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Deciphering salt and drought stress induced non-coding RNAs from French bean (*Phaseolus vulgaris*)

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Abstract

Drought and salinity stresses significantly altered microRNA (miRNA) expression in a dose-dependent manner in French bean seedlings. 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 0.4 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.

Keywords: Abiotic stress; Drought; microRNA; French bean; Salinity

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. Micro RNAs are short sequences (~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. Micro RNAs, 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 conditions such as high salt [21], sulfate starvation [26], and heavy metal toxicity [30], respectively. French bean is an important agricultural and economic crop in cultivated by more than 100 countries around the globe. In this experiment, we analyzed the effect of sodium chloride (NaCl) and drought stress on the expression levels of nine different miRNAs as well as two stress-related genes in French bean. NaCl was used to simulate salt stress, whereas withholding waterfor 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 drought have an effect on miRNA expression and on the expression of stress-related genes in French bean. One miRNA, miR395, was significantly up-regulated after exposure to high NaCl and drought conditions. Given the results of this study, we believe miRNAs may play an important role in tolerance to salt and drought stresses.

Materials and Methods

Plant material and stress treatments: French bean (*Phaseolus vulgaris cv. S-9*) seedlings were grown in a green house at 28°C; 13 h light until 6 day old and were then randomly divided into three groups. One group was used as untreated control, and other two groups were treated with salt (0.4mM NaCl for 48 h) and drought (with-holding for 1week) stresses respectively. After control and stress treatments were applied, shoots were harvested separately immediately for RNA isolation.

Total RNA Extraction: French bean seedlings were removed after stress treatment and immediately frozen in liquid Nitrogen. The seedling tissue was placed at -80 °C until RNA extraction. Total RNA was isolated from both control and stress treated seedlings using the Trizol (Invitrogen) and was then quantified and assessed for quality using a Nanodrop-2000 (Thermo Scientific, USA). RNA samples were stored at -80 °C until further analysis. Briefly, low molecular weight RNA was enriched by 5 M NaCl and 50% PEG precipitation.

Analysing microRNA Expression Changes Using RT-PCR and qRT-PCR: Applied Biosystems TaqMan, microRNA Assays were employed to detect and quantify French bean 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 salt stressed (400mM) and drought stressed sample. This was completed by reverse transcription using the Applied Biosystems TaqMan 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 French bean. 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.

Northern blotting: Total RNA (30 µg) from stressed sample along with control was probed with miRNAs probe. The blot was hybridized with five miRNAs. The rRNA bands were shown as a loading control.

Results

Effect of salt and Drought on plant growth: Seven-day old French bean seedlings were stress induced by supplementing the Hoagland media with 0 (control), 0.4 M NaCl for 48 h and stored at -80 °C until total RNA extraction. At the time of tissue removal, we

observed a gradual decrease in plant growth as the concentration of salt in the media increased (data not shown). We also noted that the roots of the seedlings exhibited a different growth pattern under high salt conditions. French bean seedlings under drought stress exhibited longer root lengths compared to control. Also, the majority of plants grown in drought conditions grew more than one root.

Salinity Stress Alters miRNA Expression Levels in French bean: Salinity treatment significantly altered miRNA gene expression; four miRNAs (miR159, miR167, miR393 and miR169) were down-regulated. In contrast, salinity stress induced the over-expression of three miRNAs (miR 172, miR395 and miR 396) and the fold changes of these miRNAs increased as the salinity concentration increased. It was observed that miR398 was not as sensitive as other miRNAs, however, it was down-regulated by salinity treatment at the tested concentration. Among the nine tested miRNAs, all miRNAs, except miR398, exhibited more than a 3-fold change under certain NaCl treatment. Of these miRNAs, miR395 was the most sensitive to salinity stress and was up-regulated by 616.37 fold at 0.4 M NaCl treatment. At the tested concentration, miR159 is the most sensitive to salinity stress with a down-regulation of 6.86 fold. 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 (Figure 1 A).

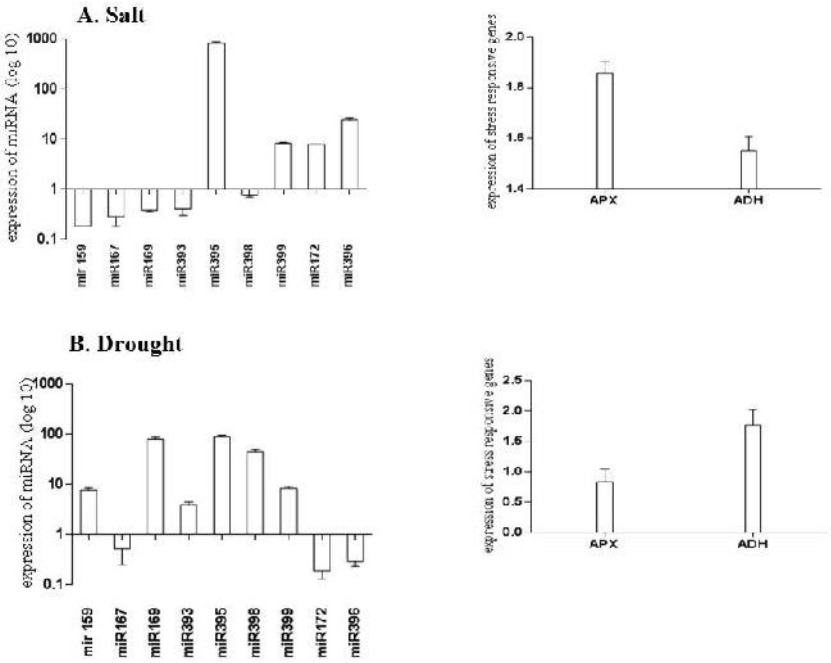

Figure 1 Altered expression of miRNAs after salt and drought stress shown by qRT-PCR

Drought Stress Alters miRNA Expression Levels in French bean: To identify drought-responsive miRNAs, the normalized expression of miRNAs was studied. miRNAs with changes in expression levels being greater than 1.5-fold in response to drought were validated by their expression patterns by real-time quantitative PCR. Expression patterns of drought responsive miRNAs showed that miR 159, miR169, miR393, miR 395, miR 398 and miR399, were up-regulated in response to the drought. Conversely, miR167 miR 172, and miR 396 were down-regulated under drought stress (Figure 1 B). These miRNAs have been reported to be involved in diverse cellular processes in plants. The known target genes of these miRNAs and their function annotations were summarized in (Table 1). For those miRNAs whose targets are not known, we predicted their targets using the Srna Target http://bioinfo3.noble.org/psRNATarget/, among these miRNAs several miRNAs have been reported to be involved in abiotic stresses. For example, miR399 and miR2111 have been reported to be up-regulated by phosphate starvation, while miR169 with target of CCAAT Binding Factor is down-regulated in response to drought stress. However, miR172, miR159, miR169, miR393 and miR398 were differentially expressed in response to salt and drought stress conditions.

Table 1. Predicted target genes of differentially expressed miRNA under drought and salt stress conditions

miRNA family	Targeted genes	Target description
miR172	TC405657	Hypothesis protein
	TC392019	Transcription factor AHAP2
	BE659941	Floral homeotic protein APETALA 2
	TC366837	APETAL2-like protein
	TC407080	Transcription factor AHAP2
	TC378006	Hypothesis protein
	TC383306	PHAP2B protein
	TC404733	Hypothesis protein
	TC352579	Hypothesis protein
	TC383335	PHAP2B protein
	TC417910	Superoxide dismutase [Cu-Zn]
miR159		MYB - Protein
miR167	GD753695	Hypothesis protein
	TC371467	Phosphatidate cytidylyltransferase
	TC379788	Hypothesis protein
	TC371879	Hypothesis protein
	BE805600	Auxin response factor 8
	BM732289	Hypothesis protein
	DB979348	Hypothesis protein
	TC389689	Hypothesis protein
miR169	TC364843	Hypothesis protein
	TC353076	Nuclear transcription factor Y subunit A-3
	TC379261	Os02g0776400 protein
	TC401273	CCAAT-box transcription factor complex WHAP12
	TC355136	Hypothesis protein
	TC383014	CCAAT-binding transcription factor
	TC366077	Hypothesis protein
	CO985073	Mitogen-activated protein kinase 10
miR393	DB989850	Auxin-responsive factor TIR1-like protein
	TC416229	Auxin-responsive factor TIR1-like protein
	TC366828	Transport inhibitor response 1
	TC365328	Transport inhibitor response 1
	TC362546	Transport inhibitor response 1
	TC398603	Hypothesis protein
	TC362758	Hypothesis protein
	FG999850	Hypothesis protein
miR395	BG789910	ATP sulfurylase
	TC359920	ATP sulfurylase
	TC358067	ATP sulfurylase
	TC360687	ATP sulfurylase
	EV282501	Hypothesis protein
	GE008734	Hypothesis protein
	TC369301	Hypothesis protein
	TC349703	Plastidiallipoyltransferase 2
	TC348882	Plastidiallipoyltransferase 2
	CF922366	Phospholipase C
	TC358694	Low affinity sulfate transporter 3

	TC358694	Low affinity sulfate transporter 3
	TC405478	Hypothesis protein
	TC411365	Cation diffusion facilitator 9
	AW759383	Glutamate dehydrogenase 2
	GR836780	Hypothesis protein
	TC369607	Zinc finger, CCCH-type; Sugar transporter superfamily
	TC357118	Hypothesis protein
	TC397169	Zinc finger, CCCH-type; Sugar transporter superfamily
	TC365139	Ketol-acid reductoisomerase, chloroplast precursor
	GD787823	Dihydroflavonol-4-reductase DFR1
miR396	TC365248	Hypothesis protein
	FK003100	Hypothesis protein
	TC373306	Cytochrome P450 monooxygenase CYP72A65
	TC366659	Hypothesis protein
	TC393538	Hypothesis protein
	TC379767	Hypothesis protein
	TC393753	Elovl2 protein
miR398	At1g08050	Cytchrome C Oxidase, zinc finger (C3HC4-type ring finger) protein
	At3g15640	CDS, Cytchrome C Oxidase, zinc finger (C3HC4-type ring finger) protein
	At3g15640	Cytchrome C Oxidase, zinc finger (C3HC4-type ring finger) protein
	At1g08050	Cytchrome C Oxidase, zinc finger (C3HC4-type ring finger) protein
miR399	At2g33770	Ubiquitin conjugating enzyme (UCE); vesicle-associated membrane protein
	At4g00170	Ubiquitin conjugating enzyme (UCE); vesicle-associated membrane protein
	At2g33770	Putative UCE2

Mature miRNA quantification by northern blotting: To confirm and validate the results obtained from the library, we examined the expression patterns of five known miRNAs. (miR 169, miR 172, miR 395, miR 396, and miR399) were individually selected and experimentally verified by northern blotting hybridization. The sequences of antisense RNA probes are listed in (Additional file). By comparing the miRNA results by sequencing to northern hybridization, three stress-responsive miRNAs (miR167,) were identified with identical expression patterns. miR395 and miR399 were up-regulated under drought and salinity. While the expression patterns of mi398 remained unchanged under drought conditions when tested by northern blotting (Figure 2). However, these were up-regulated under drought and salt stress according to the results. Therefore, the expression pattern obtained by RNA blot analysis may reflect the result from sequencing.

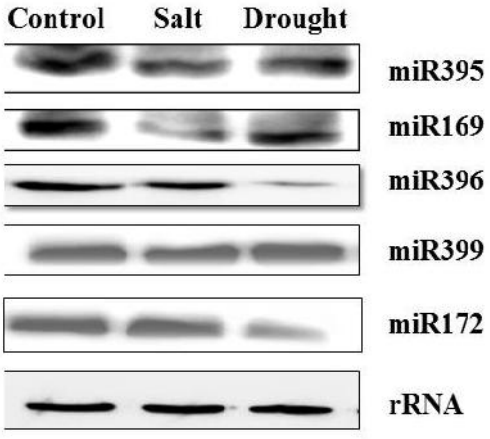

Figure 2 Northern blotting confirming differential expression of miRNAs. Total RNA (30 µg) from salt and drought conditions was loaded and probed with miRNAs probe. The blot was hybridized with five miRNAs (miR395, miR169, miR396, miR399 and, miR172). The rRNA bands were shown as a loading control.

Salt and Drought Affect the Expression of Stress-Related Genes in French bean: We also investigated changes in the expression levels of two stress-inducible genes, ADH and APX, in French bean after exposure to NaCl and drought. We found that both of these genes were up-regulated under salt stress 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 drought. ADH and APX exhibited different expression patterns under salinity and drought treatments, which suggest that French bean may have different mechanisms to handle drought and salinity stresses.

Additional file

Primers Used for Real Time – PCR

miRNA	PCR Forward Primer	RT- Primer
miR159	AGCTGCTGACTCGTTGGTTC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
miR167	CGTAGGGGAGAAGATGGGGACGAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCA
miR169	TGAGCCAAGGATGACTTGCCG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCA
miR172	GCGGCGGAGAAUCUUGAUGAU	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
miR393	GCGGCGGUCCAAAGGGAUCGCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA
miR395	GGTAATCTGCATCCTGAGGTTTA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGGT
miR396	TGAAGAAGATAGTCCCCTTAACACC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTTC
miR398	GTTGGAGGTTGCTTGTGGAAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
miR399	GCGGCGGUGCCAAAGGAGAUUU	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG

*Reverse Primer GTGCAGGGTCCGAGGT

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, Rice and Zea mays. In this report, we investigated changes in miRNA expression levels after exposure to salt and drought stress in French bean, an important vegetable crop. Using RT-PCR and qRT-PCR, we analyzed changes in nine different miRNAs in seven day old French bean seedlings after 48 h exposure to salt and drought conditions. We found that miR395 was significantly up-regulated under both salt and drought stresses. Interestingly, miR395 has only been shown to function in plant response to sulfate deprivation by targeting sulfur transporter genes [26, 37, and 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 salt and drought. 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 salt and drought 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 French bean tolerance to salt and drought conditions. Two miRNAs, miR396 and miR172, were up-regulated after exposure to 0.4 M NaCl and drought. 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 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 plant development but may also function in 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 French bean tolerance to salt and drought conditions. miR169, a miRNA known to be induced under high salinity [21], was found to be down-regulated after exposure to increasing concentrations of NaCl. It is possible that miR169 is only induced under extreme conditions greater than 0.4 M salt. Surprisingly, however, this miRNA was significantly up-regulated in French bean seedlings after exposure to drought. 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 under drought, miR159 has been shown to target MYB101 and MYB33 transcripts, two factors that positively regulate the ABA [51]. Consistent with our findings, this miRNA has also been shown to be up-regulated under drought [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 0.4 M NaCl and drought. 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 French bean 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 under drought stress conditions. 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 drought on APX and ADH expression. We found that both genes were up-regulated after exposure to 0.4 M NaCl and drought. 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. In this article, we analyzed the expression levels of nine different miRNAs in French bean seedlings exposed to 0.4 M NaCl as well as drought. We used salt and drought to stimulate abiotic 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 French bean. Therefore, we believe that miRNAs may play a key role in developing French bean plants with a greater tolerance to salt and drought stress.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper

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