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# Salt and Drought Stresses Induce the Aberrant Expression of microRNA Genes in Tobacco

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**Abstract** Drought and salinity stresses significantly altered microRNA (miRNA) expression in a dose-dependent manner in tobacco. Salinity stress changed the miRNA expression levels from a 6.86-fold down-regulation to a 616.57-fold up-regulation. Alternatively, miRNAs were down-regulated by 2.68-fold and up-regulated 2810-fold under drought conditions. miR395 was most sensitive to both stresses and was up-regulated by 616 and 2810-folds by 1.00% PEG and 0.171 M NaCl, respectively. Salinity and drought stresses also changed the expression of protein-coding genes [alcohol dehydrogenase (ADH) and alcohol peroxidase (APX)]. The results suggest that miRNAs may play an important role in plant response to environmental abiotic stresses. Further investigation of miRNA-mediated gene regulation may elucidate the molecular mechanism of plant tolerance to abiotic stresses and has the potential to create a miRNA-based biotechnology for improving plant tolerance to drought and salinity stresses.

## Introduction

Unlike animals, plants are stationary organisms that have evolved mechanisms to cope with a wide range of environmental and climate changes [1]. Over the past century, global warming has led to a rise in seawater levels [2] and a slow but gradual increase in the surface temperature of the Earth [3, 4]. This has caused previously wet regions to become more arid and the deposition of salt into low-lying grass and farm lands [5]. Aside from global warming, rain-fed plants can often times experience dry conditions and can also be naturally exposed to high concentrations of salt in soil [1]. Drought and salt stresses are two of the more severe and wide-ranging environmental stresses that significantly affect crop growth and productivity. Although much research has been dedicated to elucidating gene expression during plant exposure to dry and brackish conditions, the mechanisms underlying the regulation of gene expression remain largely unknown.

miRNAs are short sequences ($\sim 21$ nt) of endogenous non-coding RNA that negatively regulate gene expression at the post-transcriptional level [6, 7]. miRNAs have been shown to play an important role in a variety of plant biological and metabolic processes including organ maturation [8–12], hormone signaling [13, 14], developmental timing [15, 16], response to pathogens [17–19], and response to environmental abiotic stresses such as drought [20], salinity [21], heavy metals [22], and cold [23].

Recent studies have shown that drought and salinity stresses are able to induce the differential expression of thousands of protein-coding genes [24, 25]. However, the regulatory mechanisms underlying gene expression in response to drought and salt stresses are poorly understood. miRNAs, an important class of gene regulators, have been implicated to play an important role in plant tolerance to abiotic stresses [26–28]. The expression of miR393, for example, has been shown to be influenced by abiotic stress conditions [20, 27] and miR393 itself has been shown to target stress-related genes in *Arabidopsis* and rice [29]. miR169, miR395, and miR398 expression have also been shown to be induced under other environmental stress

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conditions such as high salt [21], sulfate starvation [26], and heavy metal toxicity [30], respectively.

Tobacco is an important agricultural and economic crop in the United States, as well as around the world. It is cultivated by more than 100 countries around the globe. One new study has investigated the use of tobacco as a potential biofuel crop [31]. In our previous study, we identified 259 tobacco miRNAs using a well-established in silico approach [32, 33]. In this experiment, we analyzed the effect of sodium chloride (NaCl) and polyethylene-glycol (PEG) on the expression levels of nine different miRNAs as well as two stress-related genes in tobacco. NaCl was used to simulate salt stress, whereas PEG was used to simulate drought stress. These nine miRNAs were selected based on previous studies and all of them are related to plant development and stress response. The results of this study show that NaCl and PEG have an effect on miRNA expression and on the expression of stress-related genes in tobacco. One miRNA, miR395, was significantly up-regulated after exposure to high NaCl and high PEG conditions. Given the results of this study, we believe miRNAs may play an important role in tobacco tolerance to salt and drought stresses.

## Materials and Methods

### Seed Sterilization, Media Preparation, and Tobacco Treatment

Tobacco (*Nicotiana tabacum*) seeds were sterilized by soaking in 70% ethanol for 2 min followed by 10% bleach for 15 min. The seeds were then rinsed with distilled water (approximately four times) until no bleach odor remained. Basic medium used in these experiments contained the following: Murashige and Skoog (MS) salts supplemented with Gamborg's B5 Vitamins, 1% sucrose, and 0.8% agar, and was adjusted to a pH of 5.8 after the addition of sodium chloride (NaCl) (99.85% purity, Acros Organics, Geel, Belgium) and polyethyleneglycol (PEG) 6000 (Hampton Research, Aliso Viejo, CA). Sterilized tobacco seeds were sown on Petri dishes containing MS medium and 0, 0.017, 0.043, 0.085, and 0.171 M NaCl to simulate varying degrees of salt stress. Sterilized tobacco seeds were also sown on Petri dishes containing MS medium with 0, 1, 2.5, 5, and 7.5% PEG to simulate varying degrees of drought stress. The concentration of both the salt and PEG were selected according to previous reports. Approximately 25 tobacco seeds were placed on each plate and there were a total number of five plates for each concentration of treatment (NaCl and PEG), including control plates. The plates were placed under a 16 h day/8 h night cycle for 3 weeks until seedling removal.

### Total RNA Extraction

Three week old tobacco seedlings were removed from the plates and immediately frozen in liquid nitrogen. The seedling tissue was placed at −80 °C until RNA extraction. Total RNA was isolated from the 3-week old seedlings using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Total RNA was then quantified and assessed for quality using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA samples were stored at −80 °C until further analysis.

### Analyzing microRNA Expression Changes Using RT-PCR and qRT-PCR

Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify tobacco miRNAs using stem-loop real-time PCR according to the manufacturer's instructions. There were two steps in the TaqMan miRNA Assays: (a) reverse transcription of the mature miRNA to a longer single-stranded cDNA sequence using a miRNA-specific stem-looped primer and, (b) quantitative real-time PCR. Briefly, a single-stranded miRNA cDNA was generated from 1 μg of the total RNA from three biological replicates collected from three individual NaCl plates (0, 0.017, 0.043, 0.085, and 0.171 M) as well as from three biological replicates collected from three individual PEG plates (0, 1, 2.5, 5, and 7.5%). This was completed by reverse transcription using the Applied Biosystems Taq-Man microRNA Reverse Transcription Kit and miRNA-specific stem-looped RT primers provided in the kit. Many studies show that miR159, miR167, miR169, miR172, miR393, miR395, miR396, miR398, and miR399 are important for plant growth as well for response to environmental stress [1, 7]. Thus, we selected these nine miRNAs and two stress-related genes (alcohol dehydrogenase (ADH) and alcohol peroxidase (APX)) to investigate the effect of drought and salinity stress in tobacco. In the relative quantification analysis, elongation Factor 1 α (EF1α) was used as a reference gene to normalize expression values. Three biological replicates were run for each gene for each treatment and the results were analyzed using the $\Delta\Delta C_T$ method.

## Results

### NaCl and PEG Effected Tobacco Growth

Tobacco seeds were planted on Petri dishes containing MS medium supplemented with 0, 0.017, 0.043, 0.085, and 0.171 M NaCl. Seeds were allowed to grow for 3 weeks in

each medium and were then removed and stored at −80 °C until total RNA extraction. At the time of tissue removal, we observed a gradual decrease in tobacco growth as the concentration of salt in the media increased (data not shown). We also noted that the roots of the tobacco plants exhibited a different growth pattern as the salt concentration increased. The roots grew in a more twisted and crooked pattern under higher salt concentrations as compared to the straight, uniform roots of the control plants, which were not exposed to NaCl. A decrease in root health and rigidity was observed for the plants exposed to salt as these were thinner and more prone to break when removed from the media than the control plants. The number and size of tobacco leaves per plant also gradually decreased as the salt concentration increased.

Tobacco seeds were also placed on Petri dishes containing MS medium supplemented with 0, 1, 2.5, 5, and 7.5% PEG. After 3 weeks, the plants showed consistent growth across the control and PEG concentration plates. Tobacco plants grown in higher concentrations of PEG exhibited longer root lengths compared to those grown on the control plates. Also, the majority of plants grown in 7.5% PEG grew more than one root. In contrast to the plants grown in NaCl, tobacco plants grown in PEG had the same number and size of leaves across all concentrations, including the control plates.

## Salinity Stress Alters miRNA Expression Levels in Tobacco

Salinity treatment significantly altered miRNA gene expression in a dosage-dependent manner (Fig. 1a). At the tested concentration range, three miRNAs (miR159, miR167, and miR169) were down-regulated by salinity stress and the expression inhibition was decreased as the salinity concentration increased. In contrast, salinity stress induced the over-expression of two miRNAs (miR 172 and miR 396) and the fold changes of these miRNAs increased as the salinity concentration increased. At the lowest concentration (0.017 M), salinity stress inhibited the expression of miR395 and miR399; however, both miRNAs were over expressed in a dose-response manner under the



Fig. 1 Salinity stress alters gene expression pattern in young tobacco seedlings. a Changes in nine miRNA expression levels. b Changes in two stress-related genes. The data are based on the average of three biological replicates; the bars represent the standard deviation
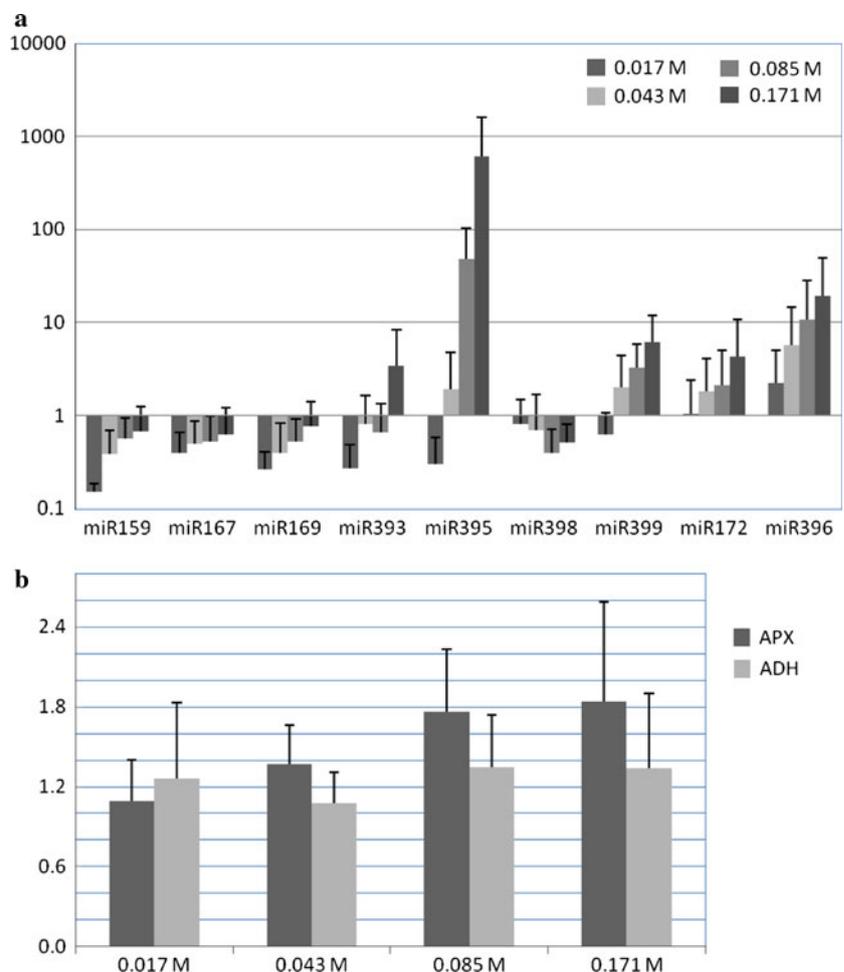
treatment of 0.043–0.171 M salinity. It was observed that miR398 was not as sensitive as other miRNAs, however, it was down-regulated by salinity treatment at all tested concentrations. The largest fold change for miR398 was observed at 0.085 M salt treatment with a 2.5-fold change.

Among the nine tested miRNAs, all miRNAs, except miR398, exhibited more than a 3-fold change under certain NaCl treatments. Of these miRNAs, miR395 was the most sensitive to salinity stress and was down-regulated 3.36-fold at 0.017 M salinity treatment while it was up-regulated by 616.37-fold at 0.171 M NaCl treatment. At low concentrations, miR159 is the most sensitive to salinity stress with a down-regulation of 6.86-fold.

One more interesting phenomenon we observed was that the down-regulated miRNAs were the most sensitive to salinity treatment at the lowest concentration (0.017 M) while the up-related miRNAs were the most sensitive at the highest concentration (0.171 M). As we know, miRNAs negatively regulate gene expression which targets specific biological function. Our data may suggest that these miRNAs have synergistic activities during salinity stress.

## Drought Stress Alters miRNA Expression Levels in Tobacco

We also analyzed the effect of PEG, a chemical that simulates drought conditions when added to plant growth media, on miRNA expression levels in tobacco (Fig. 2a). Surprisingly, all nine miRNAs were significantly up-regulated after exposure to 1% PEG. Similar to miRNA expression changes after exposure to 0.171 M NaCl, miR395 was the most up-regulated with a change in expression of greater than 2800-fold at 1% PEG. miR169 showed the second greatest change in expression level of 122-fold at 1% PEG.

miRNA expression changes showed 1 of 2 trends as the PEG concentration in the media increased: (1) a decrease in expression followed by a sudden increase at 7.5% PEG or (2) a decrease in expression over the course of all PEG concentrations. It should be noted, however, that even with a decrease in expression, all of the miRNAs were still up-regulated in comparison to the controls. miRNAs that fell into the first category included miR167, miR393, miR399,

**Fig. 2** Drought stress alters gene expression patterns in young tobacco seedlings. **a** Changes in nine miRNA expression levels. **b** Changes in two stress-related genes. The data are based on the average of three biological replicates; the *bars* represent the standard deviation
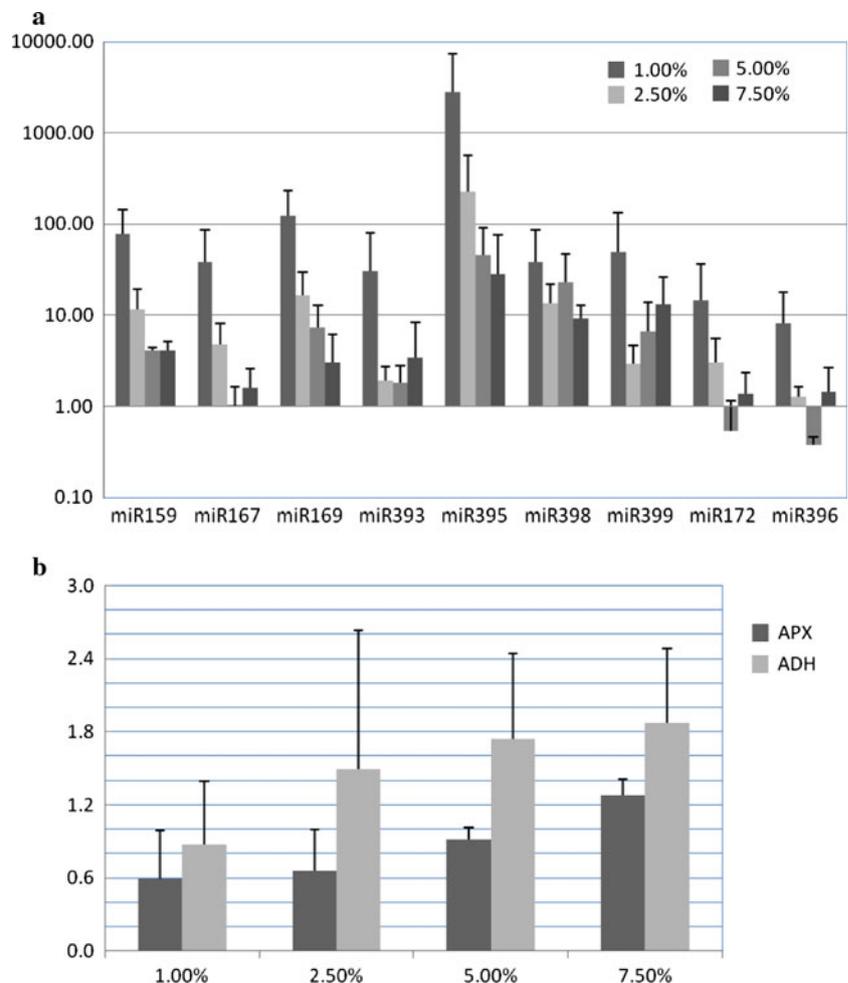
miR172, and miR396. For example, miR393 was up-regulated approximately 30-fold after exposure to 1% PEG. The expression level of miR393 decreased to a 1.9-fold up-regulation at 2.5% PEG and decreased again to a 1.8-fold up-regulation at 5% PEG. When exposed to 7.5% PEG, however, miR393 increased in expression to a 3.5-fold up-regulation. miRNAs that fell into the second category included miR159, miR169, and miR395. miR395 was up-regulated 2810.5-fold after exposure to 1% PEG. The expression of miR395 decreased to a 230-fold up-regulation at 2.5% PEG and continued to decrease to a 45-fold up-regulation at 5% PEG. Of the four drought treatments, miR395 was the least expressed at 7.5% PEG with an up-regulation of 28.5-fold. Interestingly, miR398 alternated between varying degrees of gene expression. After exposure to 1 and 5% PEG, miR398 was more up-regulated (39-fold and 23-fold, respectively) as compared to when exposed to 2.5 and 7.5% PEG (3-fold and 13-fold, respectively).

### NaCl and PEG Affect the Expression of Stress-Related Genes in Tobacco

We also investigated changes in the expression levels of two stress-inducible genes, ADH and APX, in tobacco after exposure to NaCl and PEG. We found that both of these genes were up-regulated under all four salt concentrations and that the expression of the genes was consistently the same (approximately a 1-fold increase) as the salt concentrations increased. We also found that both of these genes were up-regulated after exposure to 7.5% PEG. Interestingly, APX was down-regulated after exposure to 1, 2.5, and 5% PEG and was only up-regulated after exposure to 7.5% PEG, the highest concentration tested. ADH and APX exhibited different expression patterns under salinity and drought treatments, which suggest that tobacco may have different mechanisms to handle drought and salinity stresses.

### Discussion

Recent studies have shown that the expression of miRNAs, an important class of gene regulators, is altered after abiotic stress treatment [22, 34–36]. However, most of these studies have been performed in model organisms such as *Arabidopsis* and rice. In this report, we investigated changes in miRNA expression levels after exposure to salt and drought stress in tobacco, an important agricultural and economic crop.

Using RT-PCR and qRT-PCR, we analyzed changes in nine different miRNAs in 3 week old tobacco seedlings after exposure to salt and drought conditions. We found

that miR395 was significantly up-regulated after exposure to 0.171 M NaCl. We also found miR395 to be up-regulated after exposure to 7.5% PEG. Interestingly, miR395 has only been shown to function in plant response to sulfate deprivation by targeting sulfur transporter genes [26, 37, 38]. The results of our study suggest an alternative role for miR395 in response to high salinity and drought stresses.

miR399, a miRNA involved in regulating phosphate homeostasis in *Arabidopsis* [39, 40], was up-regulated after exposure to both NaCl and PEG. In contrast to our results, Fujii et al. [41] found that there was no significant up or down-regulation of miR399 after exposure to salt and drought stresses. However, our results show that miR399 was up-regulated 6-fold and 13-fold after exposure to 0.171 M NaCl and 7.5% PEG, respectively. The development of stem-loop RT-PCR and TaqMan qRT-PCR analysis has provided a reliable and sensitive method to determine miRNA expression in plants [42]. Therefore, small changes in the expression level of miR399 can be detected using this method. Since miR399 is only induced under stress conditions [43], we believe that miR399 may have other unconventional roles and play a part in tobacco tolerance to salt and drought conditions.

Two miRNAs, miR396 and miR172, were up-regulated in tobacco seedlings after exposure to 0.171 M NaCl and 1% PEG. miR396 has been shown to function in leaf development [44] and expression of miR396 has been shown to be induced under high salt, cold, and drought stresses [45]. Interestingly, over-expression of miR396 in tobacco leads to an increased tolerance to drought stress [46]. miR396 expression is also up-regulated in rice after exposure to high salinity and transgenic over-expression of this miRNA in rice led to plants with reduced salt tolerance [47]. Therefore, miR396 may not only play an important role in tobacco development but may also function in tobacco tolerance to environmental stress. miR172 has been shown to play a role in the phase change between vegetative and reproductive growth and contributes to floral organ identity [48, 49]. A recent study has suggested a role for miR172 in plant resistance to cold stress [45]. The results of our study suggest a novel function for miR172 in regulating tobacco tolerance to salt and drought conditions.

miR169, a miRNA known to be induced under high salinity [21], was found to be down-regulated in tobacco after exposure to increasing concentrations of NaCl. It is possible that miR169 is only induced under extreme conditions greater than 0.171 M salt. Surprisingly, however, this miRNA was significantly up-regulated in tobacco seedlings after exposure to 1% PEG. The expression levels of this miRNA, however, significantly decreased as the concentration of PEG increased. These results are consistent with those of others that show miR169a and miR169c

expression is down-regulated after exposure to extremely dry conditions [50].

Another miRNA, miR159, was shown to be highly induced after exposure to 1% PEG and then decreasingly less up-regulated in tobacco under higher PEG concentrations. miR159 has been shown to target MYB101 and MYB33 transcripts, two factors that positively regulate the accumulate of the plant hormone ABA [51]. Consistent with our findings, this miRNA has also been shown to be up-regulated under drought stress [51] and has been implicated to provide plant tolerance to environmental stress by functioning through hormone and abiotic stress signaling networks [52].

miR393 and miR398 are two additional miRNAs that have been shown to be differentially expressed under abiotic stress conditions. For example, miR393 expression levels are altered under high salinity and cold [45] as well as under drought conditions [20]. We found that miR393 was up-regulated after exposure to 0.171 M NaCl and after exposure to all concentrations of PEG. miR393 is speculated to cease plant growth and development during times of environmental stress by targeting TIR1, a positive regulator of plant growth [53]. miR398 has been found to be up-regulated in response to copper-deprivation [54]. miR398 targets superoxide dismutases, genes that scavenge free radicals, and has been shown to be down-regulated during times of oxidative stress [30, 53]. miR398 was down-regulated in tobacco seedlings exposed to all concentrations of NaCl, suggesting that the salt might have induced stress by creating an oxidative environment inside the tobacco cells. Interestingly, miR398 was up-regulated in tobacco seedlings after exposure to all concentrations of PEG. This result is consistent with the results of Trindade et al. [55] in which they found miR398 to be differentially expressed in water deficit *Medicago truncatula* plants.

APX and ADH are two genes whose expression levels have been shown to be up-regulated under environmental stress conditions. In this study, we analyzed the effect of NaCl and PEG on APX and ADH expression. We found that both genes were up-regulated after exposure to 0.171 M NaCl and 7.5% PEG. These findings indicate that these environments caused changes in the levels of gene expression as well as changes in miRNA expression.

## Conclusion

Global warming and nutrient depletion of soils due to over-farming has led to a world-wide reduction in growth and productivity of several important crops such as soybean, maize, and wheat. Tobacco, an important agricultural and economic crop, has recently been investigated as a potential biofuel crop. In this article, we analyzed the expression levels of nine different miRNAs in tobacco seedlings exposed to 0.017–0.171 M NaCl as well as 1–7.5% PEG. We used NaCl to simulate abiotic salt stress and PEG to simulate abiotic drought stress. We found that individual miRNA expression profiles varied between the two different stresses, indicating that salt and drought stresses induce differential miRNA expression through different mechanisms, such as oxidative stress or inhibition of plant growth. We also found that salt and drought conditions induced the expression of APX and ADH, two stress-related plant genes, in tobacco. Therefore, we believe that miRNAs may play a key role in developing tobacco plants with a greater tolerance to salt and drought stress.

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