity (*13*). When a miRNA is perfectly complimentary to its mRNA target, almost always the RISC complex acts to cleave the mRNA through its endonucleolytic activity. Because plant miRNAs tend to be more perfectly complimentary to their targets, this mode of action is the more common mode of action that is taken to post transcriptionally modify gene expression. On the other hand, if a miRNA is not perfectly complimentary, the RISC complex acts to prevent translation of the mRNA. The exact mechanism of this event is not currently known.

Nicotiana tabacum, or cultivated tobacco, is an important economic, agricultural, and research crop. Tobacco is widely grown across the world, but within the United States, North Carolina and Louisiana have produced the most in the past decade, according to the United States Department of Agriculture census (*18, 19*). Not only is the production of tobacco a major contributor to the economy due to the production of tobacco products such as cigarettes and cigars, but tobacco has recently been considered as a potential biofuel crop (*20*). As a result, any genetic studies that are performed to help elucidate pathways in which tobacco can be genetically modified to grow in more extreme environments or with increasing oil production for biofuel use

would be beneficial. Although much genetic research has already been dedicated to tobacco, hardly any research regarding the role of miRNAs has been performed. As a result, the goal of this project is to provide a functional analysis of miRNAs in tobacco.

The primary goals of this project are to provide a functional analysis of miRNAs in tobacco for further gene expression analyses and include the following objectives:

1. **To provide a general expression profile for miRNAs and their targets in tobacco through the use of quantitative real-time PCR.**

In a study conducted by Frazier et al -, 259 potential miRNAs were computationally identified in tobacco which were subdivided into 65 families (*21*). Out of all of the potential miRNAs that were identified in tobacco, 11 miRNAs were validated through the use of qRT-PCR. These miRNAs included miR156, miR159, miR162, miR167, miR169, miR172, miR393, miR395, miR396, miR398, and miR399 (*21*). However, no study has been performed on the expression profile of miRNAs and their targets during tobacco development. This objective will provide a follow-up study to the project completed by Frazier et al -. This study will provide greater understanding of not only the function of miRNAs and their targets, but the relative abundance in wild type *Nicotiana tabacum*.

2. **To identify and evaluate stable reference genes for use in normalizing gene expression studies in tobacco.**

To study a gene expression profile using qRT-PCR and other methods, it is important to identify a reliable reference gene that is expressed in a stable manner. A recent study provided a partial list of ideal reference genes that can serve to normalize gene expression analyses in tobacco (*22*). In that study, they evaluated the following genes in their effectiveness to serve as

normalizing reference genes: 18s rRNA, actin, elongation factor 1 α, L25 ribosomal protein, α-tubulin, β-tubulin, ubiquitin-conjugative enzyme E2, protein phosphatase 2A, and NTCP-23; in which the expression of these genes was evaluated in the leaf, stem, root, anther, and carpel in tobacco that had been exposed to differing stresses (heat, cold, salt, drought, and UV irradiation). Based on their results, they identified three genes that are sufficient for normalization of gene expression using qRT-PCR which include *L25*, *EF-1α*, and *Ntubc2*. However, it is unclear that these reference genes can be used in other cultivars under other conditions. In this objective, housekeeping genes were further tested for their effectiveness in the normalization of gene expression in qRT-PCR analyses in tobacco under nanoparticle stress conditions using the reference gene tool, RefFinder. By providing more a species specific reference gene list, the quality of normalizing gene expression in tobacco will be vastly improved and streamlined.

3. **To investigate the effects of aluminum oxide nanoparticles on the growth, development, and microRNA expression profile in tobacco.**

miRNAs are involved in the control of a wide variety of plant responses to the environment, consequently, it is vital to study the effects differing stressors have on plants and how those plants cope with the stressor. If the mechanisms by which plants respond to stress are elucidated, plants can be genetically improved for growth in environments that would not typically be ideal. Because of their increasing use in industry, nanoparticles are an emerging environmental contaminant. To date, few experiments have been performed to show the effects nanoparticles have on the growth, development, and gene expression in plants. In this objective, the effects of aluminum oxide nanoparticles on tobacco growth and development, as well the effect on the miRNA expression profile, will be investigated.

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CHAPTER 2: EXPRESSION PROFILE OF MICRORNAS AND THEIR TARGETS IN *NICOTIANA TABACUM*

Abstract

microRNAs (miRNAs) are a newly discovered class of endogenous posttranscriptional gene regulators that function to mediate gene expression by cleaving target messenger RNAs (mRNAs), or by preventing translation. Because of their importance in mediating gene regulation, identifying and elucidating the function of miRNAs has been the primary focus of many researchers. Now that many miRNAs have been identified and assessed for their functionality, the next step is to create expression profiles for miRNAs, so that gene expression studies can be further enhanced with knowledge of the basal expression levels of miRNAs and their targets. In a previous study, 259 putative miRNAs were identified in tobacco, in which 11 of them were confirmed. The primary goal of this study was to further expand on that study and create an expression profile for 9 of those miRNAs and their targets in a tissue specific manner in tobacco. We chose to study miRNAs that largely play a role in floral development and nutrient stress response. The results of our study show that all tested miRNAs and their targets were expressed in a differential manner. The results of our study also show that out of the tested miRNAs and their targets, miR159, miR157, miR167, miR172 and superoxide dismutase were expressed the highest, suggesting that these genes may play a vital role in the growth and development of tobacco.

Key Words: microRNA, gene expression, expression profile, tobacco

Introduction

Nicotiana tabacum, or cultivated tobacco, is one of the most important agricultural crops in the Southeastern portion of the United States due to its use in the production of products used for smoking. For the past decade, North Carolina and Louisiana have been the largest producers of tobacco in the Southeast (*1*, *2*). Not only is tobacco a large cash crop that is extensively used to produce tobacco products, tobacco is currently being researched for potential alternative uses. Tobacco has recently been identified as having the potential to become a biofuel plant by transgenically altering it to produce more oils that can be used for energy (*3*). Tobacco is a prime candidate for biofuel production due to its large leaf surface area, and the fact that it is not in demand for food production like soybeans and corn. Tobacco is also an ideal model organism for gene expression studies because its genome has almost completely been sequenced and because it is easy to grow and maintain. Due to the great potential of tobacco to be used for other purposes, more studies need to be conducted in order to fully understand the regulation of its growth and development in order to increase the quality and productivity of tobacco for other uses.

microRNAs are a newly discovered class of post transcriptional gene regulators that are approximately 20-22 nucleotides in length that do not code for protein (*4*-*6*). microRNAs act largely in mediating gene expression post transcriptionally by either preventing the translation of messenger RNAs (mRNAs) into proteins, or by targeting mRNAs for cleavage prior to translation (*4*-*6*). The path in which miRNAs work is determined by the sequence complimentarity of miRNAs to their target mRNAs. If the miRNA has perfect sequence complimentarity to the mRNA, the mRNA is targeted for cleavage, but if the miRNA is not perfectly complimentary to the mRNA target, translation is prevented. In plants, the primary

mode of microRNA action is through miRNA-directed cleavage of the mRNA due to the high amount of miRNAs that are perfectly complimentary to their mRNA targets.

Although miRNAs have only been recently discovered in the past 20 years, miRNAs have been shown to play key roles in the regulation of many different processes. Specifically in plants, miRNAs have been shown to aid in the regulation of leaf and root development, organ maturation, cell proliferation (*7*), flowering time (*6, 8*), and stress responses (*9-12*). Recent studies have also shown that miRNAs help to regulate approximately more than 30% of protein coding genes and this number is expected to increase as more miRNAs are discovered and their mRNA targets are identified.

The aim of this study is to provide a tissue specific miRNA expression profile for 9 miRNAs and some common protein coding genes in tobacco. Now that the specific functions of miRNAs are being discovered, it is important to now classify their basal expression levels in a tissue dependent manner. This will further provide information for the advancement of gene expression studies because if one knows a miRNA is generally highly, moderately, or not expressed in a tissue, the general expression of its protein target can be projected. The following miRNAs were chosen to study in this experiment due to the fact that they all have some commonly known functions in plants and were validated in a previous study: miR156, miR157, miR159, miR167, miR172, miR395, miR397, miR398, and miR399 (*13*). The expression levels of the following protein coding genes will also be evaluated: Squamosa-promoter Binding Protein-like (SPL), MYB Transcription Factor (MYB), Auxin Response Factor (ARF), Apetala2-like Transcription Factor (AP2), ATP-sulfurylase (ATPS), Copper Superoxide Dismutase (SOD), and Phosphate/E2 Ubiquitin-conjugating Protein (PE2U). Lastly, the relationships between some miRNAs and their targets will be evaluated. These findings will further enhance the

understanding of the roles miRNAs and their targets have in the development of tobacco which will help to advance other fields of research.

Materials and Methods

Tobacco Tissue Collection

Nicotiana tabacum c.v. NCSU seeds were planted in the East Carolina University Greenhouse Facility and were allowed to reach maturity (flowering) which takes approximately 4-5 months. Once plants reached maturity, eight differing tissue types were collected and flash frozen in liquid nitrogen. The tissue types that were collected are as follows: young leaves, old leaves, sepals, petals, ovary, stigma and style, stamen, and whole immature flowers. Leaves were deemed young if they were located at the top of the tobacco plant where the flowers emerged. Old leaves were collected at approximately the 20^{th} leaf from the top near the center of the mature plant. After flash freezing in liquid nitrogen, the tissues were stored in a $-80^{o}C$ freezer until further RNA extraction.

RNA Extraction and Quantification

After freezing, each tissue type had the RNA extracted with a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Once the RNA was extracted, a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) was used to quantify the amount of RNA per microliter. The 260/280 and 260/230 ratios were also noted to ensure that the quality of the RNA was acceptable before proceeding to the next step. Each tissue type was collected in triplicate, therefore there were three biological replicates for each tissue type.

Quantifying the miRNA and Gene Expression Levels

First, reverse transcription PCR (RT-PCR) was used to generate a single-stranged miRNA cDNA sequence from 1 µg of the total RNA collected from each tobacco tissue type. The cDNA was generated using reverse transcription with the Appled Biosystems TaqMan® microRNA Reverse Transcription Kit and the miRNA-specific stem-looped RT primers provided with the kit according to the manufacturer's protocol. Previous studies have shown that miR156, miR157, miR159, miR167, miR172, miR395, miR397, miR398, and miR399 are important in different growth and development pathways in plants, therefore these miRNAs were chosen to test. The following protein coding genes were also selected because several of them are the known targets of a few miRNAs that were screened for in this project: SPL, MYB, ARF, AP2, ATPS, SOD, and PE2U. cDNA was generated for the protein coding genes by simply using a poly-T primer and the same kit previously described for microRNAs.

Applied Biosystems TaqMan® microRNA Assays were used to detect and to quantify the miRNA and protein coding gene expression in tobacco using quantitative real-time PCR (qRT-PCR). These reactions were performed in 96 well optical plates in which each well contained cDNA, TaqMan mix, and the specific forward and reverse primer for the miRNA or protein coding gene of interest. The fluorescent probe that was used was SYBR green and each reaction plate was run on an Applied Biosystems 7300 Sequence Detection System (Foster City, CA). Tubulin, a common housekeeping gene, was used as a reference gene in order to normalize gene expression values. Three biological replicates were run for each gene within each tissue type and each biological replicate was technically duplicated to minimize the chance of error. The results were analyzed using the $\Delta\Delta C_T$ method.

Results

microRNAs are differentially expressed in differing tissue types in tobacco

In a previous study, we identified 259 tobacco microRNAs using a genome survey sequence analysis and 11 of those miRNAs were confirmed in tobacco by qRT-PCR (*13*). In this study, qRT-PCR was employed to detect the relative expression levels of these miRNAs throughout eight differing tissue types within a mature tobacco plant. These tissue types included young leaves, old leaves, sepals, petals, ovary, stigma and style, stamen, and whole immature flowers. All of the selected miRNAs have known functions in the plant and play key roles in the growth, development, and response to environmental stresses. Therefore, it is important to quantify the amount of expression these miRNAs have in a tissue specific manner to further our knowledge and understanding on how these miRNAs function.

In the floral tissues, miRNA expression profiles were very similar to each other. In the petal, stigma/style, and the sepals, miR159 was expressed the most out of all miRNAs tested. In the petal, stigma/style, and sepals, miR159 was expressed with a fold increase in expression of 41, 89, and 111, respectively, as compared to the expression of the reference gene, tubulin (See Figure 2-1). The next highest miRNA in the petal was miR157, in the stigma/style, miR172, and in the sepals, miR159 with fold changes of 4, 14, and 13, respectively (See Figure 2-1). Although not the highest in expression, it should be noted that miR157 was also one of the highest expressed miRNAs in the stigma/style and the sepals. In the petal, stigma/style, and sepals, miR399 was expressed the least out of all miRNAs tested with a fold change as compared to tubulin of 0.2, 0.5, and 0.4, respectively (See Figure 2-1). miR398 was also one of the least

expressed miRNAs after miR399 with fold changes of 0.5, 0.7, and 0.8, respectively (See Figure 2-1).

In the stamen and ovary, miR167 was expressed the most with a fold change of 10 and 41, respectively. Following miR167 in expression was miR159 and miR172 with fold changes of 8 and 20 (miR159) and 1 and 3 (miR172), respectively (See Figure 2-1). In both the stamen and ovary, miR399 was expressed the least with fold changes of 0.03 and 0.1 (See Figure 2-1). Besides miR399, miR395, miR397, and miR398 were also expressed the least out of all miRNAs tested.

The expression profiles of the immature flower, young leaf, and old leaf were similar in regards to which miRNAs were expressed the most and least. Out of these three tissues, miR159 was expressed the highest with fold changes of 31, 40, and 171, respectively (See Figure 2-1). Next, miR167 was the second highest expressed with fold changes of 24, 12, and 134, respectively (See Figure 2-1). miR172 was expressed third highest in all tissues with fold changes of 3, 3, and 34, respectively (See Figure 2-1). In all tissues, miR399 was expressed the least with a fold change in expression of 0.04, 0.08, and 0.2, respectively (See Figure 2-1).

Protein coding genes are differentially expressed in tobacco tissues

In all tissue types, superoxide dismutase (SOD) was expressed the most out of all protein coding genes tested as compared to the reference gene, tubulin (See Figure 2-2). The fold changes of SOD are as follows: petal, 10; stigma/style, 8; sepals, 5; stamen, 1; ovary, 4; immature flower, 3; young leaf, 1; and old leaf, 2 (See Figure 2-2). In the immature flower, young leaf, and old leaf, Squamosa-promoter binding protein (SPL) was expressed the highest next to SOD. MYB Transcription Factor (MYB) was expressed the least in all tissue types. The

fold changes of MYB are as follows: petal, 0.006; stigma/style, 0.1; sepals, 0.02; stamen, 0.001; ovary, 0.09; immature flower, 0.02; young leaf, 0.01; and old leaf, 0.004 (See Figure 2-2). In the stigma/style, sepals, stamen, ovary, and immature flower, Apetala2 (AP2) was expressed the lowest after MYB. In the young and old leaf, Auxin Response Factor (ARF) was expressed the lowest after MYB and in the stamen and ovary, ARF was expressed the next lowest after AP2.

Discussion

miRNAs and targets that are regulated by hormones

miR159 is a highly conserved miRNA that functions in a wide range of developmental processes such as plant growth, morphogenesis, and reproduction (*5*). miR159 is typically found to be abundant and widespread throughout entire plants, therefore it would be expected for to also be abundant in the expression profile of tobacco (*14*). The target of miR159 is MYB Transcription Factor (MYB) and their interplay has been shown to control vegetative growth, flowering time, anther development, seed shape and germination (*15-17*). In this study, miR159 was found to be one of the highest expressed miRNAs and MYB was found to be one of the least expressed protein coding gene across all tissue types. Due to the wide functionality of the relationship between miR159 and MYB, it is expected to see miR159 expressed highly and for its target MYB to be expressed much lower. Consequently, our results show that the relationship of miR159 and MYB is very important to the growth and development of tobacco plants due to the extreme inverse relationship in expression that these two genes exhibit.

miRNAs and their protein targets involving flower development and timing

miR156, miR157, and miR172 all have been identified to have a related functionality in *Arabidopsis* (*8, 18, 19*). miR156 and miR157 are classified as belonging to the same family

because of their similar sequences and their shared conserved target, Squamosa-promoter Binding-like proteins (SPL) (*18*). miR156 is one of the most highly conserved miRNAs and is known to be a master regulator of the juvenile to adult transition in plants by affecting the expression of several pathways that control differing phases of the transition (*8*). miR156 regulates the expression of miR172 via SPL which acts as a direct transcriptional regulator of miR172. As a result, if miR156 is highly expressed, the levels of the target SPL will be low, which in turn, will keep the expression levels of miR172 low (*8, 19*). If miR172 levels are low in a plant, the flowering of the plant will subsequently be delayed because the target of miR172 is Apetala 2 (AP2) which is involved in promoting flowering in plants (*8*). If a plant is flowering, one would expect for the levels of miR156/157 and SPL to be high, therefore keeping the levels of miR172 high, which in turn would cause the levels of AP2 to be low. If the levels of AP2 are low, the transcription of genes such as AP1 and LFY which are known to trigger flowering will be high (*20*).

In the mature flowering tobacco plants, we found that miR156 was only expressed in low levels in the immature flower, stamen, ovary, and young leaf and was more highly expressed in the petals, stigma and style, sepals, and the old leaf. Similarly, miR157 was highly expressed in the petals, stigma/style, and sepals. Because a high expression level of miR156 and miR157 is currently known to have a positive effect on the timing and development of flowers, through the down-stream down-regulation of AP2, the high expression of miR156 and miR157 in the petal, stigma/style, and sepals indicates that these two miRNAs may be important to the development and regulation of flowering time in these particular floral tissues.

SPL is the target of miR156 and miR157, and if the levels those miRNAs are high, one would expect that the levels of SPL would be low, therefore keeping the levels of miR172 low

which indirectly promotes flowering. Our results show that the levels of SPL were low in all tissues except for the immature flower, young leaf, and old leaf. Because low levels of SPL indirectly promote flowering in plants, the levels of SPL should be the lowest in flower tissues. The results of this study show that SPL is expressed low in the petals, stigma/style, sepals, stamen, and ovary which is what is expected. SPL was expressed high in the immature flower, young leaf, and old leaf which are not tissues that are flowering, therefore our results indicate that SPL critical to the development and timing of flowering.

miR172 is expressed low when miR156 and miR157 are expressed in a high manner via regulation through the transcription factor, SPL. In our study, we found that miR172 was highly expressed in the stigma/style, sepals, stamen, ovary, immature flower, young leaf, and old leaf. When miR172 is highly expressed, it causes its target, AP2, to be down-regulated which in turn promotes flowering. Because the expression of miR172 promotes flowering through targeting AP2, it is expected for miR172 to be highly expressed and for AP2 to be expressed low in floral tissues in particular. Our results show that miR172 was highly expressed and AP2 was expressed low in the stigma/style, sepals, stamen, ovary, and immature flower. Our results indicate that these two genes do in fact play a key role in the development of flowers because our results mirror the trend that has already been shown between these two genes in other plant species. It is possible that we see this trend in the young leaves in particular because these tissue samples were taken from very close to where the inflorescence was on the mature tobacco. Also, the high expression of miR172 in the old leaf hints that there may be an alternate role of miR172 in another capacity in plant growth and development.

miR167 has been shown to regulate both female and male floral organ development in *Arabidopsis* through the regulation in expression of auxin response factors (ARFs) (*21*). Our

results show that across all eight tissue types, miR167 was expressed the most in the sepals, stamen, ovary, immature flower, young leaf, and old leaf. ARF was shown to be least expressed in the stamen, ovary, young leaf, and old leaf. From previous studies, it is expected to see miR167 up-regulated and its target ARF to be down-regulated in parts of the flower because these two genes function in the regulation of female and male floral organ development. Out of all of the floral tissues that miR167 was highly expressed in, the immature flower followed by the ovary showed the highest amounts of miR167 expression. As a result, our results coincide with the known functions of these genes. Because miR167 was highly expressed in the young and old leaves, miR167 may possibly play an alternate role in leaf development.

miRNAs and their targets that mediate nutrient stress response

Out of all of the miRNAs and targets that we chose to study, several of them have been shown to be involved in mediating nutrient stress response in plants. miR395, miR397, miR398, and miR399 have all been shown to mediate stresses to varying levels of sulfur, copper, cadmium, and phosphate in the environment (*22-24*). Out of the mRNA targets that we studied, ATPS, SOD, and PE2U are all involved in mediating stress responses to the nutrients listed previously.

miR395 has been identified to mediate sulfur assimilation and allocation in *Arabidopsis (25)*. Sulfur assimilation and allocation is vital to the growth and development of plants because sulfur is a important component of various biological compounds such as certain amino acids, co-enzymes, and vitamins (*9*). In higher plants, most of the sulfur that is found comes from inorganic sulfate which is up taken by the roots from the soil (*9*). Once sulfate enters the roots, ATP sulfurylase (ATPS) catalyzes the initial step of converting sulfate to 5'-adenylylsulfate,

which subsequently causes the sulfur to be assimilated into the aminio acids cysteine and methionine which can be used to make proteins (*9*). ATPS is the mRNA target of miR395 and its expression is mediated through direct cleavage directed by miR395. As a result, the expression levels of APS and miR395 should show an inverse relationship. Our results show that both miR395 and ATPS are both expressed low. The tobacco plants that the tissue was harvested from were grown in a fertilized soil, therefore the plants should not have been under sulfur stress. As a result, the relative expression of miR395 should have been low because other studies have shown that sulfur starvation induces the expression of miR395, which our results indicate. Because the levels of ATPS were also low, this must indicate that there are other mechanisms that are controlling the expression of it other than miR395.

Copper is an essential micronutrient required for certain physiological processes within a plantl. It is found in copper proteins such as copper/zinc superoxide dismutase, plastocyanin, and cytochrome c oxidase, just to name a few (*26*). These three types of proteins are involved in oxidative stress response, photosynthesis, and cellular respiration through the electron transport chain, respectively (*26*). Although maintaining a proper level of copper within plants is important, too much copper can also be detrimental to plant growth and development. Excess copper has been shown to inhibit a wide range of physiological and biochemical processes such as photosynthesis, pigment synthesis, fatty acid and protein metabolism, and membrane integrity (*23*). As a result, the uptake of copper and maintaining copper homeostasis is vital to the growth and development of plants. miR397 and miR398 have both been shown to play a role in mediating copper homeostasis within *Arabidopsis (23)*. Under limited copper conditions, miR398 expression has been shown to increase in expression while its target, copper-zinc superoxide dismutase (SOD), has been shown to decrease in expression. The decrease in SOD

expression lead to the induction of iron superoxide dismutase (FeSOD), which still allowed oxidative stress response to occur while freeing copper to be used in plastocyanin to allow for photosynthesis to be maintained (*27*). Also under low copper conditions, miR397 has been shown to be involved in response to low copper levels by being up-regulated (*28*). In our study, the levels of miR398 and miR397 were relatively low across all tissue types; therefore suggesting that our plant was not copper stressed which would cause an increase in expression of miR398 and miR397. We also found that SOD levels in our study were highly expressed across all tissue types, which is what is expected if the levels of its targeting miRNA, miR398 were low.

Along with sulfur and copper, phosphate is also an important nutrient for plants which can be somewhat difficult to assimilate from the soil, therefore phosphorous levels within a plant are under tight regulation (*24*). Phosphorous is a key element in cellular components such as nucleic acids, membranes, and ATP and also plays a critical role in signal transduction through phosphorylation (*9*). miR399 has been shown to be essential in maintaining phosphate homeostasis by regulating uptake and translocation in *Arabidopsis* (*29, 30*). miR399 has been shown to be induced by low levels of phosphate in the soil in order to allow for increased phosphate uptake, translocation, and remobilization (*9*). In our study, the levels of miR399 across all tissue types were expressed low, which would suggest that our plants were not starved for phosphorous. The target of miR399 is the Phosphate/E2 ubiquitin-conjugating protein (PE2U) which directs protein recycling within cells. Since the levels of miR399 were relatively low in expression, the levels of the mRNA target, PE2U should be higher. In our study, we did not see this trend; we saw that the levels of both miR399 and PE2U were in a low state. Since PE2U is important in regulating protein levels within the cell, it is likely that this enzyme is being regulated by another pathway other than a miR399-mediated pathway.

Conclusion

In this study, we conducted a general expression profile for 9 miRNAs and their targets in eight different tissue types of tobacco. Out of the 9 miRNAs and nine mRNA targets, we found that all of them were differentially expressed in all eight tissue types in tobacco although some were expressed more or less depending on their location in the plant and the known function the miRNA or target may have. We found that miR159, miR157, miR167, miR172 and superoxide dismutase (SOD) exhibited the greatest fold change in expression, therefore indicating their great importance to the growth and development of tobacco. Providing expression profiles for validated miRNAs and their targets is important to gene expression studies because if the basal expression levels are known, then one will have a basis for further gene expression studies in which a treatment may be used.

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Figure 2-1. Average fold changes (log_2) of microRNAs in differing tobacco tissue types.

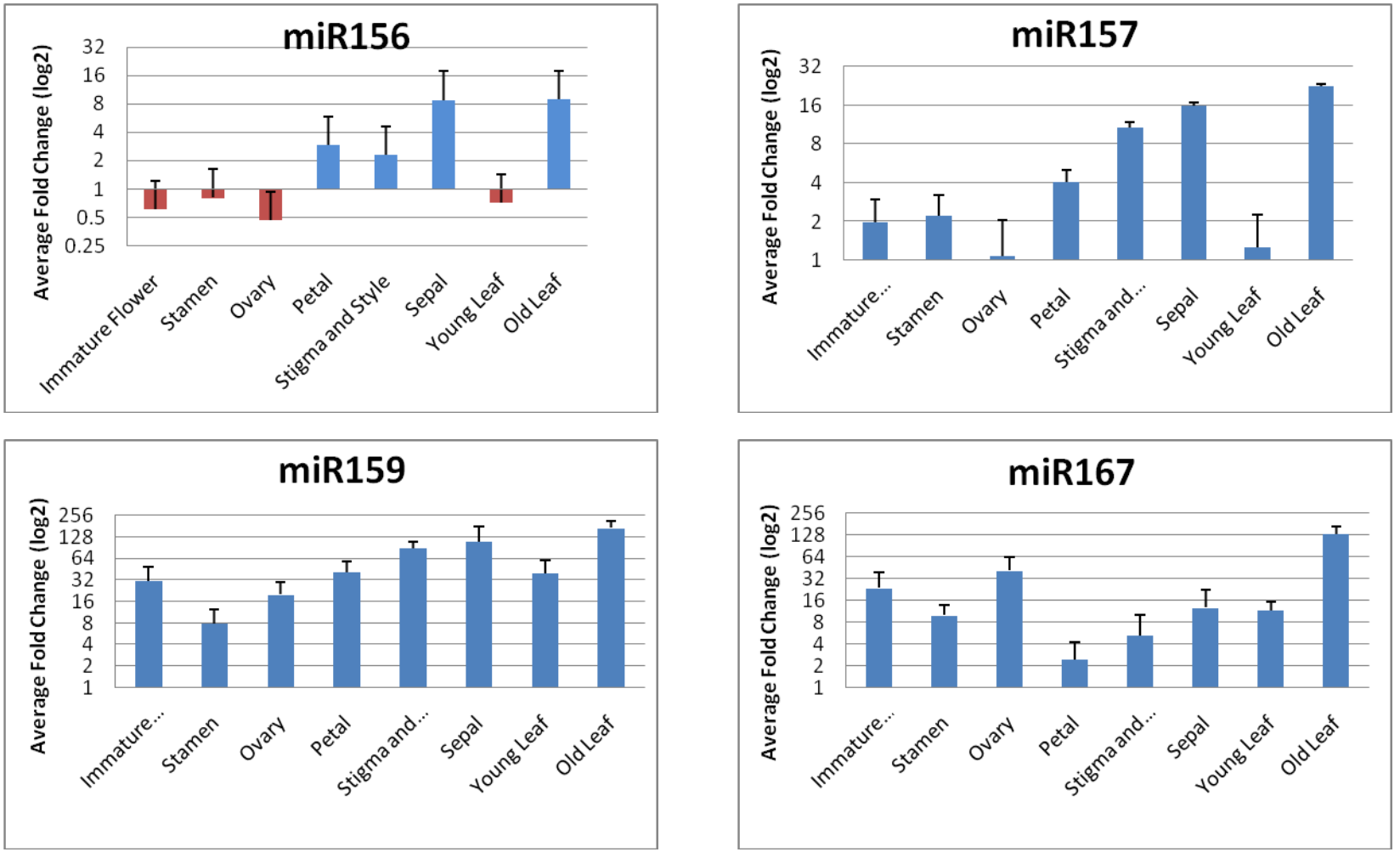
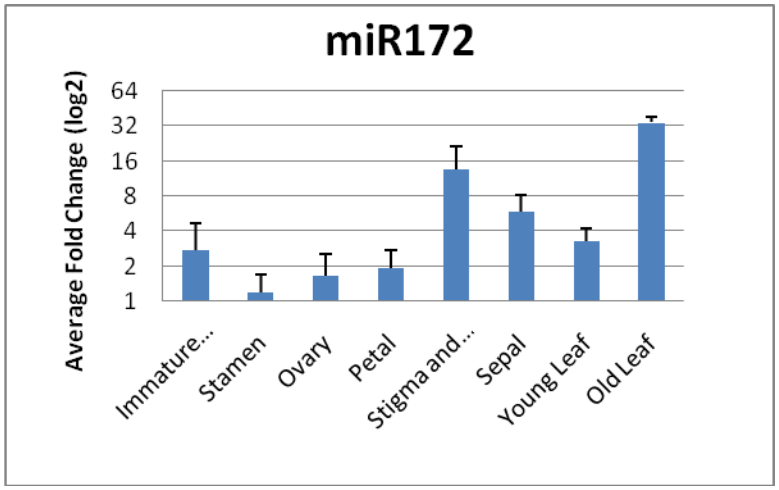
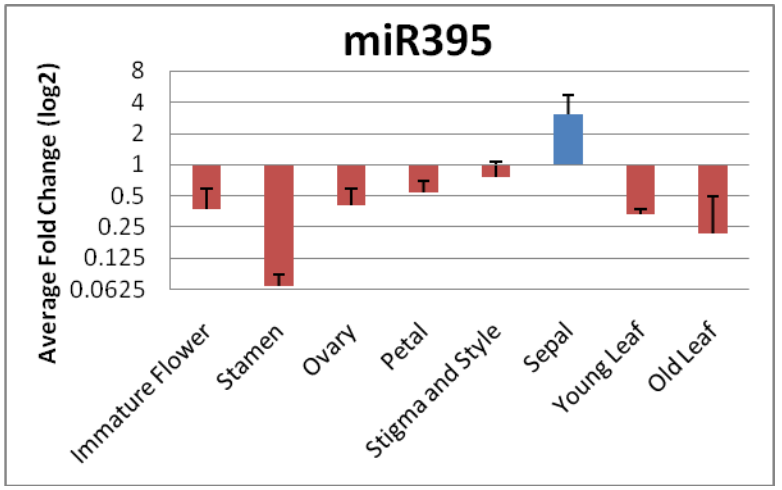
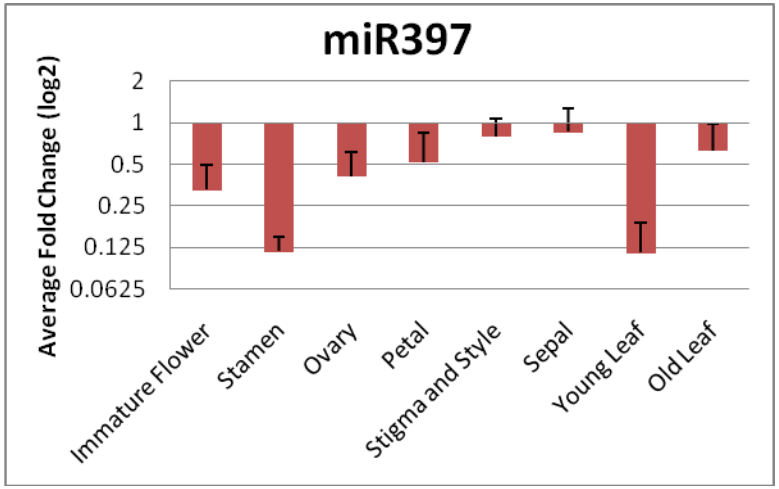


Figure 2-1 continued. Average fold changes (log_2) of microRNAs in differing tobacco tissue types.







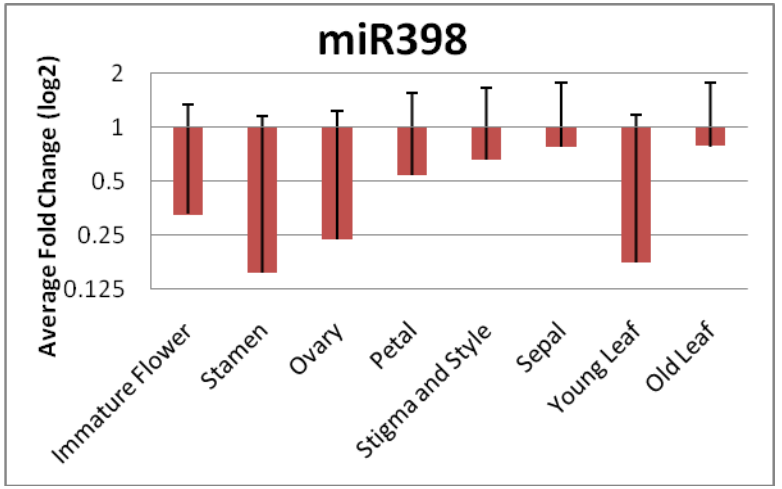


Figure 2-1 continued. Average fold changes (log_2) of microRNAs in differing tobacco tissue types.

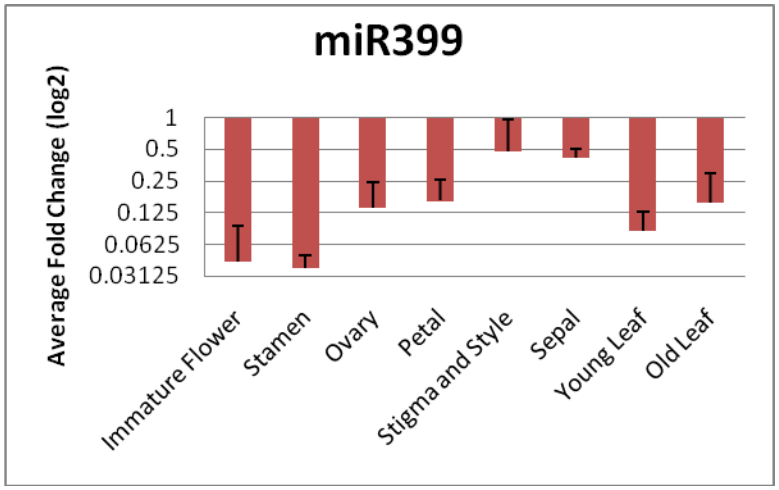


Figure 2-2. Average fold changes (log_2) of protein coding genes in tobacco that are targets of microRNAs.

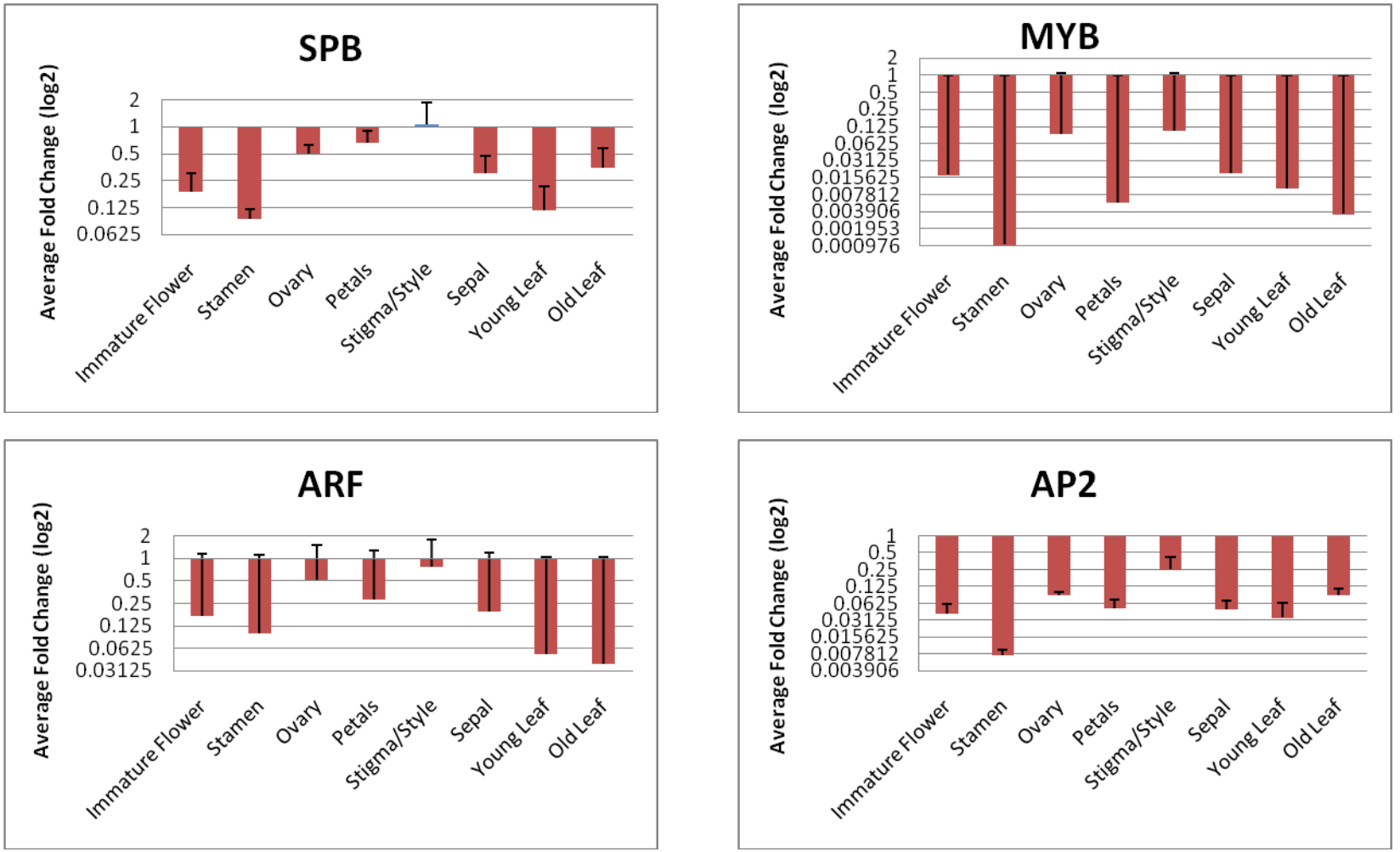
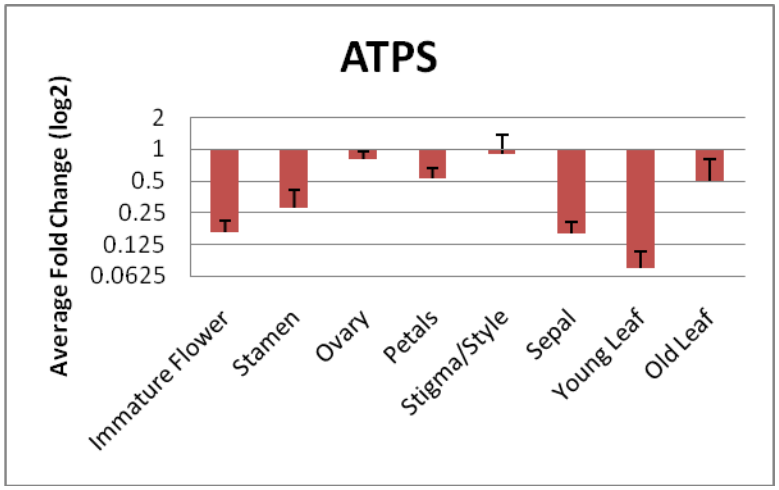
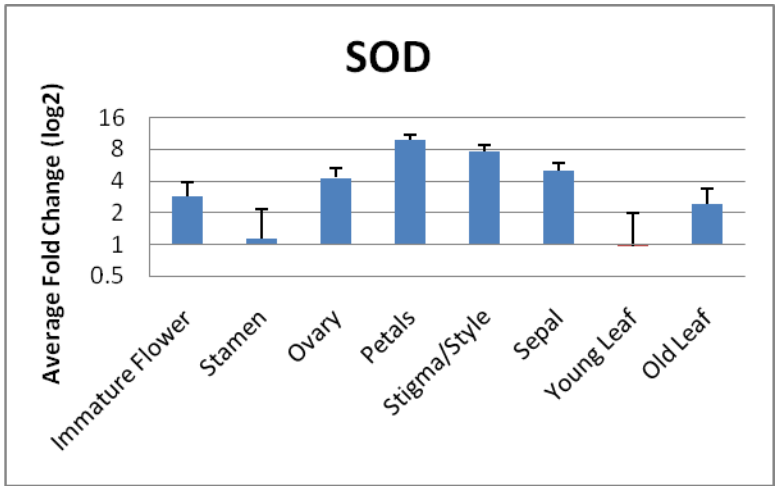
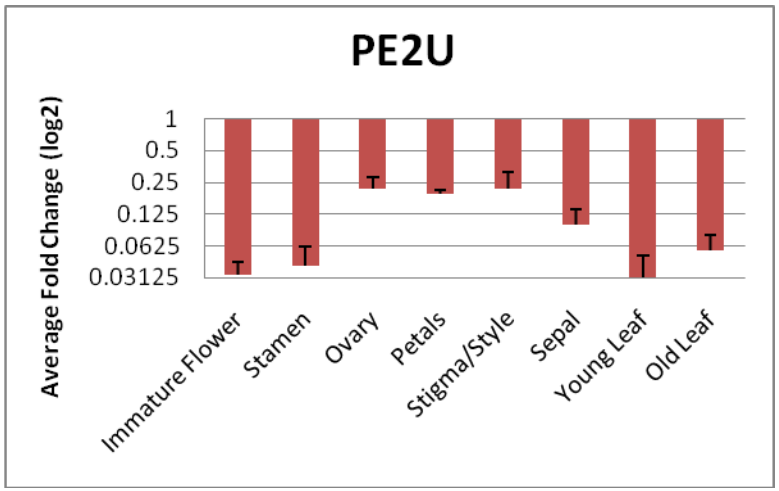


Figure 2-2 continued. Average fold changes (log_2) of protein coding genes in tobacco that are targets of microRNAs.







CHAPTER 3: EVALUATION AND IDENTIFICATION OF RELIABLE REFERENCE GENES FOR GENE EXPRESSION ANALYSES IN NICOTIANA TABACUM

Abstract

In order to complete any type of gene expression analysis, one must use a gene that is similarly expressed throughout multiple tissue types in order to normalize the gene expression data. Commonly, genes referred to as housekeeping genes are used as reference genes during gene expression analyses since their expression is not typically changed due to environmental conditions. The most common reference genes include 18S rRNA, actin, and tubulin along with many others, but some studies have shown that certain housekeeping genes that have been the standard for use as reference genes, in fact, can be differentially expressed. As a result, it is becoming vital to identify proper reference genes prior to any gene expression analysis. There are currently four major programs that have been developed for aiding in the identification of suitable reference genes which include: geNorm, NormFinder, BestKeeper, and the Comparative Delta-Ct Method. These tools employ statistical analysis to identify which genes are expressed in the most stable manner and are consequently ranked from best (most stable) to worst (least stable). Now, there is a comprehensive tool called RefFinder that combines the rankings from all four tools and makes an overall comprehensive ranking for potential reference genes. In this study, tobacco seedlings were exposed to differing levels of nanoparticles in order to simulate differing amount of stress on the seedlings. Afterwards, 12 common reference genes were tested for stability based on the overall comprehensive ranking from RefFinder. Our results show that out of the 12 reference genes tested, GAPDH and PP2A, showed the greatest amount of stability.

Key Words: Reference Genes, RefFinder, geNorm, NormFinder, BestKeeper, Comparative Delta-Ct Method, Tobacco

Introduction

Gene expression analyses are vastly becoming more important in many biological fields as research progresses. Because of the sensitivity and ease, quantitative real time PCR (qRT-PCR) is currently the most common method used to obtain data for gene expression analyses (*1*, *2*). Although qRT-PCR is extremely sensitive and reliable, one must account for errors that may occur due to variations in the initial sample amount, RNA recovery and integrity, efficiency of cDNA synthesis, and the overall transcriptional activity of certain tissues or cells (*3*). In order to normalize the expression values obtained through qRT-PCR, the use of reference genes are a common way to minimize error associated with the aforementioned issues that may arise. Reference genes, by nature, are genes that are expressed in a similar manner across differing tissue types in an organism. Subsequently, the most common reference genes are housekeeping genes because these genes are assumed to be expressed in a similar manner in all tissue types of a particular organism. Some of the best and most commonly used reference genes used in qRT-PCR include 18S rRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), elongation factor-1α (EF-1α), actin, and tubulins. Although many housekeeping genes are expressed in a more stable manner than other genes, it should be noted that in some cases, housekeeping genes can be differentially expressed (*4-6*). Consequently, in gene expression analyses, it is most ideal to identify a proper reference gene prior to the analysis in order to ensure that the most stable reference gene is chosen for that particular study.

Tobacco *(Nicotiana tabacum)* is a widely cultivated crop and is ideal for use in gene expression studies due to its hardiness and ability to be transformed easily. Although many gene expression studies have been performed on tobacco, only one study has actually surveyed the appropriateness of reference genes that are commonly use in most plant based studies (*7*).

Because ideal reference gene stability should not vary much across differing tissue types of an organism exposed to differing conditions, differing types of tobacco tissue were utilized and grown with exposure to heat, cold, salt, drought and UV radiation (*7*). Out of the eight reference gene candidates, only three of them (L25, EF-1α and Ntubc2) were deemed stable enough to be used in further gene expression studies in tobacco. It is interesting that these three reference genes were found to be the most stable because these genes are were tested alongside other common reference genes such as Rn18S, α- and β- tubulin, and actin (*7*). This study shows that it is important to verify proper reference genes for the organism and treatment before starting gene expression analysis. One should avoid choosing a reference gene that is commonly used because it may not be the best choice.

There are currently four methods that can be used to evaluate reference gene stability which include three Excel based applets, geNorm (*8*), NormFinder (*3*), and BestKeeper (*9*), and the Comparative Delta-Ct Method (*10*). Conveniently, all four of these tools have been combined by Fuliang Xie into a comprehensive reference gene tool, called RefFinder, which will not only give the rankings for all genes within each individual tool, but will give an overall comprehensive ranking for all four tools. This tool will be used to rank reference genes in our study and can be accessed at http://www.leonxie.com/referencegene.php.

In this study, our goal is to identify several reference genes that are stable to use in further *Nicotiana tabacum* studies. Because reference gene expression should be stable across differing tissues and under differing conditions, *Nicotiana tabacum* cv. NCSU seeds were placed on media that contained differing concentrations (0, 0.1, 0.5, and 1%) of either aluminum oxide nanoparticles or silicon dioxide nanoparticles to simulate differing amounts of stress. We tested the stability of expression of 12 common housekeeping genes using the online tool RefFinder

and found that the most stable genes in our study were GAPDH and protein phosphatase 2 (PP2A).

Materials and Methods

Tobacco Treatment and Growth Conditions

Nicotiana tabacum cv. NCSU seeds were sterilized by soaking in 70% ethanol for 2 minutes followed by soaking in 10% bleach for 15 minutes. After soaking in bleach, the seeds were rinsed with sterile water approximately four times until no bleach odor remained. The seeds were then sown on media that contained either aluminum oxide nanoparticles or silicon dioxide nanoparticles in the following concentrations: 0%, 0.1%, 0.5%, and 1%. Each batch of media (100mL total) contained the following: 0.44 g Murashige and Skoog Salts, 1 uL of Gamborg's B5 Vitamins (1000X), 1 g sucrose, 0.8 g agar, and the nanoparticles. The media was adjusted to pH 5.8 and autoclaved on a 20 minute cycle. Each batch of 100mL of media made 5 plates. There were 5 plates of each type of media made to ensure three biological replicates grew properly for RNA extraction. After cooling, 25 sterilized seeds were placed onto each plate. The seedlings were allowed to grow for three weeks on a 16 hr day/ 8 hr night schedule. Each plate of seedlings were then flash frozen in liquid nitrogen and were stored at -80^{o}C until further RNA extraction.

RNA Extraction

RNA was extracted from each tissue type using a mirVana microRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. A NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) was used after RNA extraction to measure the quantity and quality of the RNA. The quality was determined by the 260/280 and 260/230 ratios.

Each tissue type was collected in triplicate, therefore there were three RNA samples per tissue type. The RNA was placed in the -80^{o}C freezer until further RT-PCR.

Quantifying gene expression

First, reverse transcription PCR (RT-PCR) was used to generate a single-stranged miRNA cDNA sequence from 1 µg of the total RNA collected from each tobacco tissue type. The cDNA was generated using reverse transcription with the Appled Biosystems TaqMan® microRNA Reverse Transcription Kit and a poly T primer according to the manufacturer's protocol.

Applied Biosystems TaqMan® microRNA Assays were used to detect and to quantify the protein coding gene expression in tobacco using quantitative real-time PCR (qRT-PCR). These reactions were performed in 96 well optical plates in which each well contained cDNA, TaqMan mix, and the specific forward and reverse primer for the reference gene of interest (See Table 1). The fluorescent probe that was used was SYBR green and each reaction plate was run on an Applied Biosystems 7300 Sequence Detection System (Foster City, CA). Three biological replicates were run for each tissue type and each biological replicate was duplicated in order to minimize the potential error from biological samples or from pipetting. Once all of the Ct values were obtained, the Ct values were analyzed using RefFinder (http://leonxie.com/referencegene.php).

Results and Discussion

The comprehensive tool, RefFinder, is a new convenient way of giving an overall ranking of reference gene stability from the data obtained through four commonly used reference gene tools. These tools include geNorm (*8*), NormFinder (*3*), Best Keeper (*9*), and the Comparative

Delta-Ct Method (*10*). In this study, we looked at the gene stability assigned by each individual tool, and gave an overall comprehensive ranking comprised from the rankings of each individual tool.

geNorm

geNorm ranks reference genes based on a pair-wise comparison of the expression of a group of reference gene candidates based on the fact that the expression ratio of two ideal internal reference genes should remain relatively the same (*8*). Using this algorithm ultimately results in identifying the two most stable reference genes with the lowest M value that cannot further be ranked (*8*). The M value is defined as being the average pair-wise variation of a particular gene with all other genes, therefore genes with the lowest M values should be the most stable (*8*). This tool also can determine the optimal number of reference genes that are appropriate to be used in gene expression analysis. Out of the twelve reference genes that were used in this study, geNorm found that the two most stable reference genes were PP2A and GAPDH with a stability value of 0.157 (Figure 3-1). These were followed closely by γ-Tubulin, α-Tubulin, and L25 with stability values of 0.206, 0.258, and 0.286 respectively (See Figure 3-1).

NormFinder

NormFinder is another Excel based application that can be used to rank reference gene stability. Unlike geNorm, NormFinder uses a model-based approach to not only rank reference genes based on their stability in expression, it also takes into account the variance associated within subgroups of samples (*3*). Also unlike geNorm, NormFinder assesses the stability of each candidate reference gene independently of one another. Out of the twelve candidate reference genes, NormFinder deemed rbcmtT as the most stable reference gene with a ranking of 0.345.

Following rbcmtT were PAP, GAPDH, HrBP1, and γ-Tubulin (Figure 3-2). NormFinder ranked our reference genes slightly differently than geNorm, but overall NormFinder and geNorm both ranked GAPDH and γ-Tubulin as being stable reference genes. It should be noted that Rn18S, which is a commonly used reference gene, was ranked as least stable for both geNorm and NormFinder.

BestKeeper

BestKeeper is also another Excel based algorithm that is used to rank reference genes based on three criteria: standard deviation, percent covariance, and power of the candidates (*9*). The best suited reference genes are determined by a pair-wise correlation analysis of candidate reference genes. BestKeeper estimates the correlations between candidate reference genes, and then combines the highly correlated ones into an index based on the three critera listed above (See Table 3-2 and 3-3). Based on the results from Best Keeper, GAPDH, PP2A, L25, α-Tubulin, and γ-Tubulin were ranked the most stable, respectively (See Figure 3-3). These results coincide with the results from both geNorm, and NormFinder.

The Comparative Delta-Ct Method

The comparative delta-Ct method ranks the most stable candidate reference genes by comparing relative expression of 'pairs of genes' within each tissue sample or treatment (*10*). The delta-Ct and the average delta-Ct are calculated for every two groups within each tissue sample or treatment, and then the standard deviation for each set of delta-Cts is determined. To determine the overall stability of each candidate reference gene, the arithmetic mean of all standard deviations associated with that particular gene is found. The lower the arithmetic mean of standard deviations, the more stable the expression of the candidate reference gene is. Like

with geNorm, the two most stable reference genes that were identified with this method were GAPDH followed by PP2A (See Figure 3-4). Next were L25 and γ-Tubulin which came very close to the rankings given by geNorm, NormFinder, and BestKeeper.

Overall Comprehensive Ranking by RefFinder

The tool RefFinder (http://leonxie.com/referencegene.php), gives a comprehensive ranking to all candidate reference genes based on the geometric means of the rankings provided by geNorm, NormFinder, BestKeeper, and the Comparative Delta-Ct Method. According to RefFinder, the two most stable reference genes were GAPDH and PP2A with scores of 1.316 and 2.3, respectively (See Figure 3-5). This is to be expected because out of the four tools that were used, GAPDH and PP2A were deemed the most stable in all of the tools.

Conclusion

Finding a suitable reference gene has proven to be not as simple as choosing a housekeeping gene from a list of previously used genes due to the fact that housekeeping genes, though relatively stable, can be differentially expressed. As a result, one should go about finding a suitable reference gene that is stable prior to beginning work on any gene expression analyses. With gene expression analyses that use qRT-PCR as the data source, there are four commonly used reference gene tools that are available online to assess the stability of potential reference genes. These tools include, geNorm, NormFinder, BestKeeper, and the Comparative Delta-Ct Method. Prior to the existence of the comprehensive reference gene tool, RefFinder, one would have to use these tools individually to identify suitable reference genes. Reference gene identification is so much simpler now because RefFinder conveniently combines the individual stability rankings of potential reference genes from geNorm, NormFinder, BestKeeper, and the

Comparative Delta-Ct Method into one comprehensive ranking. In our study, we exposed tobacco seedlings to differing concentrations of aluminum oxide nanoparticles and silicon dioxide nanoparticles and tested the stability of 12 common housekeeping genes through the RefFinder program. Our comprehensive results indicate that GAPDH and PP2A were the most stable reference genes. Rn18S and actin, which are common 'go-to' reference genes, were actually ranked as being some of the least stable genes out of the 12, therefore this study shows that it is important to verify reference gene stability prior to use in gene expression analysis.

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Table 3-1. Reference Genes used in this study with their abbreviations, accession numbers, and primer sequences.

Reference Gene	Abbreviation	Accession Number	Forward Primer	Reverse Primer
18S rRNA	Rn18S	AJ236016	GTCGTCTCGTCCCTTCTGCCG	CGAATGCCCCCGACTGTCCC
Actin	Actin	X63603	TGGTCGCCCACGCCACACAG	AGTACAGGGTGTTCCTCTGGCG
Elongation Factor 1α	EF1α	Af120093	GCTGCTGAGATGCACAAGCGG	CAGGACACGACAGGCACGGG
α-Tubulin	α-Tub	AJ421411	GCCTCGAGCATGGCATTCAGC	TGCTCAGGGTGAAAGAGCTGCCT
25S rRNA-Binding Protein	L25	L18908	GCACAGGCAGCTAAGGTTGCCA	TGCAGACTCTGTGGTGAGGGG
γ-Tubulin	γ-Tub	AJ278739	ACGACTTGGTTGGCCTCCTTGC	GCCTGACTGGCTTCCTTTGTTCG
Ubiquitin	UB1	DQ830978	TGTCCTCCGTCTCCGTGGTGG	GCGAGGGTTCGGCCGTCTTC
Protein Phosphatase 2	PP2A	X97913	TGGCAGAGGACAGGCATTGGC	GCTGCATTGCCCACTCTGGACC
RUBISCO Large Subunit N-methyltransferase	rbcmtT	NTU35620	CGAACCAAGTTCAGCTCGTGATGC	TCCGCCTAGGGCAACCAGCC
Purple Acid Phosphatase	PAP	EF397753	TGCTCGGGGGATATGTCGCCTA	TCGACACCACACTCGTACAGCA
HairpinBinding Protein 1	HrBP1	AY383625	ACAGCAGTGCATTCTCAGGTCGC	AAAGTGGCAGTCAACTCAGCAGG
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH	AJ133422	ACAAATTGCCTTGCTCCCTTGGC	ACAGCCTTGGCAGCTCCAGTA

Figure 3-1. Gene stability graph generated by RefFinder for geNorm.

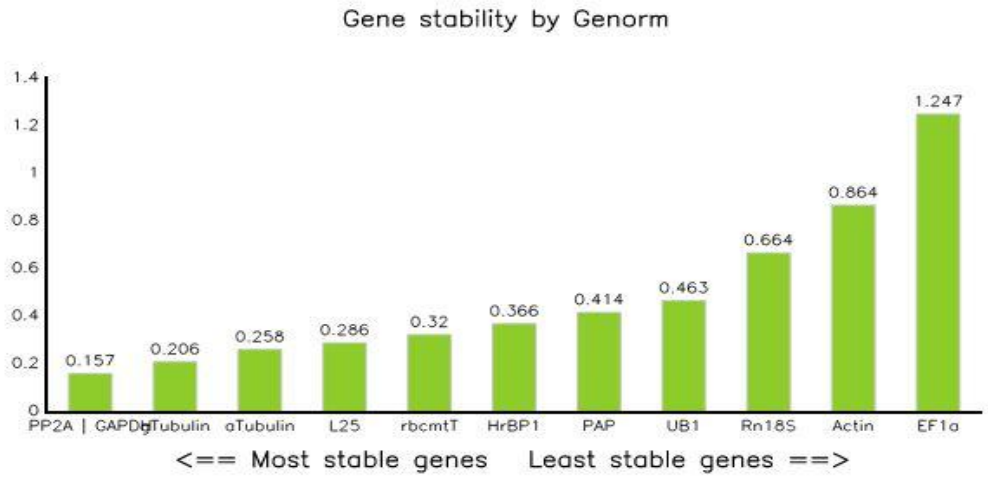


Figure 3-2. Gene stability graph generated by RefFinder for NormFinder.

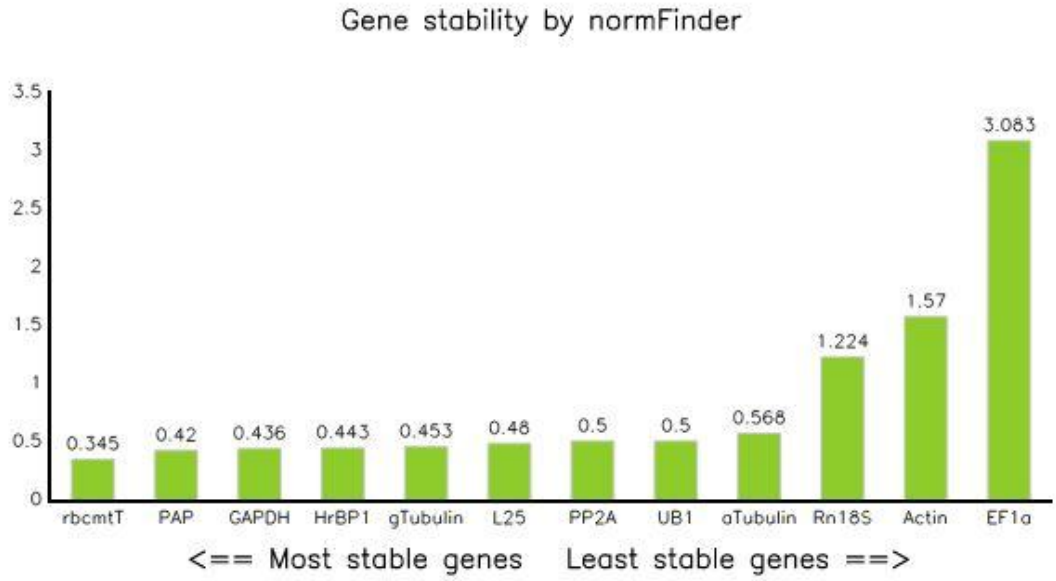


Table 3-2. Descriptive statistics of candidate housekeeping genes based on their crossing point (CP) values.

CP data of housekeeping Genes by BEST KEEPER

	Rn18S	Actin	EF1a	aTubulin	L25	gTubulin	UB1	PP2A	rbcmtT	PAP	HrBP1	GAPDH
n	48	48	48	48	48	48	48	48	48	48	48	48
geo Mean [CP]	18.25	31.27	31.94	21.02	21.05	25.55	22.59	22.26	25.25	26.31	22.59	22.16
AR Mean [CP]	18.30	31.32	32.11	21.02	21.05	25.56	22.60	22.27	25.25	26.31	22.59	22.16
min [CP]	16.59	28.15	25.39	20.28	20.29	24.88	21.63	21.53	24.53	25.36	21.52	21.43
max [CP]	22.01	35.31	38.44	22.16	22.26	26.81	24.91	23.52	26.57	27.83	24.45	23.48
std dev [+/- CP]	1.10	1.51	2.64	0.31	0.29	0.34	0.54	0.29	0.34	0.41	0.50	0.28
CV [% CP]	6.01	4.83	8.21	1.49	1.40	1.31	2.41	1.31	1.33	1.57	2.21	1.26
min [x-fold]	-3.17	-8.66	-94.01	-1.66	-1.69	-1.59	-1.94	-1.67	-1.64	-1.92	-2.09	-1.66
max [x-fold]	13.55	16.50	90.31	2.21	2.31	2.38	5.00	2.39	2.50	2.88	3.63	2.51
std dev [+/- x-fold]	2.14	2.85	6.21	1.24	1.23	1.26	1.46	1.22	1.26	1.33	1.41	1.21

Table 3-3. Correlation analysis of candidate reference genes by BestKeeper.

Pearson correlation coefficient (r)

BestKeeper vs.	Rn18S	Actin	EF1a	aTubulin	L25	gTubulin	UB1	PP2A	rbcmtT	PAP	HrBP1	GAPDH
coeff. of corr. [r]	0.549	0.759	0.772	0.414	0.536	0.575	0.658	0.499	0.701	0.632	0.668	0.588
p-value	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Figure 3-3. Gene stability ranking by RefFinder for BestKeeper

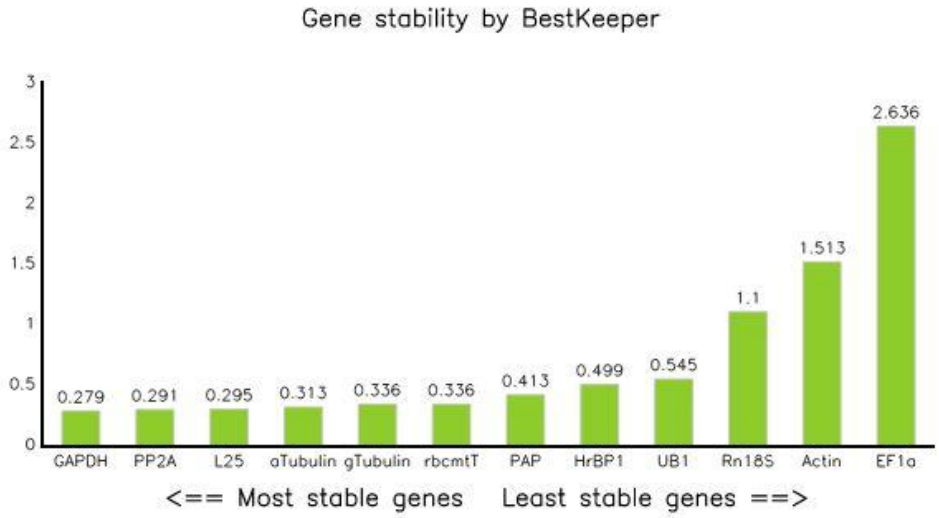


Figure 3-4. Gene stability rankings of reference genes by the Comparative Delta-Ct Method.

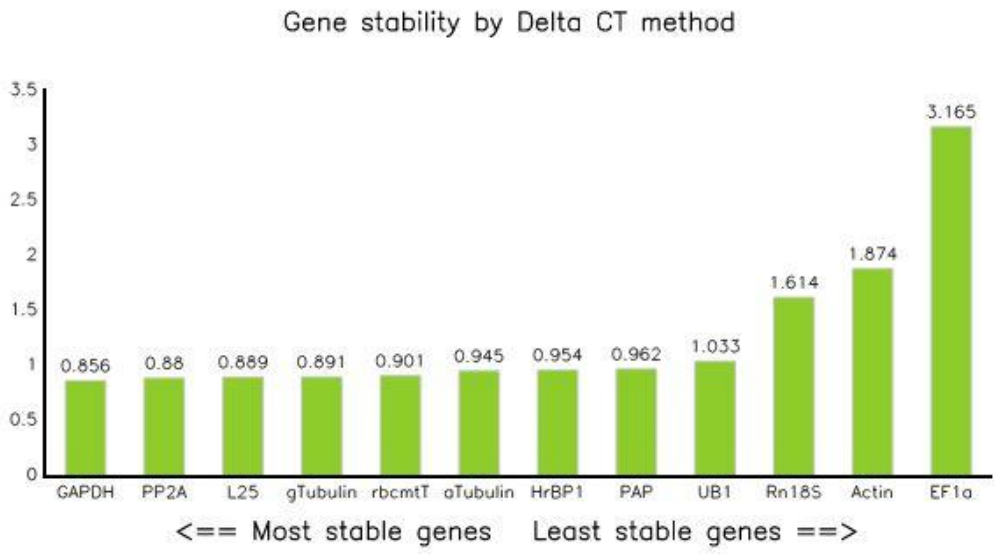
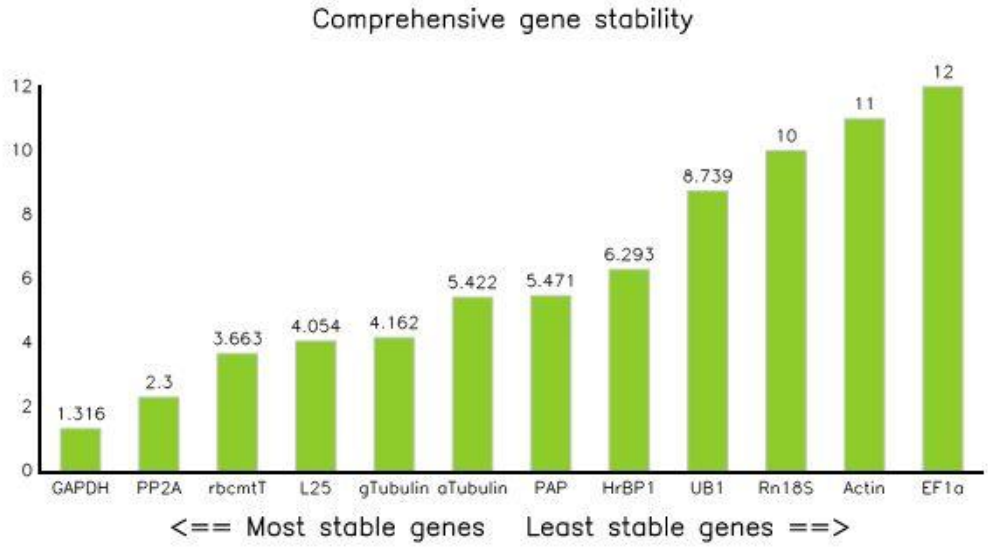


Figure 3-5. Overall comprehensive reference gene rankings by RefFinder.



CHAPTER 4: EFFECTS OF ALUMINUM OXIDE NANOPARTICLES ON THE GROWTH, DEVELOPMENT, AND MICRORNA EXPRESSION OF TOBACCO (*NICOTIANA TABACUM*)*

Abstract

Nanoparticles of 1 to 100 nanometers diameter and are being increasingly used in industry due to their unique properties, such as their small size and large surface area. Nanoparticles are used in coatings for products, colorants, food additives, pharmaceuticals, cosmetics, and paints. Therefore, residues from their use may be increasingly released into the environment as the use of these products increases. To date, few experiments have been conducted to investigate the effect nanoparticles may have on plant growth and development. It is important to study the effects nanoparticles have on plants because they are stationary organisms that cannot move away from environmental stresses like animals can, therefore they must overcome these stresses by molecular routes such as altering gene expression. microRNAs (miRNA) are a newly discovered, endogenous class of post-transcriptional gene regulators that function to alter gene expression by either targeting mRNAs for degradation or inhibiting mRNAs translating into proteins. miRNAs have been shown to mediate abiotic stress responses such as drought and salinity in plants by altering gene expression; however, no study has been performed on the effect of nanoparticles on the miRNA expression profile; therefore our aim in this study was to classify if certain miRNAs play a role in plant response to Al_2O_3 nanoparticle stress. In this study, we exposed tobacco (*Nicotiana tabacum*) plants (an important cash crop as well as a model organism) to 0%, 0.1%, 0.5%, and 1% Al_2O_3 nanoparticles and found that as exposure to the nanoparticles increased, the average root length, the average biomass, and the leaf count of the seedlings significantly decreased. We

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also found that miR395, miR397, miR398, and miR399 showed an extreme increase in expression during exposure to 1% Al_2O_3 nanoparticles as compared to the other treatments and the control, therefore these miRNAs may play a key role in mediating plant stress responses to nanoparticle stress in the environment. The results of this study show that Al_2O_3 nanoparticles have a negative effect on the growth and development of tobacco seedlings and that miRNAs may play a role in the ability of plants to withstand stress to Al_2O_3 nanoparticles in the environment.

Keywords: Aluminum oxide, nanoparticle, microRNA, gene expression, *Nicotiana tabacum*, tobacco

Introduction

Nanoparticles are classified as being materials in which at least one dimension of the material is between 1 and 100 nanometers in diameter (*1*). Nanoparticles are becoming an area of research interest due to their unique properties, such as having increased electrical conductivity, ductility, toughness, and formability of ceramics, increasing the hardness and strength of metals and alloys, and by increasing the luminescent efficiency of semiconductors (*2*). Nanoparticles are heavily used in an industrial setting because they can be used to manufacture lightweight, strong materials, as well as acting as pigments in products such as paints, sunscreens, and cosmetics (*1*). Because nanoparticles have a large surface area to volume ratio, the use of nanoparticles in both industry and daily life is greatly increasing in realms that include advancing the quality of everyday materials and processes, improving the function of electronics and information technology, allowing more sustainable energy applications, and acting as key players in environmental remediation applications (*1*). Aluminum oxide is also very wear-resistant, has good thermal conductivity, resists strong acid and alkalai containing materials, is easily shaped, and has high strength and stiffness which makes it a prime material to use in making products that include high temperature electrical insulators, high voltage insulators, thermometry sensors, wear pads, ballistic armor, and grinding media (http://accuratus.com/alumox.html).

Because the amount of nanoparticles used in industry is drastically increasing as more useful ways of integrating them into products is becoming evident, the amount of nanoparticles that are released into the environment and the effects these nanoparticles may have needs to be assessed. The focus of this manuscript is the effects of exposure of tobacco seedlings to aluminum oxide nanoparticles. Although many experiments have been performed to show the

effects aluminum toxicity has on plants, few experiments have been conducted on the effects nano-scale aluminum oxide has on plants. As a result, the focus of this project is to provide information on the effects aluminum oxide nanoparticles have on the growth, development, and gene expression in tobacco seedlings.

microRNAs, are a newly discovered highly-conserved, endogenous class of regulatory molecules that do not code for proteins (*3, 4*). microRNAs are approximately 20-22 nucleotides in length and work in post transcriptional gene regulation by either targeting messenger RNAs (mRNAs) for degradation, or by inhibiting the translation of mRNAs (*4*). microRNAs have been shown to aid in the regulation of many processes within plants such as leaf and root development, organ maturation, cell proliferation, flowering time, and abiotic stress response (*5, 6*). Recent studies have shown that microRNAs help to mediate the expression of more than 30% of protein coding genes (*7, 8*) and this number is expected to increase as more miRNAs are discovered and their target mRNAs are identified.

In this study, we chose to employ tobacco as a model species to investigate the effects of aluminum oxide nanoparticles on the growth, development, and miRNA expression in agricultural plants. We chose tobacco as our model species because its genome is almost completely sequenced, it has a relatively short generation time, and it is an important cash crop in the Southeastern portion of the United States. North Carolina produced about 40% of the total tobacco found in the United States in 2002 and about 47% of the total tobacco in 2007, therefore any research dedicated to improving the growth and sustainability of tobacco is of great importance. Although much research has been dedicated to this crop so far, no studies have been conducted to show the effects aluminum oxide nanoparticles have on tobacco growth, development, and miRNA expression during abiotic stress response to the nanoparticles. The

results of this study show that as concentrations of aluminum oxide nanoparticles increase, the root length, the average biomass, and the leaf count of each tobacco seedling decreased and the expression profile of certain microRNAs was significantly up-regulated.

Materials and Methods

Seed Sterilization, Media Preparation, and Tobacco Treatment

Tobacco (*Nicotiana tabacum*) seeds were sterilized by soaking in 70% ethanol for two minutes, followed by soaking in 10% bleach for 15 minutes. The seeds were then rinsed with sterilized water approximately four times until no bleach odor remained. After each rinsing with water, the immature tobacco seeds that floated were removed and discarded to ensure mature seeds were being sowed on Petri dishes. The basic medium used for growing tobacco contained the following: 0.44g Murashige and Skoog (MS) salts supplemented with 1X Gamborg's B5 Vitamins, 1g sucrose, and 0.8g agar per 100mL of media. Four concentrations (0, 0.1, 0.5 and 1.0%) of Al_2O_3 nanoparticles were also added to the media before the pH was adjusted. The pH of the media was adjusted to approximately 5.8 after the addition of all media components. After media preparation, 25 sterilized tobacco seeds were sowed on each Petri dish, for a total of 20 plates (5 plates per Al_2O_3 nanoparticle concentration). The plates were subsequently placed under a 16h day/8h night cycle at room temperature for exactly 3 weeks.

Total RNA Extraction

Three week old tobacco seedlings were harvested from their respective plates and were frozen in liquid nitrogen after physical measurements were recorded such as root length, leaf count, germination rate, and average seedling biomass. The seedlings were placed in a -80°C freezer until total RNA extraction. Total RNA was extracted using the mirVana miRNA

Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. The total RNA was then quantified and the quality was assessed by using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA samples were stored in a -80°C freezer until further use and analysis.

Analyzing microRNA Expression Variations Using RT-PCR and qRT-PCR

Applied Biosystems TaqMan microRNA Assays were used to detect and to quantify the miRNAs in tobacco using a stem-loop real-time PCR according to the manufacturer's instructions. There were two steps in the TaqMan Assays which included reverse transcription of the mature miRNA into a longer single-stranded cDNA sequence using a miRNA-specific stem-looped primer and secondly, quantitative real-time PCR. In brief, a single-stranded miRNA cDNA sequence was generated from 1 µg of the total RNA collected from each treatment (0%, 0.1%, 0.5%, and 1%). The cDNA was generated using reverse transcription with the Applied Biosystems TaqMan microRNA Reverse Transcription Kit and the miRNA-specific stem-looped RT primers provided with the kit. Previous studies have shown that miR156, miR157, miR159, miR162, miR167, miR169, miR172, miR395, miR396, miR397, miR398, and miR399 are important for plant growth as well as environmental stress response, therefore these miRNAs were selected to investigate the effect nanoparticle exposure has on tobacco. We also used two stress related genes, alcohol dehydrogenase (ADH) and ascorbate peroxidase (APX), to investigate the effects nanoparticle exposure has on tobacco. In the relative quantification analysis, tubulin, a housekeeping gene, was used as a reference gene in order to normalize expression values. Three biological replicates were run for each gene within each treatment and each biological replicate was technically duplicated to minimize the chance of error. The results were analyzed using the $\Delta\Delta C_T$ method.

Statistical Analysis of the Physical Characteristics and miRNA Fold Changes in Tobacco

After the physical characteristics of tobacco were measured and the fold changes in the miRNA expression levels in tobacco were quantified, the results were analyzed using the statistical software SPSS version 19. For each measurement (i.e. root length, leaf count, etc.), the measurements found for all four treatments were tested for their significance using a one-way ANOVA test with a LSD post-hoc test. All treatments were tested with a 95% confidence interval.

Results

Aluminum oxide nanoparticles affected the growth and development of tobacco seedlings

Once the tobacco seeds were plated, it took approximately three to five days for them to germinate. After ten days, the germination rates of the tobacco seedlings were calculated for each concentration of aluminum oxide nanoparticles. For the seedlings grown on control media without any aluminum oxide nanoparticles, the average germination rate was 99.2%, therefore almost all of the tobacco seeds germinated. In the media containing 0.1% aluminum oxide nanoparticles, the average germination rate was 92.8% and the seeds grown in 0.5% aluminum oxide nanoparticles had an average germination rate of 96.0%. Lastly, the seeds grown in 1.0% aluminum oxide nanoparticles had a germination rate that was much lower than the previous three concentrations at 88% (See Table 4-1). Although there was some variation between the germination rates of seeds grown in media with differing concentrations of aluminum oxide nanoparticles, this variation was not statistically significant, therefore aluminum oxide nanoparticles did not significantly affect the germination rate of tobacco seedlings.

After three weeks of culture, the seedlings grown in the presence of aluminum oxide nanoparticles showed an obvious decrease through the visualization of the root lengths, leaf count, and biomasses of the seedlings (See Table 4-1 and Figure 4-4). In this experiment, root growth and development seemed to be affected the most with a statistically significant decrease in root length as the concentration of aluminum oxide nanoparticles increased. For the control seedlings, the average root length was 29.5 mm. For the seedlings exposed to 0.1% aluminum oxide nanoparticles, the average root length decreased to 22.0 mm, which was significantly lower than the control ($p<0.05$). The seedlings exposed to 0.5% and 1.0% aluminum oxide nanoparticles had average root lengths of 5.5 mm and 2.3 mm, respectively, which were both significantly lower than the control ($p<0.01$) and the seedlings grown in 0.1% aluminum oxide nanoparticles ($p<0.01$). Although the seedlings grown in 0.5% and 1.0% aluminum oxide nanoparticles were significantly lower than the control and 0.1% aluminum oxide seedlings, the difference in root length between each other was not highly significant ($p<0.05$). Overall, as the concentration of aluminum oxide nanoparticles increased, the average root lengths of three week old tobacco seedlings significantly decreased (See Table 4-1 and Figure 4-4).

In this experiment, aluminum oxide nanoparticles affect the number of leaves in three week old tobacco seedlings (See Table 4-1). For the control, the average leaf count was 3.77. For the 0.1% and 0.5% aluminum oxide seedlings the average leaf count was 3.36 and 2.82, respectively. For the 1.0% aluminum oxide seedlings, the average leaf count was 2.39. Our results show that as the concentrations of aluminum oxide nanoparticles increased, the number of leaves per seedling also significantly decreased. As a result, aluminum oxide nanoparticles have a negative effect on the growth and development of three week old tobacco seedling leaves.

Lastly, the average biomass per tobacco seedling was assessed to see if aluminum oxide nanoparticles had any significant affect on the growth of tobacco seedlings. For the seedlings grown on control media, the average biomass per seedling was calculated to be 11.5 milligrams. For the 0.1%, 0.5%, and 1% aluminum oxide exposed seedlings, the average biomass per seedling was calculated to be 7.5 milligrams, 2.8 milligrams, and 2.1 milligrams, respectively (See Table 4-1). The average biomass of the control, 0.1%, and 0.5% aluminum oxide seedlings all significantly decreased ($p<0.05$) as the concentration of the nanoparticles increased. The average biomass of the seedlings grown in 1% aluminum oxide were significantly lower ($p<0.05$) than the control and 0.1% aluminum oxide seedlings, but was not significantly lower than the seedlings grown in 0.5% aluminum oxide nanoparticles. Overall, the average biomass of three week old tobacco seedlings decreased as the exposure to aluminum oxide nanoparticles increased.

Aluminum oxide nanoparticles altered microRNA expression in tobacco

Aluminum oxide nanoparticles significantly altered the expression levels of certain miRNAs in tobacco. In the tobacco plants exposed to 0.1% aluminum oxide nanoparticles, all of the tested miRNAs were down-regulated except for miR156, miR157, and miR172 which were up-regulated (See Figure 4-1). However, in the plants exposed to 0.5% and 1% aluminum oxide nanoparticles, all of the miRNAs that were tested were up-regulated. The expression of three miRNAs (miR156, miR157, and miR172) was up-regulated in response to increasing aluminum nanoparticle exposure, but these fold changes in expression were not significantly significant ($p>0.05$) (See Figure 1). These three miRNAs had average fold changes less than 10. Nine miRNAs (miR159, miR162, miR167, miR169, miR395, miR396, miR397, miR398, and miR399) had statistically significant increases in fold change of expression (See Figure 4-2).

miR159, miR162, miR167, and miR169 were significantly up-regulated with fold changes in the 1% Al_2O_3 nanoparticle treatment of 5.9, 5.5, 11.4, and 6.0 fold, respectively. Of all significantly up-regulated miRNAs, four miRNAs showed greater fold changes in the 1% Al_2O_3 concentration than the others. These included miR395, miR397, miR398, and miR399 with average fold changes in expression of 315, 55, 144, and 90, respectively in response to 1% Al_2O_3 nanoparticles.

Aluminum oxide nanoparticles altered the expression of stress-related genes in tobacco

In this study, we also observed a change in the expression levels of two stress related genes, ascorbate peroxidase (APX) and alcohol dehydrogenase (ADH). The trends for the expression levels in APX and ADH were slightly different from the trends in expression for the miRNAs. For APX, the expression was down-regulated 0.83 fold in the 0.1% treatment, up-regulated 2.89 fold in the 0.5% treatment, and then slightly decreases to 2.82 fold in the 1% treatment (See Figure 4-3). For ADH, the fold change is up-regulated to 1.34 fold and 3.57 fold in the 0.1% and 0.5% concentration, but decreases to 1.70 fold in the 1% treatment in a similar fashion to APX. For both APX and ADH, the fold change was not statistically significant (See Figure 4-3).

Discussion

Due to their unique properties, nanoparticles have been increasingly used in the past 20 years in electronics, biomedical applications, pharmaceuticals, cosmetics, energy applications, and various materials (*9*); therefore the potential for nanoparticles to contaminate the environment is much greater than in the past. Because plants are sessile, they must cope with environmental stressors in different ways than simply the act of relocation. Plants typically

respond to environmental biotic and abiotic stress through molecular routes. In the past decade, research has been conducted on miRNAs and they are believed to be an ancient form of post transcriptional gene regulation because of their high conservation (*10*). Because miRNAs mediate gene expression, they are thought to be one of the key factors in plant stress response. In this study, we analyzed the effects of aluminum oxide nanoparticle exposure on the miRNA expression levels in tobacco seedlings as well as how nanoparticles affect the growth and development of tobacco. We found that as aluminum oxide nanoparticle concentrations increased, the root lengths, leaf count, andthe overall biomass of each seedling significanly decreased. We also found that as the concentration of aluminum oxide nanoparticles increased, the general miRNA expression profile of selected miRNAs was up-regulated.

The results of our study showed that the germination rate of tobacco seedlings exposed to increasing amounts of aluminum oxide nanoparticles was not statistically significant. Our results were consistent with a study which investigated the effects of five different types of nanomaterials (multi-walled carbon nanotubules, aluminum oxide, zinc oxide, aluminum, and zinc) on the seed germination rate and root length of five agriculturally important crops (radish, rape, ryegrass, lettuce, corn, and cucumber) (*11*). They found that out of all five crops, that only the seed germination rate of ryegrass and corn were affected by nano-Zn and nano-ZnO, respectively. In short, Lin and Xing (2007) found that in response to 200 mg/L Al_2O_3 nanoparticles, none of the germination rates from all 5 plants they tested were significantly affected. These results mirror our results because 2000 mg/L converts to the concentration of 0.2 g of Al_2O_3 nanoparticles per 100 mL of media. This concentration fits approximately in the middle of the range of Al_2O_3 nanoparticle concentrations that we employed (0.1g, 0.5g, and 1g of Al_2O_3 nanoparticles per 100 mL of media). Lin and Xing (2007) state that the seed coat of

the tobacco seeds were most likely not permeable to the aluminum oxide nanoparticles, therefore the germination rate was not affected greatly. Thus, it would not be until after the seedlings started to emerge from the seed coat, that they would be affected by aluminum oxide nanoparticles.

Numerous studies over the past few decades have shown that the primary mode of toxicity related to exposure to aluminum ions (Al_3) is preventing root elongation in plants (*12-14*). Aluminum compromises approximately 7% of the earth's crust though most forms of aluminum are bound by ligands that make them less toxic (*12*). Although most forms of aluminum in soil are not very toxic, aluminum solubility increases as the pH of water decreases, therefore more soluble aluminum is found in acidified soils. Since approximately 40% of arable land is considered acidified, aluminum toxicity research is in great demand as it plays a large role in the potential growth of crops (*15*). Although much is known about the toxicity of aluminum ions, not much is known about the toxicity that aluminum oxide nanoparticles may have on plants, therefore one aim of this project is to assess whether aluminum oxide nanoparticles also affect the growth and development of plants.

In this study, we also found that as aluminum oxide nanoparticle concentration increased, the average lengths of three week old tobacco seedling roots decreased. Between the control seedlings and the 0.1% aluminum oxide nanoparticle-treated seedlings, the average root length decreased by 25.4%. The 0.5% and 1.0% aluminum oxide-treated seedlings decreased in length by 81.4% and 92.2%, respectively, as compared to the control. As a result, we were able to give evidence that aluminum oxide nanoparticles have a negative effect on the growth of the roots of three week old tobacco seedlings. The phytotoxicity of other metal nanoparticles has also been evaluated through other studies. Aluminum oxide nanoparticles have been shown in

previous studies to inhibit root elongation in corn, cucumber, soybean, cabbage, and carrot (*11*). Other studies have shown that metal nanoparticles negatively affect the root elongation in plants as well (*11, 16, 17*). Zinc oxide nanoparticles have been shown to greatly decrease the root lengths of ryegrass, radish, rape, lettuce, and cucumber (*11, 18*). The study of Lin and Xing (2008) also shows that the most likely contributing cause to the decrease in root growth was from damage to epidermal and cortical cells in the roots of ryegrass by zinc oxide nanoparticles. A study by Asli and Neumann (2009) (*17*) showed that titanium dioxide nanoparticles interfered with the ability of maize to uptake water in the roots by forming aggregates along the root cell walls. This ultimately blocks water uptake and therefore led to reduced root development. As a result, aluminum oxide nanoparticles may have aggregated along the roots of the tobacco seedlings, therefore impeding their proper water uptake and ultimately affecting their growth and development, but more research would need to be conducted in order to confirm the exact mode of damage.

We also found that as the concentration of aluminum oxide nanoparticles increased, the average biomass of each three week old seedling decreased (See Table 4-1). The biomass for the seedlings exposed to 0.1%, 0.5%, and 1% aluminum oxide nanoparticles significantly decreased as nanoparticle concentration increased. This drastic change in the reduction of seedling biomass is most likely correlated to the decreasing lengths of roots. Because of the reduction in root length and leaf count as nanoparticle concentration increases, the biomass would naturally decrease as well because seedlings at this stage only consist of the primary root and leaves. Little is known about the potential uptake and translocation of nanoparticles and their subsequent effect on the growth and development of plants, therefore additional studies are needed to

determine the mechanisms that aluminum oxide nanoparticles have on the phytotoxicity of tobacco seedlings.

miRNAs are a newly discovered class of small regulatory RNAs that act in post transcriptional gene regulation by either marking mRNAs for cleavage or by preventing ribosomal translation of the messenger RNA into a protein (*3, 4*). miRNAs are highly conserved, therefore they are thought to play a major role in biotic and abiotic stress responses. miRNAs have been shown to play key roles in plant response to many environmental stressors such as drought (*19, 20*), salinity (*21, 22*), and heavy metals (*23-25*). In this experiment, our goal was to identify a few miRNAs that may be involved in plant stress response to aluminum oxide nanoparticles. We chose miR156, miR157, miR159, miR162, miR167, miR169, miR172, miR395, miR396, miR397, miR398, and miR399 because they are well known miRNAs that already have known functions in plants, but our aim was to see if they may have alternative roles in abiotic stress response to aluminum oxide nanoparticles. Our results show that nine miRNAs were significantly up-regulated (miR159, miR162, miR167, miR169, miR395, miR396, miR397, miR398, and miR399) as the concentration of aluminum oxide nanoparticles increased. It also needs to be noted that in all of these microRNA expression profiles, the nine previously mentioned miRNAs are down-regulated in expression at the 0.1% aluminum oxide nanoparticle concentration. It is not known why this trend occurred throughout all nine expression profiles, but microRNAs are not the only molecules that mediate gene expression in plants. These miRNAs may have been down-regulated at this concentration because other pathways in the plant were responding to the aluminum oxide nanoparticle stress. The large amount of up-regulation in expression of the nine miRNAs suggests that these miRNAs either play an

alternative role in mediating stress response to aluminum oxide nanopartices in tobacco, or they mediate other responses as a result of aluminum oxide nanoparticle stress.

A study conducted by Reyes (2007) shows that miR159 plays a role in the response of plants to drought and during seed germination. During seed imbibitions, the levels of abscisic acid (ABA) decreases in order to allow the seeds to intake water and to begin germination (*26*). Under abiotic stress conditions, such as the conditions in our experiment, the levels of ABA within the seeds could possibly stay elevated and as a result, the growth and development of seedlings would be stunted. ABA also is known to induce miR159 which targets MYB101 and MYB33 and suppresses their expression (*26*). The consequence of MYB101 and MYB33 suppression is that it makes plants hyposensitive to ABA, therefore affecting their ability to germinate. miR159 is also up-regulated in response to drought, therefore it is believed that miR159 upregulation helps seeds to sense the environment and prevents germination if the environmental conditions are not optimal. In our experiment, miR159 was up-regulated approximately 5.8 fold in response to 1% Al2O3 nanoparticles and the seed germination rate for the same concentration of nanoparticles was lower than the other concentrations. It is possible that miR159 expression was up-regulated in aluminum oxide exposed tobacco seedlings in order to mediate a similar pathway as described above, or that the up-regulation of this miRNA plays an alternate role in allowing the mediation of tobacco seedlings to aluminum oxide nanoparticle stress.

The target site of miR162 is the Dicer-Like 1 protein that functions in the biogenesis of microRNAs in plants (*5*). Dicer-Like 1 functions to cleave pri-miRNA sequences into pre-miRNA sequences that further undergo cleavage until they become mature miRNAs (*3, 4*). In our experiment, the levels of miR162 were up-regulated, which would suggest that the amount of

miR162's target protein Dicer-Like 1 would decrease, consequently decreasing the amount of mature miRNAs that could be produced. In our study, we saw that the majority of the miRNAs screened ended up being up-regulated, not down-regulated, as suggested. This implies that miRNA162 may have an alternate role in mediating abiotic stress response to aluminum oxide nanoparticle stress.

miR167 functions mainly to target the transcription factors, Auxin Response Factor 6 and 8 (ARF), which helps to regulate the levels of the hormone auxin.(*5, 27*). Auxin is a plant hormone that plays a central role in growth and development through the regulation of reproductive organs and root development; therefore, if plants were not as sensitive to auxin, it could explain the decreased root lengths that we observed in our experiment. Most likely, miR167 plays an alternate role in mediating stress response to aluminum oxide nanoparticles.

A recent study has shown that over-expression of miRNA169 confers enhanced drought tolerance to tomatoes (*20*) through the reduction in activity of the stomata. By reducing the activity of stomata, the rate of transpiration was significantly reduced, therefore reducing the amount of water loss from the leaves. In our study, miR169 was up-regulated; therefore, it is possible that over expression of this miRNA may have a similar effect on water loss in tobacco. Whether up-regulation of miR169 plays a role in stomata activity in a similar manner in tobacco, the up-regulation of this miRNA suggests that it plays some role in mediating stress response to aluminum oxide nanoparticles. Further experiments would need to be performed to see if miR169 controls the movement of stomata in the same manner as it does in tomato.

Many miRNAs have been identified to mediate nutrient homeostasis in plants (*28*). Our results show that miR395 was up-regulated the most with a fold change of approximately 315

fold as compared to the control. miR395 is known to sense sulfate starvation and helps to assimilate sulfur in plants (*29*). Sulfur is an important element in plants because it is found in amino acids, oligopeptides, vitamins, cofactors, and many secondary products (*30*); therefore, the regulation of the amount of sulfur found within plants is important to monitor. miR395 helps to monitor this process because it targets several genes that are involved in sulfur assimilation (*29*). When sulfur levels are low within plants, miR395 expression is greatly increased; therefore, the results of our experiment indicate that the tobacco seedlings may have been undergoing sulfur starvation as a result of the up-regulation in expression of miR395. If the seedlings were undergoing sulfur starvation, that would also explain why as the concentration of the aluminum oxide nanoparticles increased, the overall growth and development of the seedlings decreased. Because sulfur is a key element in so many biologically important molecules, the growth and development of the plants would be affected if the plants were starved for sulfur. It is also possible that miR395 plays a role in mediating the stress response to aluminum oxide nanoparticle exposure through an alternative pathway.

miR396 has been shown to aid in both cell proliferation in *Arabidopsis* (*31*) and tolerance to drought in tobacco (*19*). Our findings show that miR396 was up-regulated 12 fold more than the basal expression levels in tobacco. In brief, the study conducted by Rodriguez et. al (2010) shows that *Arabidopsis* plants which have higher levels of miR396 also have higher levels of the transcription factor TCP4 and lower levels of GRFs (Growth Regulating Factors). Because of the increased miR396 levels leading to increased amounts of TCP4, cell proliferation is slowed and plant growth and development is affected as a result. Yang and Yu (2009) conducted a study to assess the effect of miR396 on tolerance to drought in tobacco (*32*). They found that if miR396 was up-regulated in tobacco, then the plants had a higher tolerance to drought through

reducing the size of their leaves and by reducing their stomatal index. Based on these two studies, since miR396 was up-regulated in the aluminum oxide nanoparticle exposed seedlings, it is possible that the growth and development of the seedlings was stunted due to a decrease in cell proliferation. It is also possible that the reason why the seedlings did not die is due to the fact that increased levels of miR396 helped in aiding the retention of water within the plants as shown in tobacco by Yang and Yu (2009). It is also possible that miR396 may have an alternate role in the response of tobacco seedlings to aluminum oxide nanoparticles.

miR397 was also significantly up-regulated in our experiment. miR397 has been shown to play a role in drought stress response, nutrient deprivation, and copper homeostasis (*33*, *34*) within plants. Copper serves as a critical cofactor for components of the electron transport chain, and as a cofactor for proteins involved in metabolism, the removal of reactive oxygen species, and in cell signaling. Since miR397 was up-regulated, this suggests that the seedlings may possibly have been deprived of copper. If this was the case, copper starvation would explain the decrease in growth of the tobacco seedlings since copper plays a key role in many cellular processes. In this experiment, miR397 was up-regulated 55 fold in the 1% Al_2O_3 concentration of nanoparticles; therefore, we believe that this miRNA may play a role in the response of tobacco to aluminum oxide nanoparticles through a similar pathway as described above, or through an alternate pathway.

miR398 has also been shown to be up-regulated in response to water deficit in *Medicago truncatula* (*35*)and has also been shown to be down-regulated in response to oxidative stress or low copper levels (*23*). Another study has also shown that miR398 is up-regulated in the presence of 1% sucrose (*36*). Our seedlings were grown in media supplemented with 1% sucrose; therefore, it is a possible that miR398 could have been up-regulated simply due to

exposure to sucrose in our growth media. More likely, miR398 plays an alternate role in mediating stress to aluminum oxide nanoparticles in tobacco seedlings. More research needs to be conducted to elucidate the exact mechanism of the miR398 response to aluminum oxide nanoparticles exposure.

miR399 has been shown to play a key role in regulating phosphate starvation in plants (*37*). Phosphate plays a key role in metabolism and signaling, therefore levels in plants needs to be highly regulated. In our experiment, miR399 was greatly up-regulated (90 fold), third behind miR395, therefore the seedlings may possibly have been under the influence of phosphate starvation which would explain the decrease in growth and development of the seedlings. Because miR395 (sulfate regulation) and miR399 (phosphate regulation) were both up-regulated the most in response to 1% Al2O3 nanoparticles, these two miRNAs may play a key role in response to stress to aluminum oxide nanoparticles exposure besides their known roles in sulfate and phosphate regulation.

Conclusion

Overall, we found through our study that Al_2O_3 nanoparticles have a negative impact on the growth and development of three week old tobacco seedlings. We saw an overall significant reduction in root length, average biomass, and leaf count of each tobacco seedling after being exposed to 0.1%, 0.5%, and 1% Al_2O_3 nanoparticles, but no significant change in the germination rates of the seedlings. We have not conducted any studies to show the exact mode of phytotoxicity of aluminum oxide nanoparticle exposure to tobacco seedlings. The two common possibilities are either the nanoparticles will adhere to the roots impeding any uptake of water and nutrients, or that the nanoparticles are taken up and translocated within the plant

therefore causing toxicity internally. Aluminum oxide exists commonly in the soil as aluminum is one of the most abundant elements found in the earth's crust. It is not known how much of the aluminum oxide found in the soil exists as nanoparticles, therefore more research needs to be conducted to determine if the increasing use of aluminum oxide nanoparticles in industry is drastically affecting the concentrations of aluminum oxide that is already present in the soil. More research also needs to be conducted in order to elucidate the mechanisms of aluminum oxide nanoparticle toxicity and to determine the exact mode of phytotoxicity in plants.

miRNA expression levels in tobacco were also altered as a result of exposure to aluminum oxide nanoparticles. In particular, miR395, miR398, and miR399 exhibited the greatest increase in fold changes of 315, 144, and 90, respectively. These miRNAs play a key role in nutrient starvation and resistance to drought, therefore this study suggests that these miRNAs may either play a similar role in nutrient starvation and resistance to drought as shown in other experiments or that these miRNAs play alternate unknown roles in response to stress caused by exposure to aluminum oxide nanoparticles. Our study analyzed the changes in expression levels of only twelve conserved miRNAs, therefore in order to identify novel tobacco miRNAs that may play a role in plant tolerance to aluminum oxide nanoparticle stress, other experiments such as high through-put sequencing will need to be performed. We found that aluminum oxide nanoparticles affected miRNA expression levels in tobacco and since they play a key role as gene regulators, miRNAs may play a vital role in tobacco tolerance to stress caused by aluminum oxide nanoparticle exposure. As a result, more research needs to be conducted to identify the roles of miRNAs in mediating plant stress responses in order to subsequently improve the ability of plants to withstand stresses in the environment.

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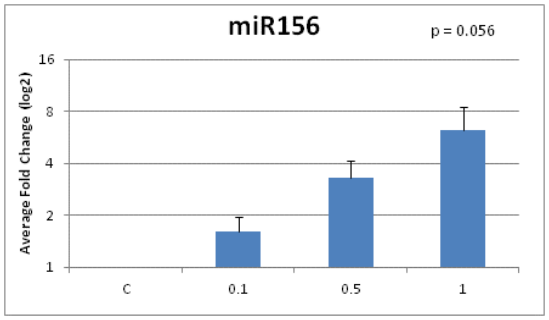
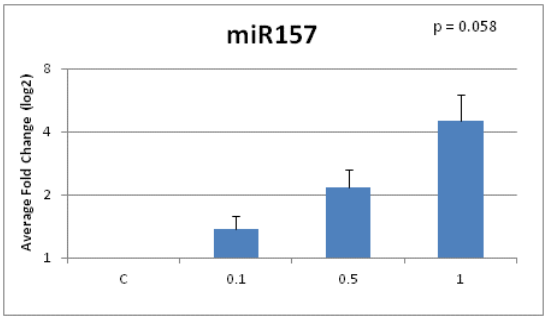
Table 4-1. Nanoparticles effect the growth and development of three week old tobacco seedlings. All physical measurements were averaged for each treatment (control, 0.1% Al_2O_3, 0.5% Al_2O_3, and 1% Al_2O_3). Each average is stated with the standard error associated with that treatment and the significance levels are denoted with the letters, a-d. Different letters dentote statistical significance within the physical measurement.

	MS Media (Control)	**0.1%**	**0.5%**	**1%**
Root Lengths (mm)	29.54 ± 2.09 a	22.04 ± 3.01 b	5.52 ± 0.24 c	2.31 ± 0.13 c
Leaf Count	3.77 ± 0.09 a	3.34 ± 0.14 b	2.82 ± 0.08 c	2.39 ± 0.14 d
Germination Rate	24.80 ± 2.33 a 99.2%	23.20 ± 1.24 a 92.8%	24.00 ± 0.55 a 96%	22.00 ± 1.00 a 88%
Biomass per seedling (mg)	12.0 ± 0.0015 a	7.5 ± 0.0015 b	2.8 ± 0.00056 c	2.1 ± 0.0003 c

Figure 4-1. Average miRNA fold changes in expression in response to aluminum oxide nanoparticles (control, 0.1%, 0.5%, and 1%) that do not fall within the 95% confidence interval.





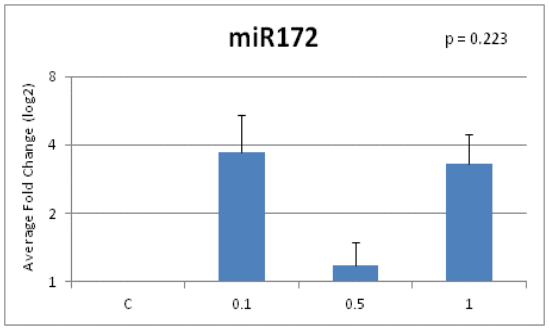


Figure 4-2. Statistically significant average fold changes of microRNA expression levels in tobacco plants exposed to aluminum oxide nanoparticles (control, 0.1%, 0.5%, and 1%). Statistical significance is denoted by letters a-d.

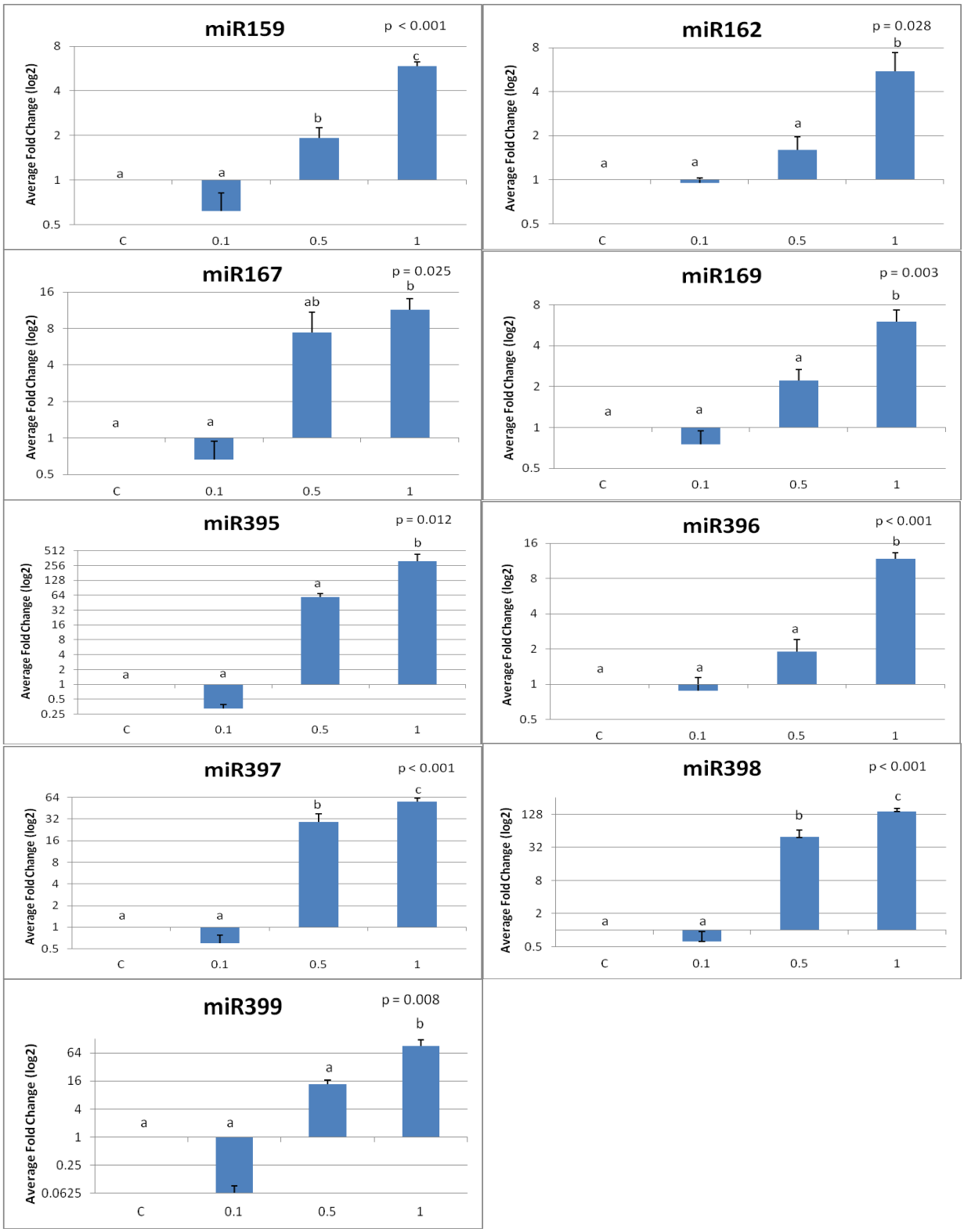


Figure 4-3. Average fold changes in expression of two stress genes, APX and ADH in tobacco plants exposed to aluminum oxide nanoparticles (control, 0.1%, 0.5%, and 1%).

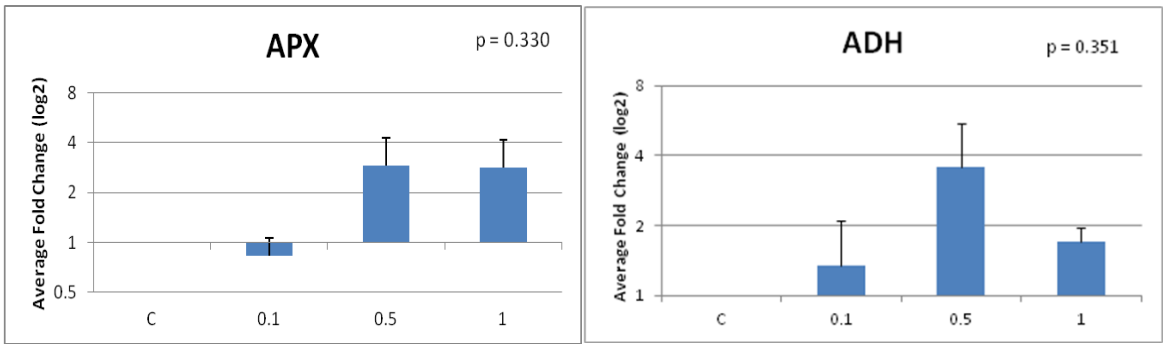


Figure 4-4. Phenotype of aluminum oxide treated tobacco seedlings. From left to right, control, 0.1%, 0.5%, and 1% aluminum oxide treated seedlings.