Potassium deficiency had a significant effect on the growth, development and microRNA-mediated mechanism in wheat (*Triticum aestivum* L.)

by

Thomas Thornburg

December, 2020

Director of Thesis: Dr. Xiaoping Pan

Major Department: Biology

Potassium is a crucial nutrient in the growth and development of plants and lack of available potassium was found to have significant affects in altering the morphology and gene expression of wheat. Wheat plants were grown from seeds in nutrient rich solutions, one with a standard concentration of potassium and one completely deficient in potassium. Root morphology of wheat was analyzed by comparing root volume, diameter, area, length, and branching zones. Dry biomass was recorded to compare root and shoot growth. Leaf and root respiration data was collected by measuring oxygen consumption of various samples. Root vitality measurements were gathered with a fluorescent analyzer at 485nm, but no significant results were found. Chlorophyll content was measured via spectrophotometry at 645nm and 663nm wavelengths. Gene expression of numerous miRNAs and target genes was measured and analyzed with qRT-PCR. Potassium deficiency resulted in a reduction in biomass and size of seedlings that was significant in shoots but had minimal influence on root vigor and biomass. The roots of potassium deficient plants, however; did suffer significant effects on root development. The emergence of seminal roots was delayed but had enhanced root length at a later time point. Increased total root surface area was also observed in potassium deficient samples along with extended non-branching zones. Results of

gene expression analysis indicated that potassium deficiency altered the expression of many miRNAs and their target genes, allowing for the study of gene expression changes in response to abiotic stress. The changes in gene expression, when compared with alterations in wheat morphology and physiology, offer new insight into the actions and mechanisms of miRNA mediated gene regulation.

Potassium deficiency had a significant effect on the growth, development and
microRNA-mediated mechanism in wheat (*Triticum aestivum* L.)

A Thesis

Presented To the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

M.S. Molecular Biology and Biotechnology

by

Thomas Thornburg

December, 2020

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by

Thomas Elliott Thornburg

APPROVED BY:

DIRECTOR OF THESIS:

_____

Xiaoping Pan, PhD

COMMITTEE MEMBER:

_____

Baohong Zhang, PhD

COMMITTEE MEMBER:

_____

Yumin Li, PhD

COMMITTEE MEMBER:

_____

CHAIR OF THE DEPARTMENT OF BIOLOGY:

_____

David Chalcraft, PhD

DEAN OF THE GRADUATE SCHOOL:

_____

Paul J. Gemperline, PhD

ACKNOWLEDGEMENTS

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INTRODUCTION

Triticum aestivum, or common wheat, is the third most planted crop in the United States in terms of acreage and overall production according to the United States Department of Agriculture (USDA) (*1*). Wheat is also a major crop grown widely across the world, making it an especially important and widely used resource. The issue of potassium deficiency in agriculture has a surprisingly wide reach, affecting crops around the world (*2*). Studies on potassium concentration in soil have revealed that in China as many as 75% of paddy soils are K^+ deficient, and that in Southern Australia as many as 67% of wheat fields are as well (*3*) (*2*). Generally, soil potassium levels are found below 0.3mM (*2*). Considering the prevalence of agricultural plots across the world with a potassium deficiency problem further research should be conducted to determine the cause of the issue and address it.

Potassium (K^+) is a crucial nutrient in many plant processes including photosynthesis, osmoregulation, and activating enzymes in important metabolic pathways (*4*). This allows K^+ concentration levels to regulate these enzymes both transcriptionally and post-transcriptionally (*4*). It is also important in plant growth and development making it a point of interest in maximizing crop yield efficiency (*5*). Research has shown that a deficiency of K^+ in plants is a form of abiotic stress that triggers a variety of responses resulting in reduced growth and productivity (*6*). Coping strategies such as morphological and physiological modifications and regulation of K^+ transport systems exist; however, these mechanisms are not fully understood (*6*). By further investigating the effects of K^+ deficiency and availability on plant systems and

identifying the mechanisms involved in driving the observed changes crop yields and quality can be maximized.

MicroRNAs (miRNAs) are small regulatory RNA molecules that bind to target mRNA sequences upon formation of an mRNA-inducing silencing complex (RISC) (7). They are a class of small non-coding RNAs responsible for regulating targeted genes in metabolic and developmental processes (8). miRNAs are approximately 22 nucleotides long and in plants are made from *Dicer-like 1* (*DCL1*) from precursor sequences (pre-miRNAs) (9). They are also involved in the regulation of stress responses, such as abiotic and biotic stress (8). Gene expression is inhibited by miRNAs in two ways: either by the targeted degradation of mRNA molecules or by acting to inhibit protein translation (9). Research into identifying new miRNAs and determining their functions is growing rapidly in a multitude of plant species.

Research into the effects of K^+ deficiency on wheat has identified variant transcription features in metabolic processes, cation binding, transferase activity, and ion transporters (2). There have been reports made demonstrating that plants in varying concentrations of nutrient solutions also exhibit variance in miRNA expression levels (10). Considering the findings of previous studies, it seems fairly likely that alteration of miRNA expression could be one of the methods by which K^+ deficiency changes transcription features in wheat. This information raised a question, do miRNAs exhibit an influence over the changes in transcription features of *Triticum aestivum* upon abiotic stress induced by K^+ deficiency and if so, what is the mechanism involved?

MATERIALS AND METHODS

Wheat Seed Sterilization and Growth Conditions

Wheat seeds of the *AK-58* genotype were collected, and surface sterilized in 9% H_2O_2 solution for 5 minutes followed by ten washes with sterilized water. Sterilized seeds were then carefully placed roughly 1 centimeter apart and 5 centimeters from the top of germination paper soaked in nutrient solution with differing potassium concentrations. The papers were rolled and placed in a plastic tub of nutrient solution in dark conditions at 28°C for four days until germination was observed. Tubs were covered with a plastic sheet punctured with a needle to prevent evaporation of solution while allowing airflow. Plant nutrient solution consisted of 1 mM NaCl, 1.25 mM $Ca(NO_3)_2$, 0.25 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 0.05 mM EDTA FeNa, 0.02 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $MnSO_4$, 0.2 µM $CuSO_4$, and 0.005 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Potassium concentration of the nutrient solution was manipulated to yield a high potassium solution as a control, and a low potassium solution. The high potassium control contained 1.25 mM KCl, while the low potassium solution contained 0.01 mM KCl and 1.24 mM NaCl.

Upon germination 25 seedlings were collected from each treatment group for analysis and the rest were transferred to a hydroponic system under lamplight at 28°C. The system contained a nutrient solution that consisted of 2 mM NaCl, 2.5 mM $Ca(NO_3)_2$, 0.5 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 0.1 mM EDTA FeNa, 0.02 mM H_3BO_3, 0.001 mM $MnSO_4$, 0.2 µM $CuSO_4$, and 0.005 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The potassium concentrations were manipulated to create a high concentration control with 2.5 mM KCl, and a low concentration treatment with 0.02 mM KCl and 2.48 mM NaCl.

Root Morphological Analysis

Morphology of the plants was observed at two time points, 4 days and 8 days after treatment to account for the number of roots. Scans and images of the various regions of the plants were then taken with an EPSON scanner. Analysis of the length, area, volume, and diameter of the roots was then done using the WinRHIZO software. The length of the main root was measured along with the length of regions where no branched roots were present.

Dry Biomass

Samples taken 4 days after treatment were divided into shoots and roots and dried in an oven at 80ºC. The samples were then ground, and the dry mass measured in a balance.

Measurement of Root and Leaf Respiration

Samples were collected 4 days and 8 days after treatment from the treatment and control groups. From the middle of the first fully opened leaf 2cm were cut out and weighed along with a section of roots (5-7 cm from the stem). A Clark Chlorolab2 instrument was used to collect and measure data from the respiration assay. Samples were placed into the incubator of the device along with 2 mL of saturated $CaSO_4$ solution. The instrument then measured oxygen consumption of the samples for 10 minutes. The rate of O_2 consumption was determined from the data collected.

Root Vigor

The TTC method was used to determine root vigor for six biological replicates of each sample at each time point. Root samples were cut into 1cm length fragments and soaked in a solution of 0.6% TTC in 0.1 M phosphate buffer solution. The samples were

dyed for 24 hours in TTC before washing with ddH$_2$O three times. Extraction of triphenylformamidine (TTF) was done by soaking the samples in 95% ethanol for 10 min at 85ºC. Root vigor measurements were measured with a fluorescent analyzer at 485 nm wavelength.

Measurement of Chlorophyll

Samples of the first fully opened leaf at each treatment and time point were collected and broken in mortar with CaCO$_3$ and acetone and transferred to a 15 mL centrifuge tube. The mortar was washed with acetone to collect leftover residue and was added to the centrifuge tube. Samples were then centrifuged at 10,000 rpm for 10 min. Acetone was added to a total volume of 5 mL and chlorophyll content measured in a spectrophotometer at 663 and 645 nm wavelengths. Six biological replicates were collected for each treatment and time point.

RNA extraction, cDNA synthesis, and qRT-PCR gene expression analysis

RNA extraction and isolation were performed as outlined in the manufacturers protocol of the MirVana miRNA Isolation Kit. Extracted RNA was quantified and quality tested with a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). Extracted RNA was stored at -80°C until needed.

Reverse transcription of isolated RNA was performed as outlined in the manufacturers protocol of the TianGen miRcute miRNA First-strand cDNA Synthesis Kit. miRNA specific stem-loop primers from the kit were used to synthesize cDNA from the previously extracted RNA samples. Synthesized cDNA was then used in qPCR.

Analysis of miRNA and target gene expression was done as outlined in the manufacturers protocol of the TianGen miRcute Plus miRNA qPCR Detection Kit. The

previously synthesized cDNA was amplified and quantified to determine expression levels. 50xROX Control Dye and 50xROX Reference Dye were used along with SYBR Green to facilitate measurement of expression levels. Expression values were normalized against the reference gene *EF1A*. Fold change was calculated using the $\Delta\Delta C_T$ method. Three biological and technical replicates were run for each gene and miRNA to reduce error.

A selection of 20 miRNAs (miR160, miR164, miR165, miR166, miR167, miR169, miR171, miR172, miR390, miR393, miR396, miR847, miR857, miR156, miR162, miR319, miR395, miR778, miR399, miR827) and 9 target genes (*NAC1, NAC2, HD_Zip, IAR3, NFYA2, ARF2, GRF1, bHLH, SPL1*) involved in plant development and stress response were chosen for analysis.

RESULTS

Potassium deficiency significantly affects wheat growth and biomass

Deficiency of potassium was shown to have a significant effect the growth of wheat above ground while having no significant effect on root biomass. Potassium deficient seedlings were observed to be noticeably impacted after 4 days of treatment. Compared to wheat with normal potassium availability the treatment group developed smaller leaves, a change in leaf color from dark green to light yellow, and a smaller overall size. At the 4-day time point the biomass of the treatment group significantly reduced from 50.78 mg to 39.34 mg, a 22.53% reduction (Table 1). This difference in biomass was consistent with the 8-day time point as well with a significant reduction from 67.22 mg to 53.83 mg, a 19.92% reduction (Table 1).

No significant differences were observed between the root masses of the control and treatment groups at either the 4-day or 8-day time points (Table 1). Though a significant difference in root mass would be expected after enough time in potassium deficient conditions no such trend was observed within 8 days after transfer (Table 1). Despite no significant difference in root biomass, the difference in the biomass of the shoots was great enough to cause a significant difference between the total plant biomass of the control and treatment at both time points (Table 1). A relevant significant difference was observed in the shoot area of the treatment and control groups at both time points as well (Table 1).

Table 1: Effect of potassium deficiency on wheat seedling biomass and shoot area *

DAT (d)[&]	Treatments	Dry shoot weight (mg)	Dry root weight (mg)	Dry plant weight (mg)	Shoot area (cm^2)
4	Control	36.16±3.49a	14.62±1.61a	50.78±4.65a	33.41±4.09a
	K deficiency	24.6±2.19b	14.74±1.21a	39.34±2.91b	28.01±3.18b
8	Control	48.1±8.13a	19.12±4.24a	67.22±11.61a	55.03±8.18a
	K deficiency	35.4±4.48b	18.43±3.03a	53.83±7.47b	37.94±4.54b

* Different lower-case letters indicate significant difference at P<0.05 level
[&] Days after transfer (DAT) of wheat seedlings into potassium treatment solution

Potassium deficiency significantly affects wheat root development

Though no significant difference in root biomass was observed at the 4-day and 8-day time points, significant differences in several areas of root development were observed. Potassium deficient conditions resulted in the significant delay in emergence of seminal roots at the 4-day time point, the control producing 5.4 per plant as opposed to the treatment producing 4.2 (Table 2). By the 8-day time point the treatment plants had recovered producing 5.2 seminal roots to the control's 5.5 (Table 2). Though the treatment recovered in number of seminal roots after 8 days of treatment, there was a significant difference at the 8-day time point in seminal root length that was not present at the 4-day time point (Table 2). Seminal roots in the treatment group were observed to be significantly longer at 18.55 cm/SR compared to the control group at 13.15 cm/SR at the 8-day time point (Table 2).

A significant difference was also observed in the non-branching zone length at the 8-day time point with the potassium deficient plants growing longer roots than the control, 7.27 cm compared to 4.04 cm (Table 2). After 8 days of treatment the total root

length of the treatment samples was also found to be significantly greater than the roots

of the control samples, 370.76 cm/plant compared to 256.73 cm/plant (Table 2). Along

with total root length, total root surface area was found to be significantly greater in

treatment samples at the 8-day time point compared to the control (Table 2). Potassium

deficient samples at 8 days had an average surface area of 30.79 cm^2/plant versus the

control's 22.21 cm^2/plant (Table 2). No significant differences were observed at any time

point in total root volume, average root diameter, or branching zone length (Table 2,

Figure 1).

Table 2: Effect of potassium deficiency on morphological parameters of wheat seedling roots from germination to emergence at various days after transfer (DAT) *

DAT (d)	Treatments	TRL (cm/plant)	TRS (cm²/plant)	TRV (cm³/plant)	ARD (mm)	SRN (No/ plant)	SR length (cm)		FRN (No.)		BZL (cm)	NBZL (cm)
							Cm/SR	Cm/plant	No/SR	No/plant		
4	Control	144.60±10.77a	14.53±1.14a	0.12±0.01a	0.32±0.32a	5.4±0.89a	12.34±4.67a	66.63±25.21a	15.89±4.97a	85.8±26.86a	8.13±2.37a	5.29±1.60a
	K deficiency	153.55±22.56a	15.52±1.57a	0.13±0.02a	0.32±0.32a	4.2±0.45b	13.27±1.91a	55.74±8.04a	10.43±5.86a	43.8±24.64b	6.97±1.58a	8.12±1.15a
8	Control	256.73±46.67 b	22.21±4.12b	0.16±0.03a	0.26±0.26a	5.5±1.29a	13.15±1.19b	72.34±6.55b	34.18±5.45a	188.0±29.97a	10.62±1.68a	4.04±0.22b
	K deficiency	370.76±63.54a	30.79±4.43a	0.20±0.03a	0.27±0.27a	5.2±0.84a	18.55±2.25a	95.94±11.71a	41.81±7.29a	217.4±37.92a	11.59±0.62a	7.27±0.30a

* Different lower-case letters indicate significant difference at P<0.05 level. – No corresponding root parameters at this time point. Total root length: TLR; Total root surface area: TRS; Total root volume: TRV; Average root diameter: ARD; Seminal root: SR; First order roots number: FRN; Branching zone length: BZL; Non-branching zone length: NBZL.
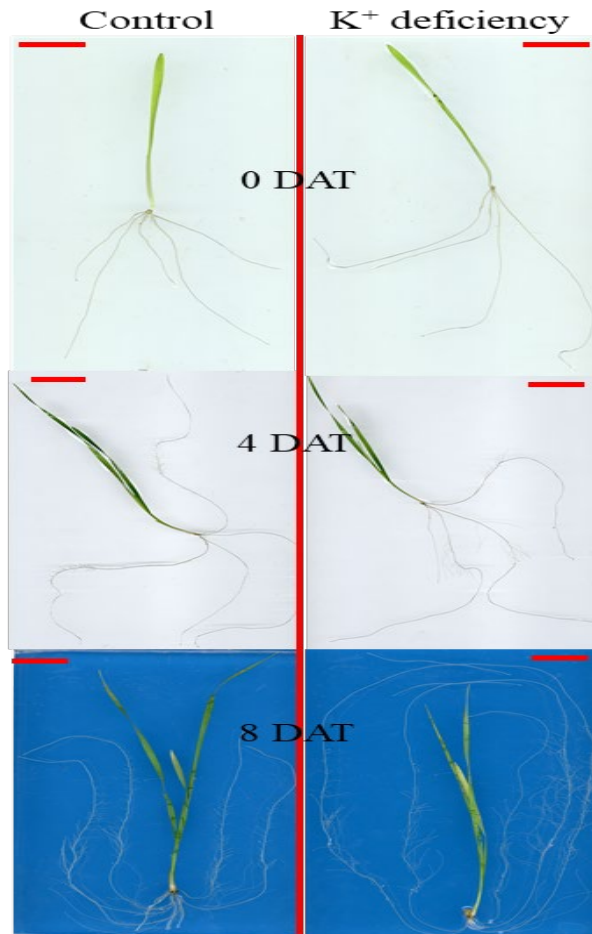
Figure 1: Morphological variances between wheat seedlings of potassium control and potassium deficient groups at 0, 4, and 8 days after transfer (DAT).

Potassium deficiency affects wheat respiration and root vitality

Potassium deficiency was observed to have a significant effect on root and leaf respiration, but only at the 4-day time point (Table 3). The potassium deficient plants demonstrated significantly heightened respiration in both the leaves and the roots at the 4 days after transfer time point (Table 3). The rate of oxygen consumption of the control root was 204.27 nmol L^{-1}/min/g FW which was lower than the potassium deficient treatment at 291.74 nmol L^{-1}/min/g FW (Table 3). Oxygen consumption in the leaves was determined to be 712.70 nmol L^{-1}/min/g FW in control group samples and 1371.04

nmol L^{-1}/min/g FW in the potassium deficient treatment group (Table 3). As treatment

continued to the 8-day time point the plants displayed no significant difference in the

rate of Oxygen consumption indicating they may have adapted to the low potassium

environment.

Table 3: Effect of potassium deficiency on oxygen consumption rate to evaluate root
vitality, root respiration, and leaf respiration at various days after transferring (DAT) *

DAT(d)	Treatments	Root vitality (OD g^{-1} FW)	Oxygen (O$_2$) consumption rate (nmol/min/g FW)	
			Root	Leaf
4	Control	2.64±0.31a	204.27±27.65b	712.70±463.90b
	K deficiency	2.81±0.61a	291.74±45.31a	1371.04±457.35a
8	Control	1.07±0.23a	174.34±24.01a	538.35±153.64a
	K deficiency	1.33±0.27a	129.23±36.98a	695.13±246.75a

* Different lower-case letters indicate significant difference at P<0.05 level.

Potassium deficiency affects chlorophyll biosynthesis in wheat seedlings

The biosynthesis of chlorophyll a and b was significantly inhibited in the

potassium deficient treatment group, resulting in significantly lower total chlorophyll

content (Table 4). The presence of Chlorophyll a was significantly reduced in the

treatment group compared to the control at both the 4-day and 8-day timepoints with

reductions of 17% and 18% respectively (Table 4). Chlorophyll b was similarly affected

at both the 4-day and 8-day time points with reductions of 14% and 20% respectively

(Table 4). As such the total chlorophyll content was similarly reduced at both time

points. Despite the significant changes in biosynthesis the ratio of chlorophyll a to

chlorophyll b remained consistent throughout all treatments and time points (Table 4).

Table 4: Effect of potassium deficiency on chlorophyll and b content at various days after transfer (DAT)

DAT (d)	Treatments	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Chl a : Chl b	Chlorophyll content (mg/g)
4	Control	1.27±0.18a	0.35±0.05a	3.63:1a	1.62±0.23a
	K deficiency	1.05±0.07b	0.30±0.02b	3.50:1a	1.35±0.08b
8	Control	1.28±0.11a	0.35±0.03a	3.66:1a	1.63±0.14a
	K deficiency	1.05±0.11b	0.28±0.04b	3.75:1a	1.34±0.15b

* Different lower-case letters indicate significant difference at $P<0.05$ level.

Expression analysis of miRNAs and target genes in root tissue with qRT-PCR

Low K^+ concentrations induced a significant change in the expression of certain genes and miRNAs compared to the reference gene *EF1A*. Root samples of wheat seedlings at 4 days and 8 days post germination were collected for RNA extraction and analysis with qRT-PCR. A total of 20 miRNAs and their 9 target genes were observed at the 4-day and 8-day timepoints. The results of gene expression analysis can be broken down into two groups: miRNA expression 4 and 8 days after transfer (Figure 2) and target gene expression 4 and 8 days after transfer (Figure 3).

A clear trend was observed in the expression of miRNAs at 4 days post germination in which all 20 were upregulated (Figure 2). The expression of each miRNA ranges from 1.1-fold change to 5.4-fold change with miR847 and miR171 demonstrating the lowest and highest expression respectively (Figure 2). A majority of these, 13 out of 20, were upregulated by 2-fold or greater. Of these 6 miRNAs (miR156, miR164, miR166, miR169, miR171, and miR390) showed greater than a 3-fold upregulation (Figure 2). The expression of many miRNAs was found to be statistically significant at this timepoint (miR156, miR160, miR162, miR164, miR165, miR166, miR167, miR169, miR171, miR172, miR319, miR390, miR399, miR827, miR847 and miR857) (Figure 2).

As opposed to the 4-day samples, the changes in miRNA expression at 8 days after transfer were greatly reduced. Of the 20 miRNAs analyzed 2 were downregulated (miR172 and miR778) (Figure 2). Of the remaining miRNAs the highest upregulated fold change was observed in miR171; however, none of the miRNAs at 8 days after transfer was upregulated by greater than 2-fold (Figure 2). At 8 DAT the expressions of miR164 and miR847 were found to be statistically significant compared to the reference gene.

Change in expression of the target genes at 4 days after treatment displayed a different trend from what was observed in expression of the target genes. The expression of 8 of the 9 target genes (*NAC1, NAC2, IAR3, NFYA2, ARF2, GRF1, bHLH* and *SPL1)* was upregulated at the 4 DAT timepoint (Figure 3). Of these 8 *GRF1* was determined to be statistically significant with upregulated expression over 2-fold (Figure 3).

At the 8 days after transfer timepoint 7 of the 9 target genes were found to be upregulated (*NAC1, NAC2, HD-Zip, IAR3, ARF2, bHLH* and *SPL1*), and 2 downregulated (*NFYA2* and *GRF1*) (Figure 3). *NAC1* and *HD-Zip* were significantly upregulated by over two-fold, whereas *GRF1* was significantly downregulated at this timepoint (Figure 3).

The relationship between gene expression changes can also be observed by looking at the delta-C_t values which have been compared to the reference gene *EF1A*. At 4 days post germination two significant differences were observed ($p<0.05$) in genes *miR390* and *GRF1* (Figure 4). Only three genes (*miR167, miR393, bHLH*) expressed more highly than the reference gene (Figure 4). At 8 days post germination an

increased number of genes showed greater expression than the reference gene, but no observed differences between control and treatment were significant (Figure 5).

The dry mass of wheat root and leaf samples were collected and analyzed at the moving (0 days post germination) and 4-day post germination time points. Significant differences were observed in the leaf mass samples at both the moving and 4-day time points (Figure 6). At the 4 day timepoint the high potassium control was observed to have a much greater mass than the low potassium group (Figure 6). Significant differences were not observed in the root mass of samples, which remain fairly equal between the high and low potassium samples at both time points (Figure 7).

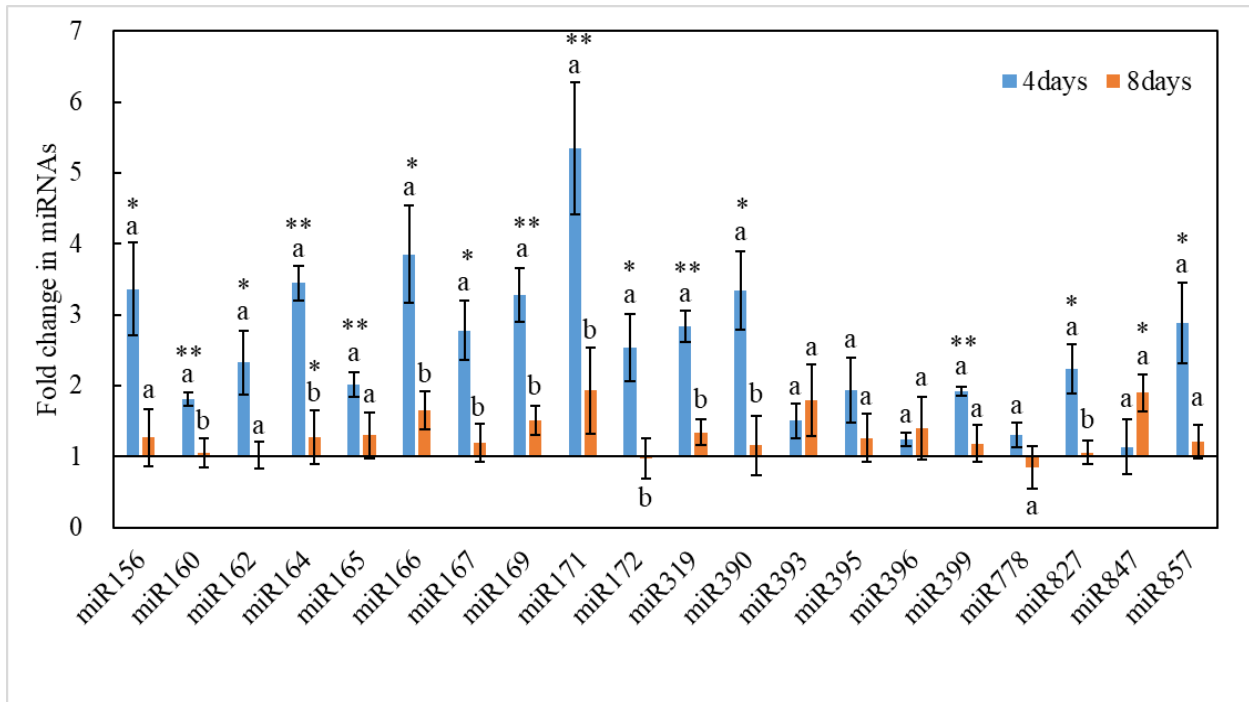**Figure 2:** Fold change of miRNAs tested at 4 and 8 DAT. TwoWay ANOVA statistically significant groups signified by * = p<0.05 and ** = p<0.005. Different letters indicate significant difference in fold change between 4 and 8 DAT according to LSD test (p<0.05).
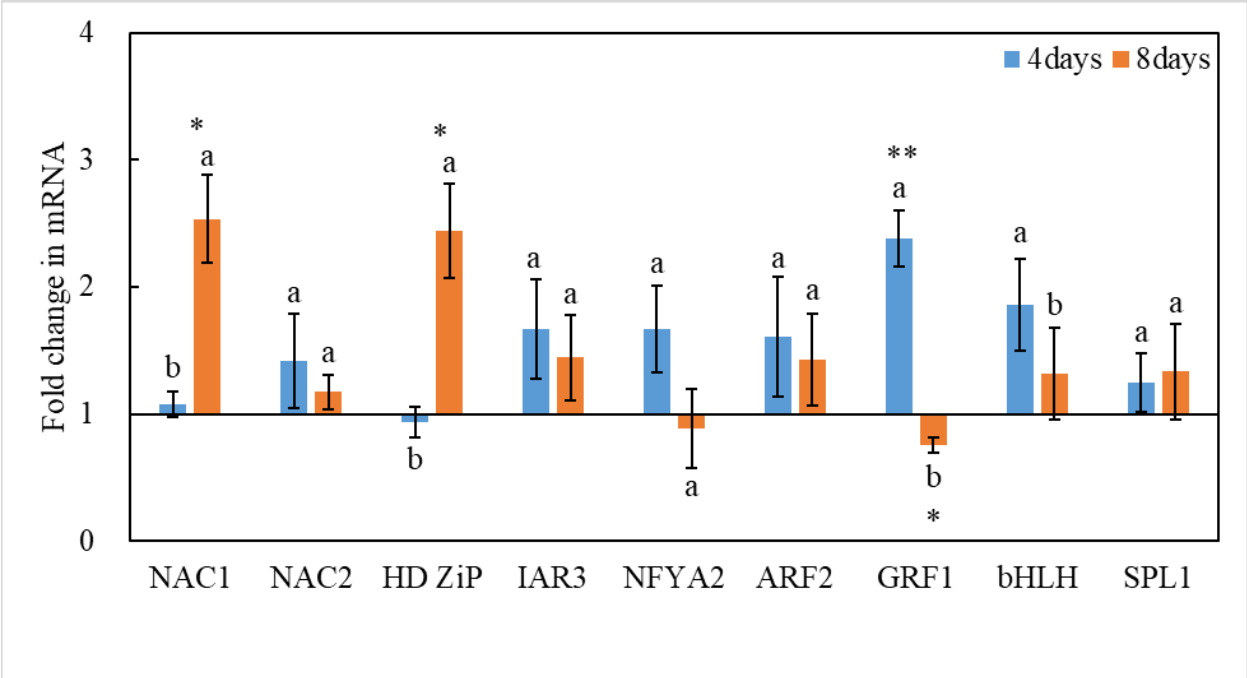
Figure 3: Fold change of target genes tested at 4 and 8 DAT. TwoWay ANOVA statistically significant groups signified by * = p<0.05 and ** = p<0.005. Different letters indicate significant difference in fold change between 4 and 8 DAT according to LSD test (p<0.05).

Figure 4: ΔC_t values from qRT-PCR analysis after comparison to reference gene *EF1A* at the 4-day post germination time point. High potassium control (Blue) and low potassium treatment (Orange) shown side by side for each gene analyzed. OneWay ANOVA statistically significant groups signified by * = $p<0.05$.

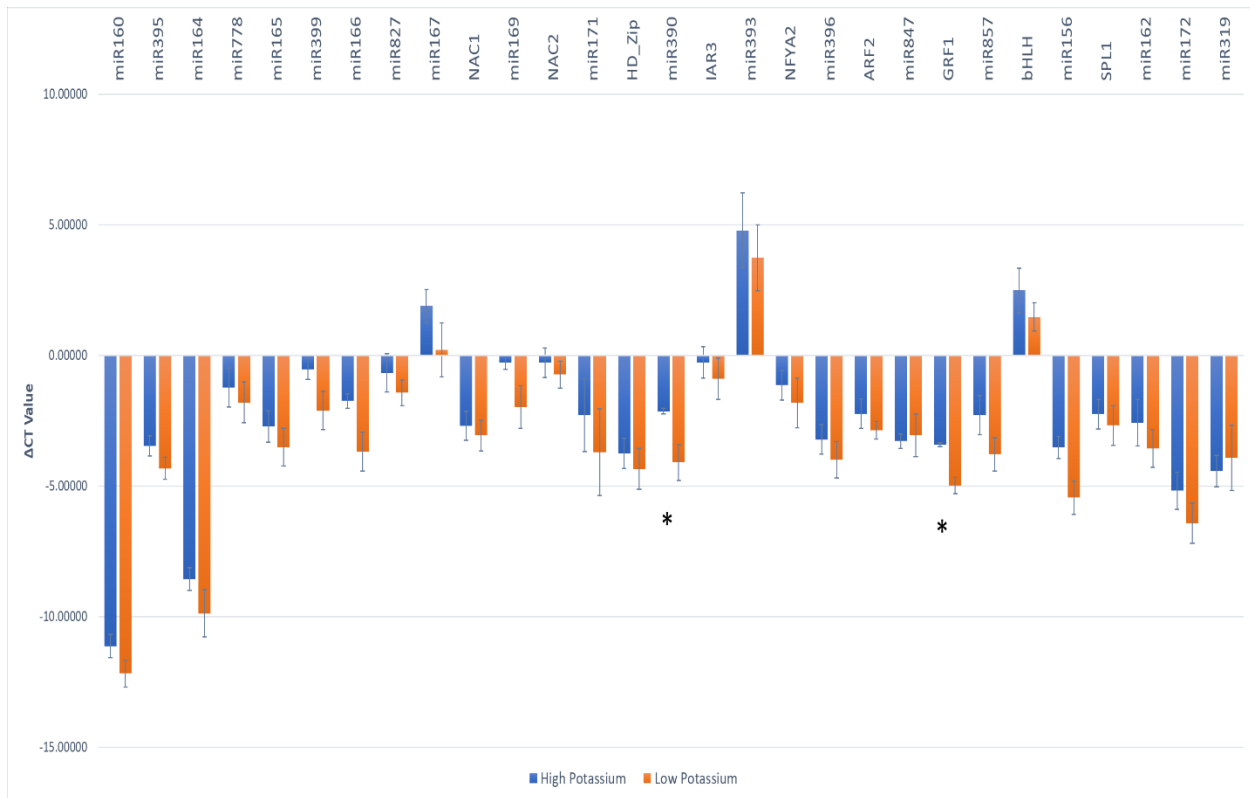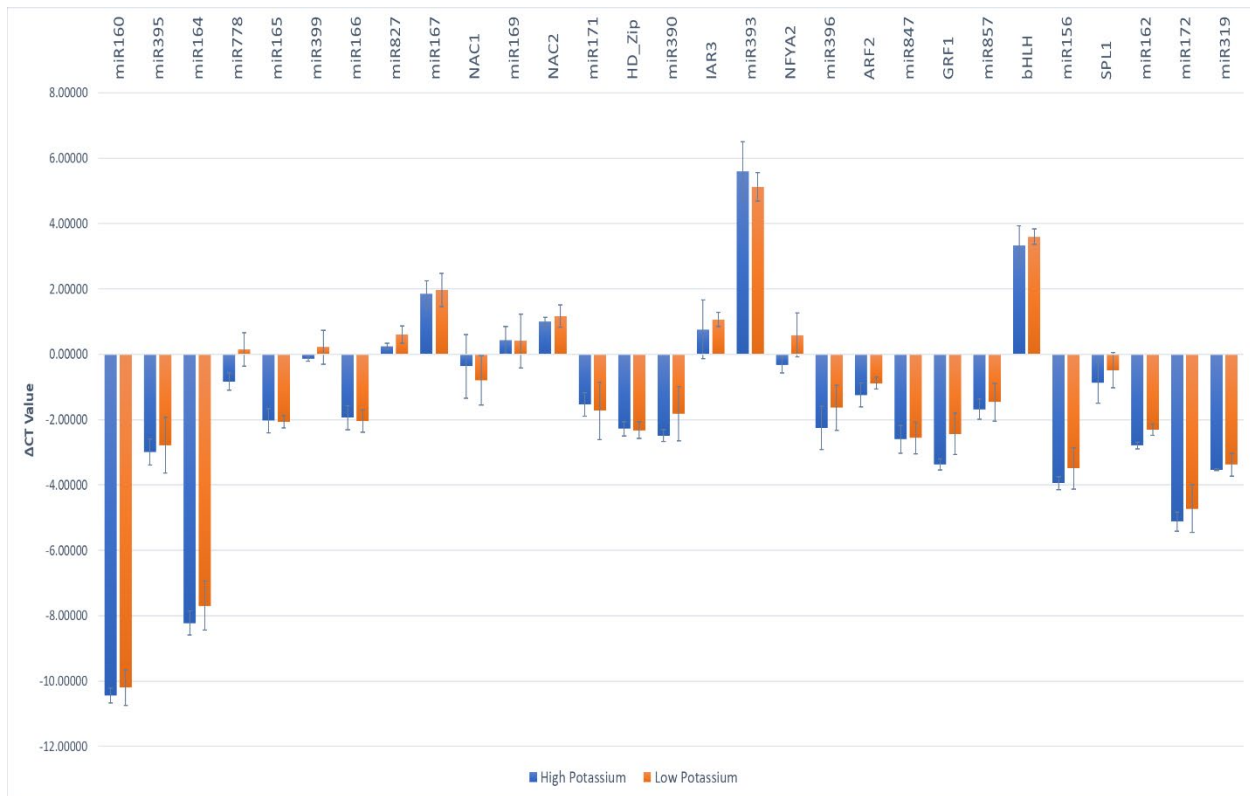**Figure 5:** ΔC_t values from qRT-PCR analysis after comparison to reference gene *EF1A* at the 8-day post germination time point. High potassium control (Blue) and low potassium treatment (Orange) shown side by side for each gene analyzed. OneWay ANOVA statistically significant groups signified by * = $p < 0.05$.

Figure 6: Leaf mass of wheat samples collected at 0-days PG (Moving) and 4-days PG at high potassium (Control) and low potassium. OneWay ANOVA statistically significant groups signified by * = $p<0.05$ and ** = $p<0.005$.

Figure 7: Root mass of wheat samples collected at 0-days PG (Moving) and 4-days PG at high potassium (Control) and low potassium. OneWay ANOVA statistically significant groups signified by * = $p < 0.05$ and ** = $p < 0.005$.

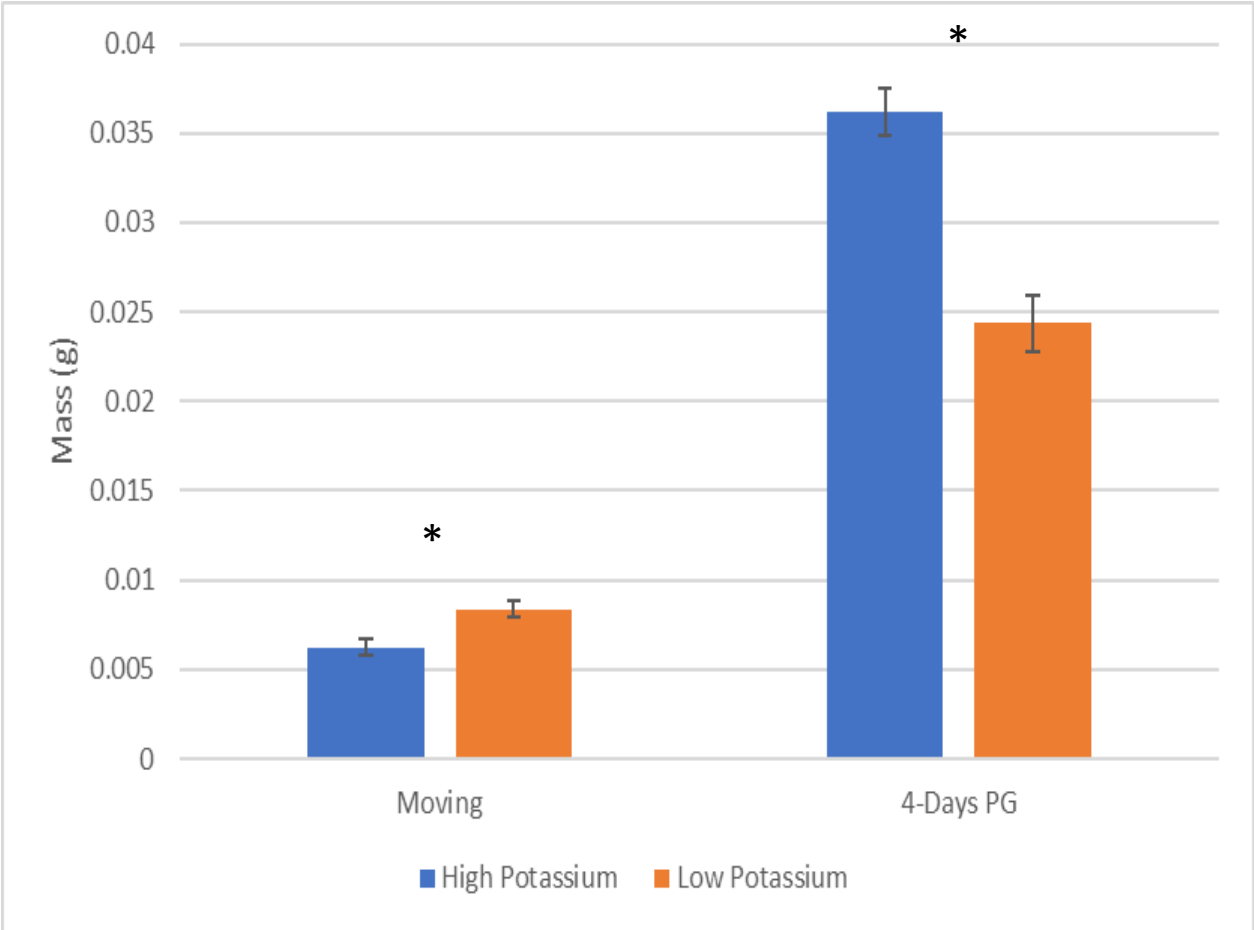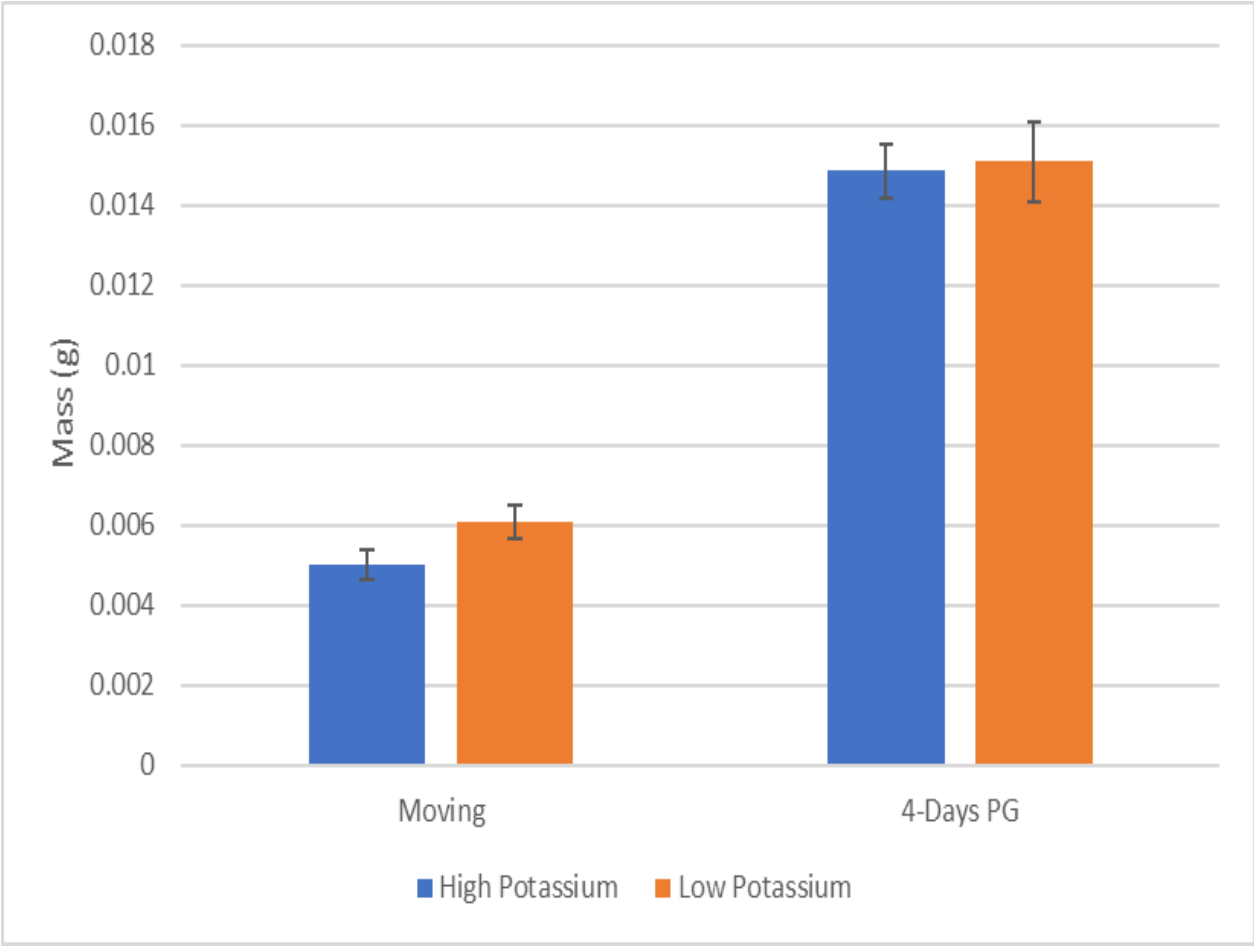**miRNA regulates wheat plant response to potassium deficiency by mediating the expression of protein genes**

The expression of target genes is regulated by the expression levels of the miRNAs that target it. This means that the greater the expression of the miRNA the more highly regulated and inhibited the associated target gene will be. This implies that an inverse relationship between the miRNA's expression and the target gene's expression can be observed, where target gene expression will decrease as miRNA expression increases. There are, however; many more factors in gene regulation other than miRNA-mediated regulation which complicates the relationships between regulatory factors and their target genes. To better understand the interactions between the chosen miRNAs and their target genes, a model of the observed regulatory effects was created (Figure 8). The relationship between the fold changes of the miRNAs and target genes from 4 to 8 days of treatment were graphed in order to demonstrate a linear regression (Figure 8). A rough negative slope can be drawn through data points once graphed.
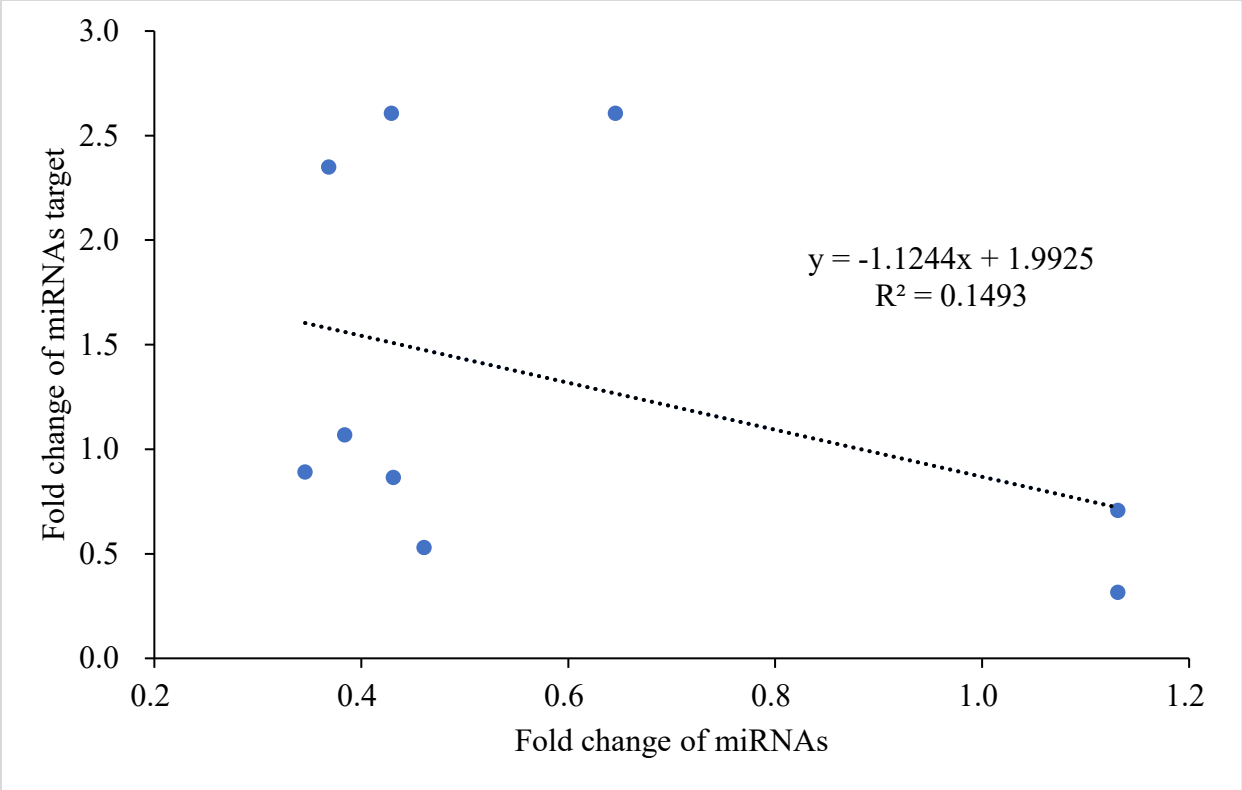
$$y = -1.1244x + 1.9925$$
$$R^2 = 0.1493$$

Figure 8: Model of a linear relationship between the fold change inhibition of miRNAs and their target genes.

DISCUSSION

Deficiency of K^+ in plants triggers reductions in a myriad of physiological processes such as NO_3^- uptake, photosynthesis, and stomatal conductance, all of which lead to a decrease in crop yield and quality (*5*). In order to maximize the efficiency of crop production it is important to gain a better understanding of the mechanisms driving the physiological changes associated with K^+ deficiency. This study was conducted with the goal of gaining a systematic understanding of the phenomenon induced by potassium deficiency in the development and growth of wheat. Emphasis was placed on root development including an investigation into miRNA-mediated gene regulation in response to potassium deficient and potassium rich environments. In this study, the change in expression of miRNAs and associated target genes in *Triticum aestivum* were analyzed under high and low K^+ conditions. This was done in order to ascertain the relationship between potassium deficiency and gene regulation via miRNAs, as well as to gain a better understanding of the miRNA mechanisms themselves. We determined that low K^+ availability likely had an impact on the expression of some miRNAs and target genes, but not on others.

Potassium deficiency was observed to have an affect on many of the physiological processes in wheat and altered the morphology of seedlings in various ways. The biomass of potassium deficient wheat shoots as well as the shoot areas were significantly altered at both the 4-day and 8-day time points while the root biomass was unchanged. The treatment groups roots were, however; found to have significantly longer seminal roots after 8 days of treatment, as well as greater total root length, surface area, and longer non-branching zones. Longer root growth is likely an attempt

to spread further in search of the macronutrients necessary for the seedlings to thrive. At the 4 DAT timepoint miR164 was found to be significantly upregulated, over 3-fold, while *NAC1* showed almost no change in expression compared to the reference gene. By the 8-day timepoint miR164 expression was reduced to an insignificant level, whereas *NAC1* expression was significantly upregulated at this time. This could indicate that in response to a deficiency of potassium 8 days after treatment miR164 expression was reduced, allowing an increase in *NAC1* expression to support root growth.

Potassium deficiency was also found to significantly increase the rate of oxygen consumption in both leaves and roots at the 4-day timepoint, however; by the 8-day timepoint consumption rates had balanced out. Despite these findings chlorophyll a and b content was found to be significantly reduced in treatment groups at both time points. The amount of reduction of each chlorophyll was consistent at both timepoints as shown by the ratio of chlorophyll a to b. This may indicate aberrant regulation by a miRNA and target gene pair with influence over chlorophyll production and plant respiration. This may warrant further investigation into the expression of genes related to chlorophyll biosynthesis and function.

Auxin Response Factor (*ARF*) genes have been shown to play key roles in the development and root growth of plants such as Arabidopsis (*8*). According to other studies the *ARF* family of genes are targets for regulation by several miRNAs, namely miR160 (*8*) (*11*), miR167 (*12*), and miR390 (*13*). miR160 is an important root development regulator and has been found expressed at high levels in *Gossypium hirsutum* (*14*). miR160 has also been documented to exhibit negative regulation on the *ARF-10*, *ARF-16,* and *ARF-17* genes, and to be regulated itself by endogenous target

mimics (eTMs) (*11*). The target gene analyzed, *ARF2*, was not observed to be targeted for inhibition by miR160 at 4 days after transfer. miR160 was significantly upregulated by approximately 2-fold while *ARF2* was also slightly upregulated. At the 8-day timepoint miR160 did not display any significant change in expression compared to the reference gene. The expression of *ARF2* was also not significantly changed, which indicates no inhibitory effect by miR160. miR167 was found to regulate the expression of *ARF6* and *ARF8* in *Arabidopsis* (*12*). In our study miR167 was not found to have a regulatory effect on expression of *ARF2* 4 days post germination since both are upregulated, and it is unlikely that there was an effect at 8 days since miR167 shows very little change in expression compared to the control. miR390 does not directly target and bind to *ARF* family genes, but has been shown to act as a regulating factor by targeting *TAS3* which results in the production of Trans-acting small interfering RNAs (tasiRNA) (*13*). These tasiRNAs then target *ARF* genes, acting to suppress them (*13*). At 4 days post germination miR390 was significantly upregulated, implying that it likely did not act to reduce expression of the also upregulated *ARF2* target gene. At 8 days miR390 was not found to be significantly changed similarly to *ARF2*.

The homeo-domain leucine-zipper (*HD-ZIP*) family of genes have been shown to be targets of both miR165 (*15*) and miR166 (*16*). At 4 days post germination miR165 and miR166 were upregulated approximately 2-fold and 4-fold respectively. *HD_Zip* showed very slight downregulation at this timepoint but was not found to be statistically significant. At the 8-day timepoint miR165 and miR166 each show very little change in expression, a significant reduction from the expression at 4 days. *HD_Zip* at 8 days was significant upregulated. Since a significant reduction in miR165 and miR166 expression

occurred alongside a significant increase in expression of *HD-Zip* one or both of these miRNAs may have had an inhibitory effect on gene expression.

Genes of the *NAC* family have been identified as targets of miR164 in *Arabidopsis*, and that *TaNAC21/22* is a target gene for miR164 in wheat (*17*). Both miR164 and *NAC1* are upregulated at 4 days post germination, with miR164 approximately 3-fold upregulated. Though *NAC1* is upregulated, its expression is extremely low and not significant compared to the reference gene. At 8 days post germination miR164 expression is significantly reduced, whereas *NAC1* shows a significant increase in expression. Though NAC1 expression was not downregulated at any point, this may demonstrate an inhibitory relationship between miR164 and *NAC1*.

Studies have shown that miR396 targets the *GRF* gene family in plants and may also act to regulate bHLH74 (*18*). miR396 was slightly upregulated at 4 days after transfer, though was not significantly different in comparison to the reference gene. At this timepoint *GRF1* was significantly upregulated by over two-fold, whereas *bHLH* was also upregulated but not significantly. At 8 days after transfer miR396 did not display a significant change in expression. *GRF1* was significantly downregulated at 8 days, as opposed to *bHLH* which showed a small degree of upregulation. miR156 targets have been identified in *Arabidopsis* among the *SPL* gene family, and have been studied alongside miR172 targets in the *AP2-like* family in order to better understand their functions (*19*). miR156 displayed over 3-fold upregulation at 4 days after transfer, while miR172 was upregulated by over 2-fold. The *SPL1* target gene was slightly upregulated at 4-days and showed almost no change in expression by 8 days. miR156 and miR172 each displayed almost no change in expression by 8 days, as opposed to the slight

upregulation of *SPL1*. A study in *Arabidopsis* revealed that miR171 may act antagonistically towards miR156 targeting *SPL2* and *SPL9* genes, with respect to the regulation of trichome distribution (*20*) (*21*). miR171 showed the highest expression level of day 4 miRNAs and had largely reduced expression by 8 days, dropping from over 5-fold to under 2-fold upregulation. Considering the lack of change in expression by *SPL1* between the 4 and 8-day timepoints it is unlikely that any of the previously discussed miRNAs had an inhibitory effect on *SPL1* expression.

Arabidopsis root architecture is influenced by the regulation of a target gene, *NF-YA2,* that is targeted by an miR169 isoform (*22*). In the gene expression data gathered miR169 was over 3-fold upregulated at 4 days but showed a large reduction in expression by 8 days where it was less than 2-fold upregulated. The *NFYA2* target gene was found to be upregulated at 4 days, but by 8 days expression was reduced to a slightly downregulated state. The expression of *NFYA2* was not statistically significant at either timepoint. This shows that the expression of *NF-YA2* fell as miR169 expression was reduced.

The expression of a number of other miRNAs that have not yet been found to be associated with the observed reference genes was analyzed to investigate potentially undiscovered interactions. miR162, miR319, miR399, miR827 and miR857 were all significantly upregulated in wheat root cells 4 days after transfer and demonstrated reduced expression at 8 days after transfer. miR847 each demonstrated very low levels of expression relative to the other miRNAs analyzed at 4 days after transfer but demonstrated significant upregulation at the 8-day timepoint. miR393, miR395 and miR778 did not demonstrate significantly altered expression at either timepoint.

Most of the miRNAs observed followed a similar trend. Most were significantly upregulated at the 4-day timepoint and then saw significantly reduced expression at 8 days after transfer. The target genes did not follow a consistent trend in the same way as the miRNAs. Differences in expression between 4 and 8 days after transfer varied with each specific target gene and may correspond to the miRNAs observed, or to other factors of regulation that were not observed.

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