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Expression of miRNAs regulates Growth and Development of French bean (Phaseolus vulgaris) under Salt and Drought Stress conditions

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Abstract

Identification of stress-regulated miRNAs is crucial for understanding how plants respond to environmental stimuli. We are interested in the identification of miRNAs in French bean (Phaseolus vulgaris) to uncover different plant strategies to cope with adverse conditions and because of its relevance as a crop in developing countries. In this study, we investigated the effect of salt and drought stress in expression of small regulatory RNAs. Both salt and drought stresses altered the expression pattern of miRNA in a dose-dependent manner. However, each miRNA responded to drought stress in a different pattern. Salt and drought stress changed the expression level of miRNAs mainly from 0.9-fold up-regulation to 0.7-fold down-regulation. Micro RNAs were less sensitive to drought than salinity, as evidenced by the narrow fold change in expression levels. Although the range of change in expression level of miRNAs was similar under salt and drought stress, no miRNAs displayed significant change in expression level under all tested salt conditions. Micro RNAs, miR156 and miR162, showed significant change in expression level under high drought stress. This suggests that miR156 and miR162 may attribute to the adaption to drought stress and are good candidates for improving the vegetable crop by transgenic technology.

Keywords: Drought, French bean, miRNA, salt stress.

Introduction

MicroRNAs (miRNAs) are an extensive class of newly discovered non-coding small RNAs that regulate gene expression at the post-transcription levels by mRNA cleavage or translation repression. By regulating their target proteins, miRNA have been reported to be involved in diverse biological processes, including organ development hormone signaling, defense against pathogens, and response to abiotic and biotic stresses[1]. Important abiotic stresses in this regard include salinity, drought cold and heavy metals, nutrition, and other stresses[2]. More than 40 miRNA families have been associated with abiotic stress in plants, 13 of which have been found to be responsive to salt and drought stresses. These 13 miRNAs include miR156, miR159, miR165, miR167, miR168, miR169, miR319, miR393, miR395, miR396, miR398, miR399, and miR402. Recently, miR172 and miR397 were also reported to be implicated in drought stress in *Solanum* and rice. Almost all of these stress-induced miRNAs are evolutionarily conserved, which suggests that miRNAs-mediated regulatory mechanism may be evolutionarily conserved for corresponding environmental stresses in plants. However, the same miRNAs reported to respond abiotic stress in one certain species may not have the same function in other species. To date, opposite expression in Arabidopsis and rice under drought stress has been observed for at least 10 miRNAs that involve in stress response. This raises the question whether these reported stress responsive miRNAs still play tolerance roles in other plant species[3].

Several studies have demonstrated that miRNAs play important roles in the responses to biotic and abiotic stimuli. Abiotic stress-regulated miRNAs were first investigated in *Arabidopsis*. miR393, miR402, miR397b, and miR319c were induced by at least one of the treatments including drought, cold, salt and ABA, whereas miR398 was down-regulated. Further study showed that miR398 mediates the post-transcriptional induction of two *superoxide dismutase* genes involved in the first line of defense against toxic superoxide radicals and is also down-regulated by oxidative stress in *Arabidopsis.* Also in *Arabidopsis*, miR169 is down-regulated by drought through an ABA-dependent pathway to control the expression of the NFYA5 transcription factor, which mediates tolerance to drought. To discover stress-regulated miRNAs, it is necessary to compare the expression of miRNAs in plants grown under normal and stress-treated conditions. This was achieved by Northern blot analyses when digital expression analysis was not effective because traditional sequencing technology provided only very low coverage. With the application of next-generation sequencing and microarrays, it became much easier and cost-effective to perform genome-wide expression profiling to identify stress-regulated miRNAs. As a result, discovery of stress-regulated miRNAs has expanded from the model dicot *Arabidopsis* to model monocot rice and other non-model

plants, and many more stress-regulated miRNAs were found. French bean is a major vegetable crop cultivated all over the worldwide. Recently extensive efforts have been devoted to the discovery of conserved and novel miRNAs, as well as the analysis of miRNAs in stress responses. Micro RNAs that are regulated by various stresses were identified. Most of previous studies on miRNAs that are regulated by abiotic stresses in French bean have been focused on antioxidant responses in early growth stages. However, the onset of salt and drought stresses during early stage of growth can dramatically compromise in the crop yield. No investigations have been performed on the expression patterns of miRNAs and their potential roles under stress conditions in this important biofuel feedstock. In this study, we chose 12 miRNAs to study and these 12 miRNAs are conserved in dicots and monocots. Except miR162, 11 of the 12 miRNAs have been reported to be involved in salt or drought stress in previous studies in model plant species[4]. miR162 was also selected because of its important role in miRNA processing by negatively regulating the dicer-like 1 (DCL1) gene. Therefore, there is a need to expand our knowledge on miRNA expression under abiotic stresses. In this study we investigated how salt and drought stresses affected the germination and altered the expression levels of miRNAs.

Material 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 7day old and were then randomly divided into three groups. One group was used as untreated control, and other two groups were treated with salt (400mM NaCl for 48 h) and drought (with-holding for 2weeks) stresses respectively. After control and stress treatments were applied, shoots and roots were harvested separately immediately for RNA isolation.

RNA isolation and real-time RT-PCR analysis: Total RNA was isolated from different tissues of control and stressed seedlings using miRNA Isolation kit (Ambion) according to manufactures instructions. The quality of miRNA was assessed by 15% Urea PAGE, using 14, 24, and 39nt oligos as size standards. Twelve miRNAs were selected for this study, which included miR156, miR157, miR159, miR162, miR167, miR169, miR172, miR395, miR396, miR397, miR398, and miR399. A majority of these miRNAs have been reported to play a role under stress conditions in model plant species. Table-1 listed the primers for these 12 miRNAs. Real time RT-PCR was used to characterize the expression of 12 miRNAs in both shoot and root under salt and drought stress. First, the RT reaction was carried out using TaqMan micro RNA reverse transcription kit (Applied Biosystems). The mixtures of 12 miRNA specific primers were used to obtain the cDNA.

RNA gel blot analysis: For miRNAs quantification, northern blot hybridization was conducted using high sensitive miRNA Northern blot assay kit (Signosis, USA). 30 µg total RNA of each sample was electrophoresed on 15% polyacrylamide gel and transferred to membrane. Antisense RNA biotin labeled in the 5' end (Invitrogen) was used for hybridization probes. The Cyber Green® II stained (Biotech) rRNA bands in the polyacrylamide gel are used as a loading control.

Results and Discussion

Salt stress altered the expression pattern of miRNAs in French bean: Salinity treatment affected the miRNA expression. The expression of miR162 was increased at maximum 0.9-fold up-regulation under salt stress condition (400mM); in contrast, miR397 showed 0.7-fold down-regulation under salt stress conditions. The expression level of miR156 and miR159 was down-regulated, whereas it was up-regulated under higher salt concentration; in contrast, the expression level of miR172, miR395, and miR399 was up-regulated under 400mM salt concentration while down-regulated under higher salt conditions. The expression level of miR157 and miR398 was down-regulated under lowest and highest salt concentration while up-regulated in the moderate salt stress (200mM, data not shown). Of special interest, the expression level of miR167 was down-regulated under 200mM or lower salt concentration while was up-regulated by 0.3-fold when exposed to 400mM salt condition. Although salinity treatment affected the expression of all tested miRNAs, the changes in miRNA expression were small. The highest fold change in expression was miR157 and only 3 fold up-regulation was observed under moderate salt treatment. All the other 11 miRNAs only showed less than 0.8-fold up-regulation in expression level and the 0.8-fold up-regulation was observed in miR162 under the most severe salt stress. miR397 was the most down-regulated of all miRNAs evaluated with a change in expression of 0.7-fold at 400mM salt treatment; miR157 showed the second greatest change in expression of 0.4-fold down-regulated at 200mM salt stress (figure 1).

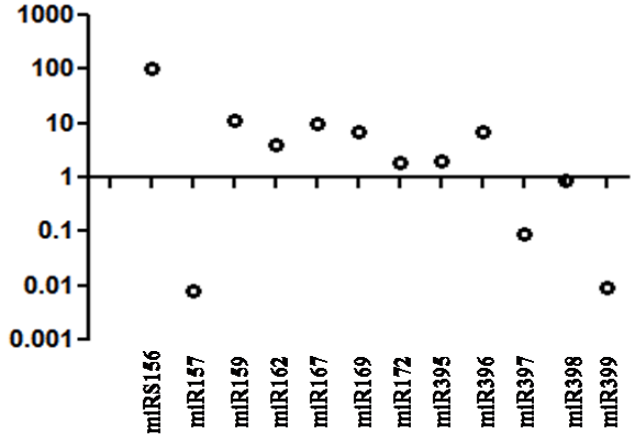

Figure-1
Relative expression levels of 12 miRNA. U6 small RNA was used as a control. Total RNA (1ml) from each of three conditions (control, Salinity and Drought) were used for verification

Response of known miRNAs to drought stress: To identify drought-responsive miRNA expression, miRNAs in the two libraries (control, and drought) were compared. The results of real-time PCR and gel blot analysis of miRNAs expression levels reveals that all 12 tested miRNAs were expressed in French bean seedlings, but their expression level varied from each other (figure 1). Among the assayed miRNAs, miR156 was the miRNA with the highest expression level; compared with other miRNAs, the expression level of miR156 was 124-fold of the average of the 12 miRNAs. miR159, miR167, miR169 and miR396 were also highly expressed. However, the expression level of miR157, miR399 and miR397 were relatively low and their expression levels were less than 10% of the average expression level of the 12 tested miRNAs; of them, miR157 and miR399 were the miRNAs with the lowest expression. miR156 and miR157 were grouped into one miRNA family because of their high sequence similarity and shared targets. Results from both the methods showed that 22 members in 4 miRNA families, i.e., miR399, miR2089, miR2111 and miR2118, were up-regulated in response to drought stress (figure 1). The significant difference of miR156 and miR157 in French bean indicates that they may be involved in different development stages and play different roles in growth and development.

To confirm and validate the results obtained from the expression patterns of miRNA were individually selected and experimentally verified by northern blotting hybridization. The sequence of antisense RNA probes. By comparing the miRNA results by Real time-PCR patterns to northern hybridization, five stress responsive (Salt and Drought) miRNAs (miR157, miR397, miR399, miR396 and miR156) were identified with identical expression patterns (figure 2).

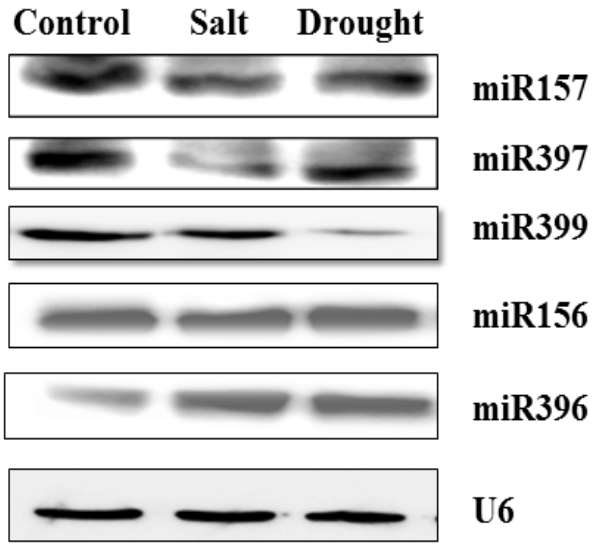

Figure-2
Northern blotting confirming differential expression of miRNAs, Total RNA 30µg from each of three conditions (Control, Salt and Drought) was loaded and probed with miRNAs probe. U6 was used as control of equal loading

Nowadays, characterization of the vital roles of miRNAs play in plant stress responses is an active research field. Although many studies have demonstrated that the plant miRNAs function as important regulators in development and morphogenesis processes, more reports are indicating that plant miRNAs are also involved in environmental stress tolerance. One reason why French bean has gained attention as a dedicated vegetable crop is that it can grow on suboptimal land under salt and drought conditions. Our results indicated that salt stress had a significant effect on the germination rate and growth of French bean in almost all tested abiotic stresses. Interestingly, drought stress had no obvious effect on the germination rate of French bean; the significant effect of drought stress on French bean growth was observed at high water stress conditions. Barney and colleagues also reported that French bean demonstrated great tolerance to drought stress. This result suggests that French bean has evolved a more effective mechanism to cope with drought stress as opposed to salt stress. Therefore, it is interesting to further investigate the change in gene expression, especially the gene expression regulators, under such stress conditions.

Micro RNAs are an extensive class of newly discovered gene regulators. They have been reported to play important roles under abiotic stress in model plant species. Plant miRNAs have been reported as having a strong propensity towards regulating responses to abiotic stress, including dehydration, freezing, salinity, alkalinity, and other stresses by transcriptional factors or proteins. Expression levels of miRNAs induced by environmental stresses vary. Some miRNAs may be involved in response to several abiotic stresses, while others seem to be specific to an individual stress. Differences in expression patterns could also be an effect of the nature and severity of individual stress and the level of impact that it has on the tissue under study. Using qRT-PCR, we studied the expression change of 12 conserved miRNAs in 6 day-old French bean seedlings exposed to salt and drought stress. Of the 12 miRNAs, 11 have been demonstrated to be involved in salt or drought stress in previous study, eight of these in both the *Arabidopsis thaliana* and *Oryza sativa*. It is interesting that, in general, we did not find dramatic changes in expression of miRNAs in response to abiotic stresses and only a small fraction of miRNAs showed some level of regulation. Overall, the slight changes observed in the expression of miRNAs points to the existence of a fine-tuning mechanism rather than a dramatic control of expression exerted by miRNAs under drought, cold and salt stress. This fine-tuning mechanism may be important in plants to regulate gene expression without impacting negatively growth and development.

Our results indicate that both salt and drought stresses altered the expression pattern of miRNAs in a dose-dependent manner. Salt and drought stress changed the expression level of miRNAs mainly from 0.9-fold up-regulation to 0.7-fold down-regulation, and drought stress altered the expression of miRNAs from 0.9-fold up-regulation to 0.6-fold down-regulation. Although the range of change in expression level of miRNAs was similar

under salt and drought stress, no miRNAs displayed significant change in expression level under all tested salt conditions, however, two miRNAs, miR156 and miR162, showed significantly change in expression level under high drought stress. This suggests that miR156 and miR162 may attribute to the adaption of French bean to drought stress and are good candidates for improving French bean as a vegetable crop by transgenic technology. miR156 is one class of conserved miRNAs, which play an important role in multiple biological processes. By targeting squamosal promoter binding protein-like (SPL) genes, miR156 has been demonstrated to temporally regulate shoot development[5], control the development timing from juvenile to adult transition together with miR172, secure male fertility, regulate anthocyanin biosynthesis, and is involved in flowering control. Overexpression of miR156 in *Arabidopsis*, *rice,* and *maize* led to a prolonged vegetative phase together with the production of significantly higher number of total leaves, which resulted in enhanced biomass accumulation, miR156 was demonstrated by microarray-based analysis to response to salt stress but not to drought stress in *Arabidopsis*; miR156 was induced by 1.6-fold by salinity stress. In rice, miR156 was found to respond to drought stress and was down-regulated by 2.1-fold by drought stress. However, study on salt stress of maize showed that miR156 was not involved in salt response[6]. Our results indicate that the expression of miR156 was significantly induced by 1.7 fold under high drought condition. Further studies on the expression change of downstream genes would help us to illustrate the mechanism of tolerance of French bean to drought stress. miR162 has been reported to involve in miRNA biogenesis by negatively regulating dicer-like 1 (DCL1) gene. It was also implicated to play a role in cotton fiber development, although it was reported to be significantly down-regulated under cadmium stress in rice. In our study, miR162 was down-regulated under all drought stress treatments, while the expression change was statistically significant only under high drought conditions. This suggests that miR162 plays an important role during drought stress and feedback regulation of miRNAs also functions in French bean to adapt the drought stress. Given the multiple functions of miR156 and miR162, it would be interesting to investigate how the numerous phenotypes would play out in overexpressed transgenic French bean and whether these over-expressed miRNAs would confer higher tolerance to French bean, on the other hand, miR396 regulates a family of growth factors in rice. Up-regulation in our drought and salt stress libraries suggests the down-regulation of growth factors, perhaps to redirect resources to other parts of the plant in response to drought. This confirms that growth regulation is a mechanism highly sensitive to abiotic stresses.

Conclusion

Our results suggest that miRNAs play important roles in plant growth under abiotic stresses in vegetative tissues. Further functional analysis of stress regulated miRNAs and their targets will allow us to dissect the complex miRNA-mediated pathways and networks in plant stress responses.

Acknowledgements

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Table-1
Primers used in reverse transcription (RT) for amplifying 12 miRNAs. The reverse primer is provided by the kit. The nucleotides in green are the same as or complementary to the miRNA sequences. RT and FP in the primer name indicate that the primer is reverse transcription primer or forward PCR primer respectively

miRNA	Sequence	Primer names	Primer sequence
miR 156	UGACAGAAGAGAGUGAGCAC	miR156-7RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
		miR156FP	GCGGCGGTGACAGAAGAGAGT
miR 157	UUGACAGAAGAUAGAGAGCAC	miR156-7RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC ACTGGATACGACGTGCTC
		miR157FP	GCGGCGGTTGACAGAAGATAGA
miR 159	UUUGGAUUGAAGGGAGCUCUA	miR159RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
		miR159FP	GCGGCGGTTTGGATTGAAGGG
miR 162	UCGAUAAACCUCUGCAUCCAG	miR162RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGAT
		miR162FP	GCGGCGGTCGATAAACCTCTG
miR 167	UGAAGCUGCCAGCAUGAUCUA	miR167RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGATC
		miR167FP	GCGGCGGTGAAGCTGCCAGCA
miR 169	CAGCCAAGGAUGACUUGCCGA	miR169RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCA
		miR169FP	GCGGCGGCAGCCAAGGATGAC
miR 172	AGAAUCUUGAUGAUGCUGCAU	miR172RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
		miR172FP	GCGGCGGAGAATCTTGATGAT
miR 395	CUGAAGUGUUUGGGGGAACUC	miR395RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGTTC
		miR395FP	GCGGCGGCTGAAGTGTTTGGG
miR 396	UUCCACAGCUUUCUUGAACUG	miR396RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTTC
		miR396FP	GCGGCGGTTCCACAGCTTTCT
miR 397	UCAUUGAGUGCAGCGUUGAUG	miR397RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA
		miR397FP	GCGGCGGTCATTGAGTGCAGC
miR 398	UGUGUUCUCAGGUCACCCCUU	miR398RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
		miR398FP	GCGGCGGTGTGTTCTCAGGTC
miR 399	UGCCAAAGGAGAUUUGCCCUG	miR399RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGC
		miR399FP	GCGGCGGTGCCAAAGGAGATT