Identification of MicroRNAs as Biomarkers for Stress Conditions in Maize (*Zea mays*)

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Abstract. Over 2,000 microRNAs (miRNA) have been discovered in plants and animals, and these miRNAs regulate various biological processes. However, there is no definitive conclusion regarding the certain types of miRNAs expressed under different stress conditions in plants and animals. Although, one study has made efforts to construct artificial miRNA in potatoes (*Solanum tuberosum*) [7], adequate knowledge of miRNAs prevalent in drought stresses was lacking. This study aims to identify miRNAs expressed under different stress conditions in *Zea mays*. TotalRNA was isolated from *Zea mays* under different stress conditions, namely, darkness (etiolation), salinity, drought, and control (no stress). Isolated totalRNAs were reverse-transcribed (RT) into cDNA ends, using stem-loop pulsed RT protocol, and were tested using end-point PCR protocol for the presence of targeted zma-miRNA, using designed primer pairs. It was found that plants under stress conditions declined in average height (cm) and

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number of leaves approximately 16 days after planting, with exception to control (no stress). Zma-miRNA 167a, 168a, and 169a were prevalent under abiotic stress treatments. Amplicons obtained under control (no stress) were lower than the 600 base pairs in comparison to other stress conditions. Prevalence of these zma-miRNAs in abiotic stresses further imply that they play a crucial role in abiotic stress regulation and assist in future efforts of constructing artificial miRNA for stress tolerances in plants, providing knowledge of novel miRNAs. However, unusual trends in control (no stress) amplicons may be the result of possible error and further analysis will be done to examine this trend.

Introduction

MicroRNA (miRNA) can be found in plants, animals, and viruses. These non-coding ribonucleic acids average 22 nucleotides long, and are found to be circulating at certain levels in the blood [5]. There are now over 2,000 miRNAs that have been discovered in plants and animals. They take part of a significant regulatory role in animals and plants by targeting specific mRNAs for translation repression or degradation [3]. This process is known as RNA interference (RNAi) (Figure I). There are two types of small classes of RNA; small interfering RNA (siRNA), which is always expressed when there is presence of antigen/foreign substance, and miRNA, which is expressed under different developmental stages. Genes can either be silenced by nuclear-encoded miRNA or synthetic double stranded RNA (dsRNA) [3]. DICER, an enzyme

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in plants that is encoded by the DICER1 gene, enables the stimulation of the RNA-induced silencing complex (RISC), which is a multiprotein complex and is vital for the process of RNA interference [3]. This enzyme is essential for the development of miRNA from pre-microRNA (pre-miRNA) in nuclear-encoded miRNA and the development of small interfering RNA (siRNA) from short hairpin (shRNA) in synthetic dsRNA. The miRNA or siRNA binds to an enzyme-containing molecule known as RNA-induced silencing complex (RISC) [3]. Then, the miRNA-RISC or siRNA-RISC complex binds to target, or complementary, messengerRNA (mRNA) sequences. This results in the enzymatic cleavage of the target mRNA. The cleaved mRNA is rendered nonfunctional and hence is “silenced” [8]. This process is important both in gene regulation by microRNAs and in defense against viral infections, which often use double-stranded RNA as an infectious vector [8].

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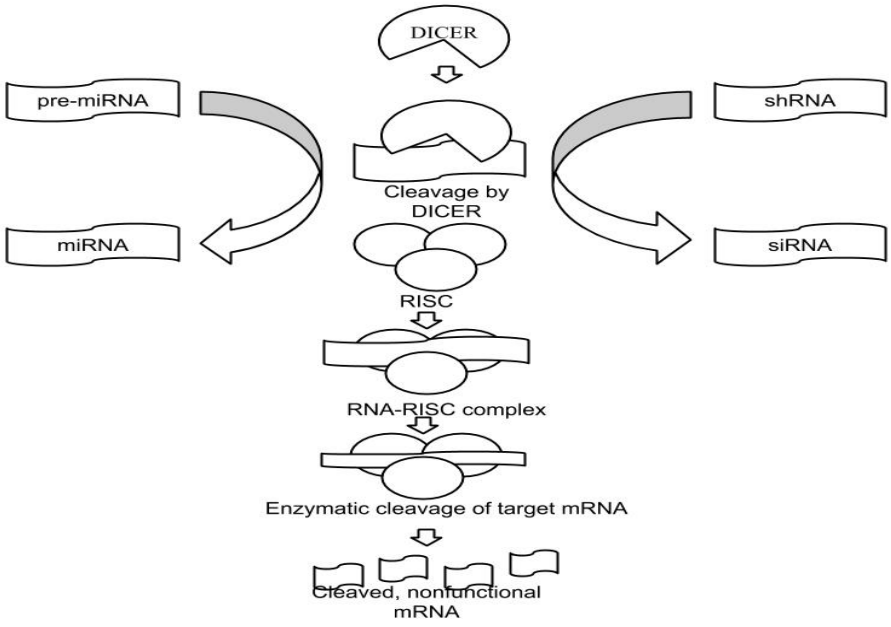

Figure I. Process of RNA Interference (RNAi) for gene silencing.

Recent scientific advances have revealed the synthesis pathways and the regulatory mechanisms of miRNAs in animals and plants, furthering the implications of miRNA-based regulation in novel miRNAs that are differentially expressed in drought-tolerant and drought-sensitive inbred maize lines [6], messaging for communication [3], therapeutic intervention of cancer [4], clinical research [1], and quantitation [2]. In particular, one study attempted to construct artificial miRNA to silence drought stress in *Solanum tuberosum* [7]. However, in order to construct these artificial miRNAs, knowledge of miRNAs prevalent in drought stress for *Solanum tuberosum* is required. This served as a reason for the identification

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of miRNAs expressed under different stress conditions in *Zea mays*. The present study seeks to identify miRNAs expressed under different stress conditions in *Zea mays* in order to provide knowledge about certain types of miRNAs found prevalent in abiotic stresses (drought, darkness (etiolation), and salinity) and assist in future efforts of constructing artificial miRNA for stress tolerances in plants.

Materials and Methods

Materials. Sweet corn (Carolina Biological Supply Company), Adaptis growth chamber cmp6050 (Conviron), autoclave, RNeasy plus mini kit (Qiagen), 2-Mercaptoethanol, liquid nitrogen, mortar/pestle, 1% denaturing agarose RNA gel electrophoresis (agarose, formaldehyde, formamide, electrophoretic tank, gel plate, gel comb), centrifuge, spectrophotometer, saline water (1 molar nuclease-free water salt concentration), thermal cycler for polymerase chain reaction (PCR), deoxynucleoside triphosphate (dNTP) mix, stem-loop RT Primer, 5x first-strand buffer, dithiothreitol (DTT), RNase-OUT (40 units/µl), SuperScript III RT (200 units/µl), and primer designs were utilized throughout the duration of this study.

Methods. *Zea mays* seedlings (1-2 weeks after planting) were subjected to 4 different treatments, namely, drought, darkness (etiolation), salinity, and control (no stress). Treatments were placed in a growth chamber (Adaptis cmp6050) following standard procedures, at 27° Celsius (C),

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55-60% relative humidity, and a 16-hr light/dark cycle. 500 milliliters of water was added to each treatment group, with exception to drought stress,every week. Growth parameters (average plant height (cm) and number of leaves) were taken every 3 day-interval. TotalRNAs from the treatments were isolated approximately 27 days after planting. This was a long and intensive process. Liquid nitrogen was used to effectively grind leaves during the disruption phase of totalRNA isolation. The lysate was homogenized in Buffer RLT Plus and pipetted into QIAShredder placed in a 2 mL collection tuber then centrifuged for 2 minutes at maximum speed. Then, the test substance was transferred to the gDNA eliminator spin column placed in a 2mL collection tube and centrifuged (30 s at ≧ 8000 x g (≧ 10,000 rpm)), being repeated until all liquid had passed through the column membrane to eliminate the DNA. 1 volume (600 µL) of 70% ethanol was added to the flow-through and mixed well through pipetting. 700 µL of sample (including any precipitate) was transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged (15 s at ≧ 8000 x g (≧ 10,000 rpm)) and the flow-through was discarded. Isolated totalRNA was quantified with the use of a spectrophotometer. Sample mix of isolated totalRNA was loaded onto the 1% denaturing agarose RNA gel electrophoresis. After approximately 45 minutes, the gel was visualized under UV light in the gel imaging system. The isolated totalRNAs were reverse-transcribed into complementary DNA (cDNA) ends, using stem-loop pulsed RT protocol. The cDNA ends were now tested in end-point PCR protocol to test the presence of targeted zma-miRNA, using designed primer pairs.

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Results

Zea mays under Darkness (etiolation) stress, drought stress, and salinity stress, were observed to have declined in average height (cm) at approximately 16 days after planting (Figure II). However, control (no stress) experienced average linear growth and no declines throughout the period of data collection. (Figure II).

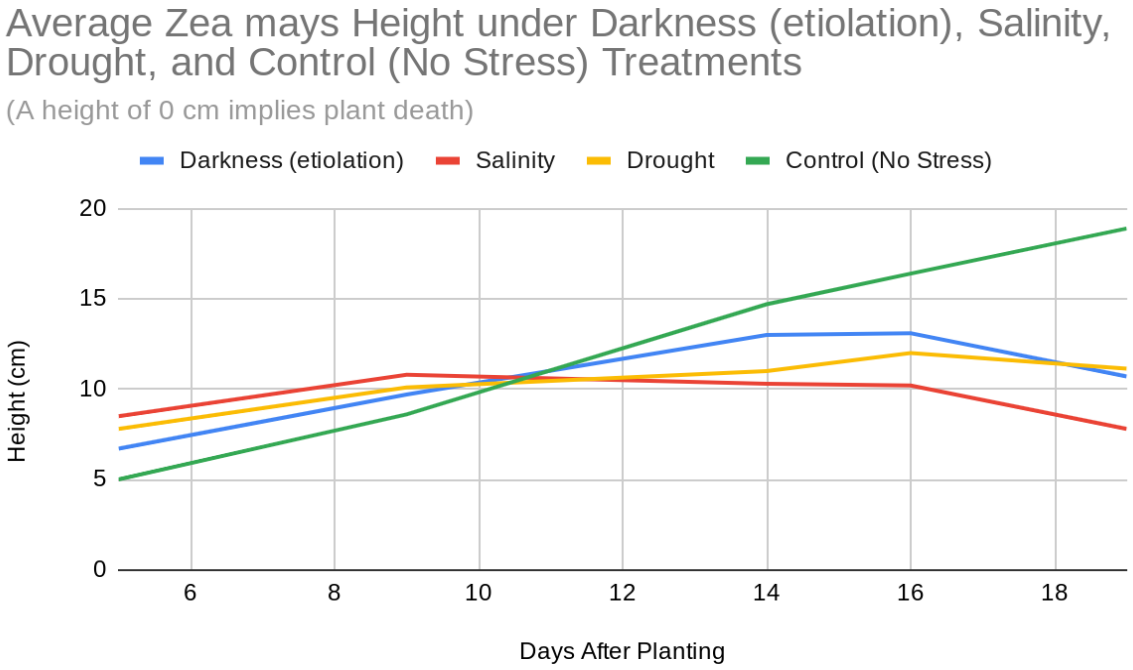

Figure II. Line graph of the average height (cm) of *Zea Mays* under darkness (etiolation), salinity, drought, and control (no stress) treatments.

Similar to the declines recorded for average height (cm) under darkness (etiolation), salinity stress, and drought stress treatments experienced, a decline in the average number of leaves approximately 16 days after planting (Figure III) was recorded. In contrast, control (no stress) experienced average linear growth and no declines throughout the duration of sample recordings (Figure III).

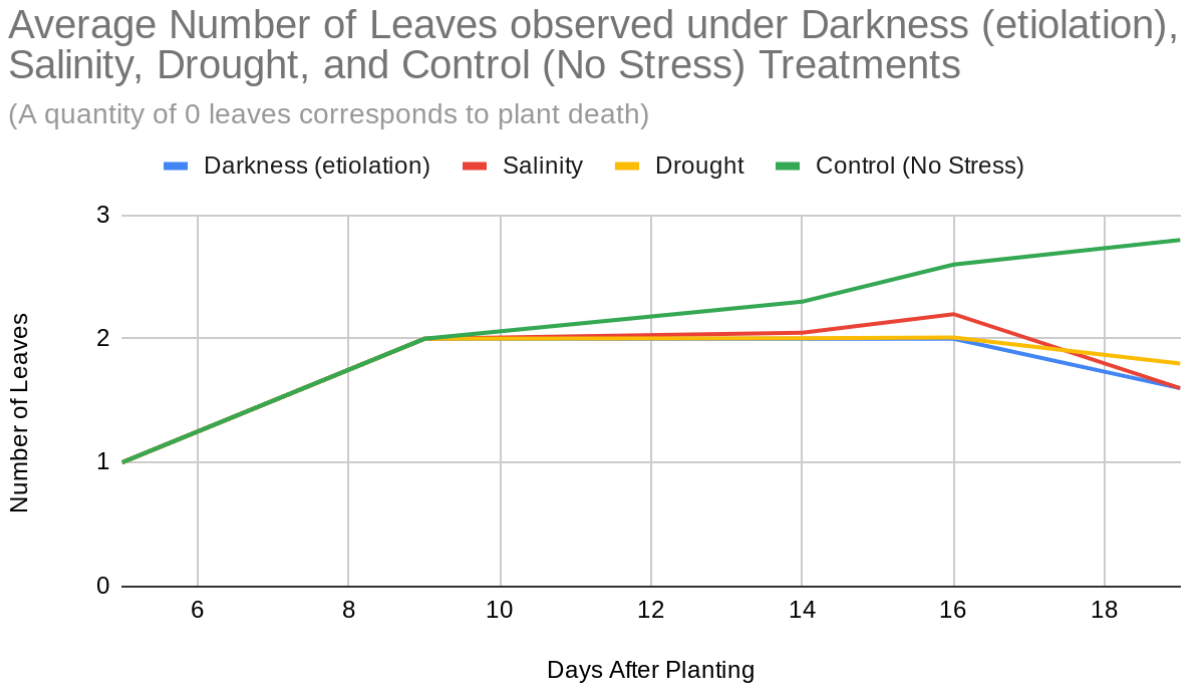

Figure III. Line graph of the average number of leaves of *Zea Mays* under darkness (etiolation), salinity, drought, and control (no stress) treatments.

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Plants from the control (no stress) yielded the highest amount of totalRNA compared to those obtained from the stress conditions (drought, darkness (etiolation), and salt treatments) (Plate I). Drought stress and control (no stress) were found to have comparable amounts of totalRNA while the amount of totalRNA from *Zea mays* under darkness (etiolation) and salinity treatments were remarkably lower (Plate I).

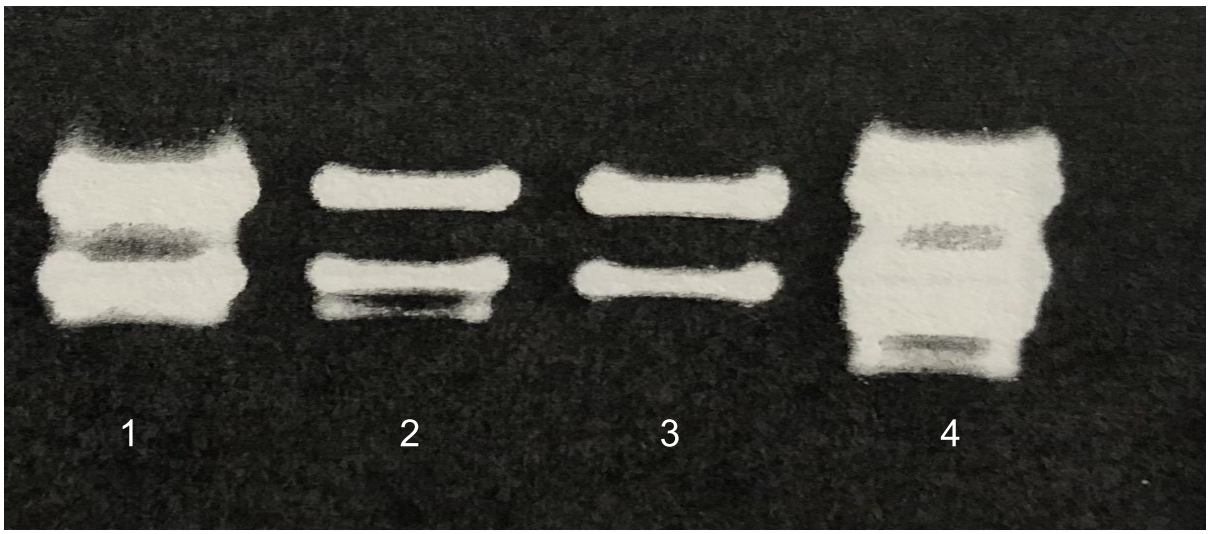

Plate I. Image of the 1% denaturing agarose RNA gel electrophoresis taken on gel imaging system. TotalRNA yields from *Zea mays* in drought, darkness (etiolation), salinity, and control (no stress) treatments. Lane 1 represents the drought stress treatment. Lane 2 represents the darkness (etiolation) stress treatment. Lane 3 represents the salinity stress treatment. Lane 4 represents the control (no stress) treatment.

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Amplification fragments were observed from the PCR, using primers designed for zma-miRNA 167a, performed on cDNA ends obtained from *Zea mays* under drought, darkness (etiolation) and salt treatments. However, amplicons obtained under control (no stress) were slightly lower than the 600 base pairs used for the gel imaging system in comparison to the other stress conditions (Plate II).

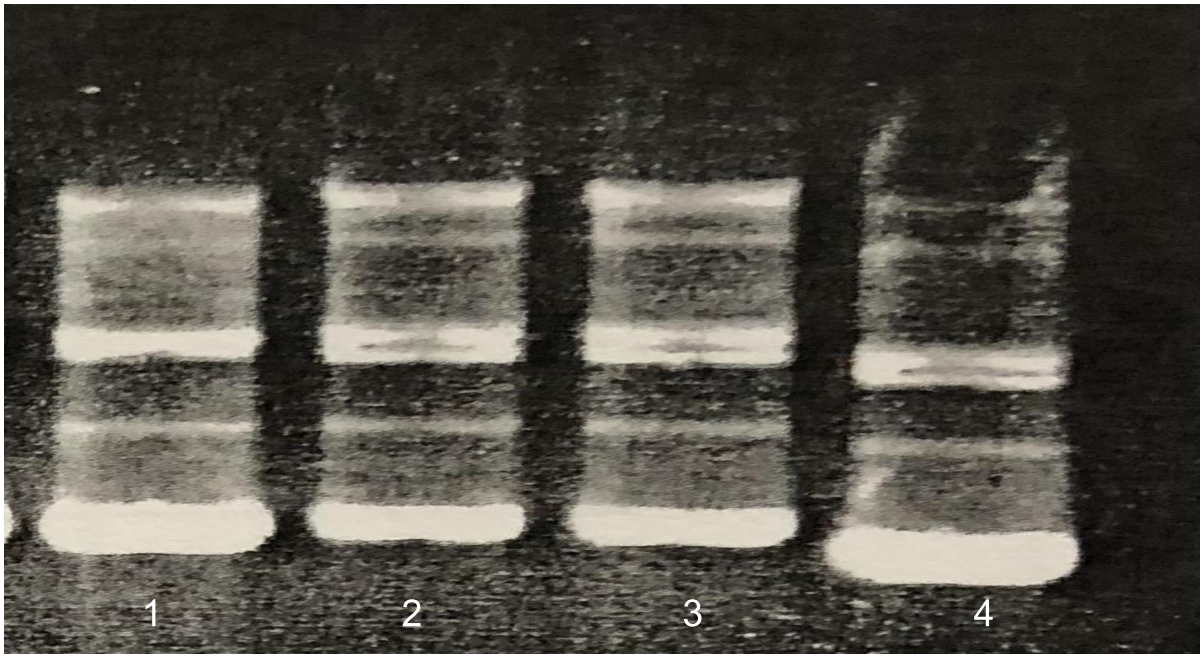

Plate II. Image of amplified zma-miRNA 167a from cDNA libraries generated from *Zea mays* under different stress conditions. Expression patterns of zma-miRNA 167a from *Zea mays* in drought, darkness (etiolation), salinity, and control (no stress) treatments. Lane 1 represents the drought stress treatment. Lane 2 represents the darkness (etiolation) stress treatment. Lane 3 represents the salinity stress treatment. Lane 4 represents the control (no stress) treatment.

As for zma-miRNA 168a, amplification fragments were also observed from the quantitative PCR, using primers designed for zma-miRNA 168a, performed on cDNA ends obtained from *Zea mays* under drought, darkness (etiolation) and salt treatments. In contrast, amplicons observed under control (no stress) were slightly different size in comparison to the other stress conditions with approximately fragment sizes of 300-500 base pairs (Plate III).

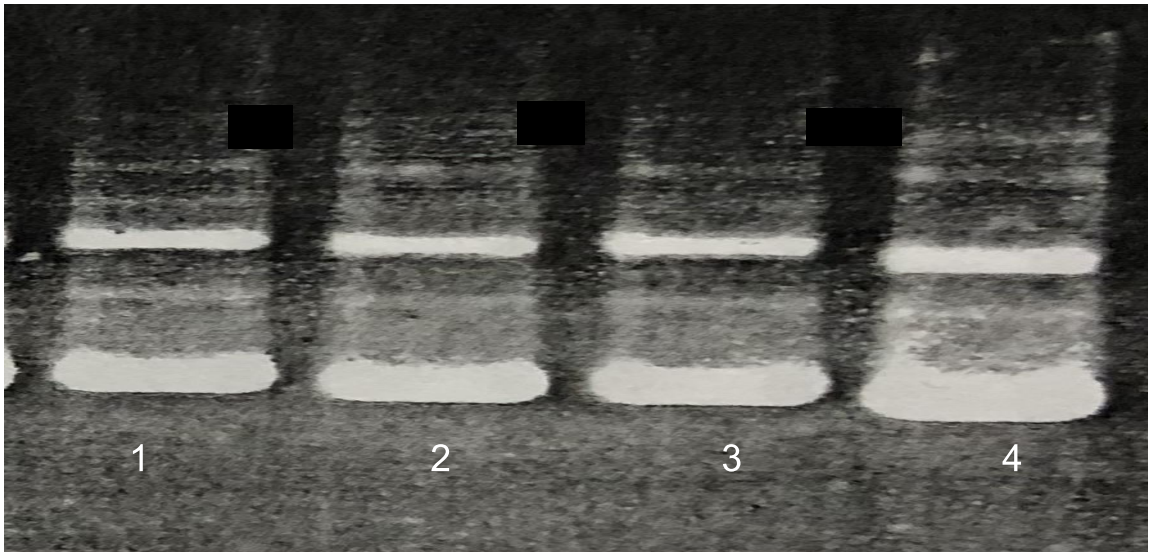

Plate III. Image of amplified zma-miRNA 168a from cDNA libraries generated from *Zea Mays* under different stress conditions. Expression patterns of zma-miRNA 168a from *Zea mays* in drought, darkness (etiolation), salinity, and control (no stress) treatments. Lane 1 is the drought stress treatment. Lane 2 is the darkness (etiolation) stress treatment. Lane 3 is salinity stress treatment. Lane 4 is control (no stress) treatment.

For zma-miRNA 169a, amplification fragments were observed from the PCR, using primers designed for zma-miRNA 169a, performed on cDNA ends obtained from *Zea mays* under drought, darkness (etiolation) and salt treatments. Similar to zma-miRNA 168a, amplicons

obtained under control were slightly different size compared to the other stress conditions at 300 base pairs (Plate IV).

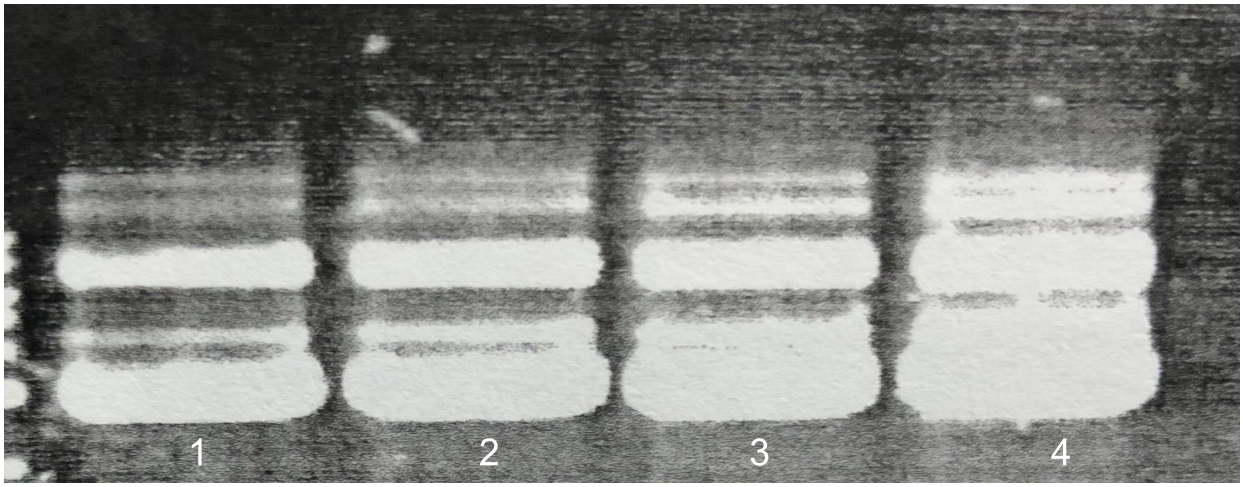

Plate IV. Image of amplified zma-miRNA 169a from cDNA libraries generated from *Zea Mays* under different stress conditions at 300 base pairs. Expression patterns of zma-miRNA 169a from *Zea mays* in drought, darkness (etiolation), salinity, and control (no stress) treatments. Lane 1 represents the drought stress treatment. Lane 2 represents the darkness (etiolation) stress treatment. Lane 3 represents the salinity stress treatment. Lane 4 represents the control (no stress) treatment.

Discussion

Decline in *Zea mays*' average heights and number of leaves under stresses compared to control (no stress) implies the negative effect these conditions can have on Zea mays. (Figure II) (Figure III). These results showed that stress induced by darkness (etiolation), salinity, and drought weaken the potential height and amount of leaves on *Zea mays* approximately 16 days

after planting. This finding is supported by one study that has found stress treatments to have a negative effect on *Solanum tuberosum* [7].

The images of isolated totalRNA indicated that control (no stress) and drought stress had no significant effect on the amount of totalRNA in *Zea Mays*. As a result, higher amounts of totalRNA were recorded under this treatment (Plate I). However, it appears that darkness (etiolation) and salinity stresses affected the amount of totalRNA, which resulted in lower amounts of totalRNA. One study has observed similar trends to this finding [6]. These findings correlate to the study's focus of miRNAs expressed under *Zea mays*.

The cDNA ends were tested in quantitative PCR for the presence of targeted zma-miRNA 167a, 168a, and 169a, using designed primer pairs. Amplification products indicated that zma-miRNA 167a, 168a, and 169a are prevalent under drought, darkness (etiolation) and salt treatments (Plate II) (Plate III) (Plate IV). In particular, one study has observed similar results [6]. However, amplicons obtained under control (no stress) were slightly lower than the 600 base pairs in comparison to the other stress conditions and different sizes in comparison to the other stress conditions at 300-500 base pairs. These findings correlate to the study's focusing of identifying miRNAs expressed under different stress conditions in *Zea mays* and support the idea that zma-miRNA 167a, 168a, and 169a may play a role in abiotic stress regulation.

In the literature, zma-miRNA 156 and zma-miRNA 172 were found prevalent in abiotic stresses [6]. Prevalence of these zma-miRNAs in abiotic stresses allow for further implications

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that these zma-miRNA may play a crucial role in abiotic stress regulation and assist in future efforts of constructing artificial miRNA for stress tolerances in plants, providing background knowledge of novel miRNAs. Future studies will focus on sequencing the identified zma-miRNA under drought conditions in *Zea mays* with the goal of transforming high yielding corn with these zma-miRNAs. Such that the newly transformed high yielding *Zea mays* can withstand drought stress if adopted in arid or semi-arid environments. However, the unusual trends in control (no stress) amplicons may be the result of a possible error and further analysis will be done to examine this trend.

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