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RESEARCH ARTICLE

Regulation of mir159 and mir396 Mediated by *Piriformospora indica* Confer Drought Tolerance in Rice

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Abstract: Drought stress is one of the most determinative factors of agriculture and plays a major role in limiting crop productivity. This limitation is going to rising through climate changes. However, plants have their own defense systems to moderate the adverse effects of climatic conditions. MicroRNA-mediated post-transcriptional gene regulation is one of these defense mechanisms. The root endophytic fungus *Piriformospora indica* enhances plant tolerance to environmental stress based on general and non-specific plant species mechanisms. In this work, we investigated the effects of drought and *P. indica* inoculation on the expression of two important miRNAs, miR159 and miR396, in rice plants. To this end, leaf samples were harvested at control (F.C.) and severe drought stress (25% F.C.) in *P. indica*-colonized and non-inoculated rice plants 4 weeks after fungal inoculation. We have observed contrary expression patterns of miR396 (down-regulated) and miR159 (up-regulated) under drought stress condition. However, both miRNAs showed up-regulation by *P. indica* inoculation. We have observed significant up-regulation of miR396 and miR159 by treatment of *P. indica* under drought stress condition. Regulation of growth, hyposensitivity response and bio-water saving pathways directly affected by MYB and GRF transcriptional factors. So, remarkable change of miR156 and miR396 could lead plant to be tolerable under drought stress by the fine regulation of MYB and GRF, respectively.

Keywords: Endophyte, Drought stress tolerance, miRNAs, Post-transcriptional gene regulation

INTRODUCTION

Piriformospora indica, a root endophyte similar to arbuscular mycorrhizal fungi, which has been isolated from rhizosphere soil of plants growing in extreme hot conditions of Thar desert of Rajasthan, India. *P. indica* grows inter- and intracellularly, and forms pear shaped chlamydospores in the cortex of the host roots. This fungus increases the uptake and metabolism of nitrate and phosphate [1, 2] and allows plants to survive under abiotic stress [3, 4], besides stimulating growth and seed production in a large number of diverse host plants [5, 6, 7] . Also, *P. indica*-root colonization led to drought stress tolerance in several plant species including Arabidopsis and barley [8, 9]. As of recently, the gene and the metabolite regulatory networks that promote the growth of *P. indica*-colonized plants are still largely unknown. Nonetheless, some studies have shown that various factors induced by *P. indica* in host plants were responsible for its positive effects [10, 11].

The most important environmental stress by which the production of many crop species is severely affected and limited worldwide is drought stress. One of the molecular changes that plants utilize to cope with drought stress is

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regulation of small 21 nucleotides RNAs, called miRNAs. miRNAs play essential roles in gene expression regulating through degradation or translation inhibition of the interested mRNAs [16, 17, 18, 19].

miRNAs are key factors in regulating many biological events, like growth and development. Researches demonstrated changes in miRNAs levels in response to environmental factors such as biotic and abiotic stresses. There has been an increasing attention to unravel the role of miRNAs in environmental stress response in recent years [22].

Our previous studies revealed several prominent miRNAs involved in biotic and abiotic stresses [20, 26]. Using deep sequencing, 9 new miRNAs in rice from miR11336 to miR11344 were identified in response to fungal elicitors [27]. In a study performed on rice under drought condition, miR474 and miR854a up-regulated [28]. When searching for possible rice miRNAs engaged in drought response, miR167 and miR413 were found to be regulated by ABA, and miR166 as well as miR319 were reported to be controlled by GA, which both are plant phytohormones and ABA is believed to play crucial role against several stresses[29]. Furthermore, miR167 showed interference in auxin signaling responses [30]. In a recent study, 71 new miRNAs were found to show differential expression in drought stress in different tissues of rice, in which out of them 18 miRNAs were up-regulated, but the remaining were down-regulated [31]. There are several reports that emphasized the role of miR159 in stress responses such as response to drought and ABA [33], salt [34] , cold [35] and Hypoxia stress[36]. miR159 could regulate MYB transcription factors transcripts [37, 38]. It has been also shown that miR159 over-expression led to reduction of MYB transcription factors followed by plant hyposensitivity, but conversely cleavage resistant forms of MYB resulted to plant hypersensitivity [38].

miR396 is the other important stress responsive miRNA. The role of miR396 in drought stress [34], salt [34] , cold [35] and Hypoxia stress [36] has also been reported. Growth regulating factors (GRFs) showed to be regulated through miR396 targeting and play essential role in cell division and differentiation during leaf development [24, 39]. Thus, these miRNAs could be introduced as essential miRNAs in stress response regulation.

In the present study, we tried to analyze of expression patterns of two important miRNAs including miR156 and miR396 under drought stress condition in rice plant with and without *P. indica* inoculation.

MATERIALS AND METHODS

Fungal growth, plant inoculation and growth condition

P. indica was grown on a complex medium (CM) at 24°C [40]. The spore suspension was collected after 4 weeks by gently scratching the fungus surface on the Petri dishes with a spatula until the spores were released. The spore concentration was adjusted to 5×10^5 spores per ml as described by Ghabooli *et al.* (2013).

For plant inoculation, rice seeds of the cultivar "Sadri" were surface-sterilized with 70% ethanol (v/v) for 30 s followed by 6% sodium hypochlorite (NaOCl) for 15 min and then thoroughly rinsed with water. 3-4 days old rice seedlings were inoculated by immersing in the spore suspension solution with gentle shaking for 1h. The control seedlings were dipped in sterile water. Inoculated and non-inoculated rice seedlings were later transferred into pots, filled with 5 kg of garden soil and then placed in the greenhouse at 27/18 °C day/night cycle, 60% relative humidity and a photoperiod of 16 h. The experiment was conducted in a completely randomized design under two treatments (*P. indica*-inoculated and non-inoculated plants), and two levels of drought treatments [Filed capacity (F.C.) and 25% F.C.] in three replications. Before drought treatment, root samples were tested for *P. indica*-colonization according to Ghabooli et al. (2013). Drought was initiated 2 weeks after plant inoculation by withholding water and drought-stressed pots were re-watered, when the soil moisture reached 25% F.C. The well-watered treatment was maintained near F.C. The shoot samples from *P. indica*-colonized and non-inoculated plants under well-watered and water-deficit conditions were harvested 4 weeks after inoculation. Three plants from each pot were merged together, and considered as a single replication and used for physiological analysis. The collected samples for RNA extraction were immediately frozen in liquid N_2.

Fresh weight, dry weight and RWC analysis

The shoot samples from inoculated and non-inoculated plants were harvested 4 weeks post initial inoculation. The fresh shoot samples were then placed in paper bags and incubated in an oven at 70 °C to constant weight, after which the dry weights were determined. RWC (Relative Water Content) was determined in the leaf tissue according to Bars and Weatherly (1962) using the below equation:

$$RWC\ (\%) = [(FW - DW)/ (TW - DW)] \times 100$$

Where FW is fresh weight of the leaf tissue taken, TW is the turgid weight after rehydration at 25°C for 24 h and DW is the dry weight after oven drying at 60°C for 48 h.

RNA extraction and Quantitative Real-Time PCR analysis

For each treatment, total RNA was extracted from the leaf of each three independent biological replicates using Trizol reagent (Invitrogen) according to manufacturer's instructions with some modification as previously described [41]. The quantity and quality of extracted RNAs were evaluated by NanoDrop 2000 (Thermo Scientific) and the integrity was also assessed by electrophoresis on a 1.0 % agarose gel. The sequences of mature miRNAs have been obtained from online miRNAs database (mirbase.org; release 21). Stem–loop RT–PCR and miRNA gene-specific real time PCR Primers were designed according to Chen et al. [42] and 18srRNA and *eEF*-1 were used as reference genes [43] (Table 1). miRNA stem–loop reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to Varkonyi-Gasic et al. [44] qRT-PCR reactions were performed (three for each treatment by three technical replicates per treatment) using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne) on a Rotor-Gene Q (5-plex) according to the manufacturer's instructions. A two-tailed t test of significance (Microsoft Office Excel 2007) was used to compare the treatments [25].

RESULTS

Effects on fresh weight, dry weight and RWC

Microscopic analyses showed that *P. indica* colonized rice roots as measured by production of the number of chlamydospores at 2 weeks after spore inoculation (colonization rate was more than 80%). No colonization was observed in non-inoculated plant roots. Previous microscopic analyses showed that *P. indica* colonized rice roots efficiently [45, 23]. *P. indica* had a growth-promoting effect on colonized rice plants as they grow faster and more vigorously than control plants under both well-watered and drought condition. *P. indica* increased shoot fresh and dry weight of *P. indica*-colonized rice plants by 6% and 5% compared to non-inoculated plants under well-watered condition, respectively at 4 weeks after inoculation (Figure 1). As expected, drought had a negative effect on growth rates of both *P. indica*-colonized rice and non-inoculated plants.

Table 1. Sequence of Real-Time PCR primers

miRNA	Forward (5' → 3')	Reverse (5' → 3')
miR159	ggagtttggattgaaggga	gtgcagggtccgaggt
miR369	ggatccacaggctttcttg	gtgcagggtccgaggt
eEF-1 a	gacttccttcacgatttcatcgtaa	tttcactcttggtgtgaagcagat
18srRNA	ctacgtccctgccctttgtaca	acacttcaccggaccattcaa
	Stem-loop RT-PCR primers (5' → 3')	
miR159	gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccagagc	
miR369	gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccagtt	

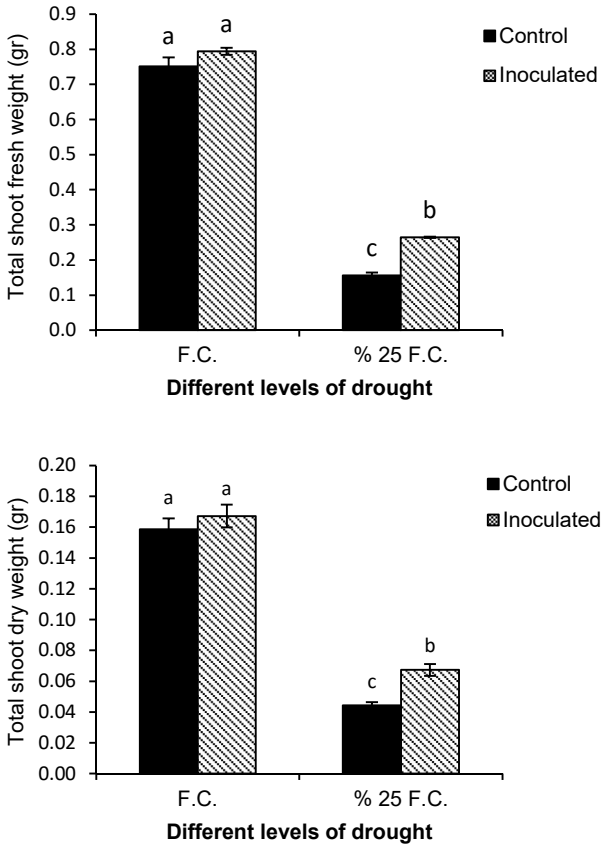

Figure 1. Effect of *P. indica* on rice shoot fresh and dry weights under well-watered (F.C.) and drought (25% F.C.) conditions. Rice fresh and dry weights were measured 4 weeks after fungal inoculation. Bars represent the standard error of the mean. Statistically significant differences ($P < 0.05$) between are indicated by different letters.

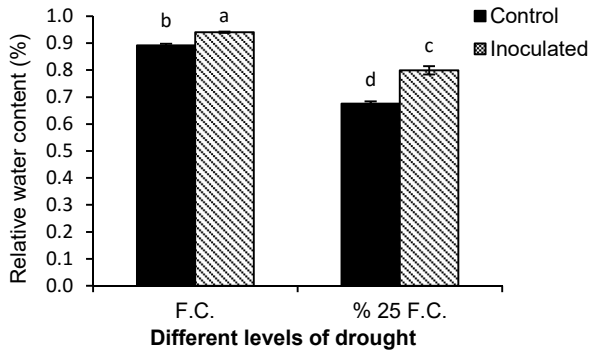

Figure 2. Effect of *P. indica* on relative water content (RWC) in inoculated and control plants under well-watered (F.C.) and drought (25% F.C.) conditions. RWC was measured 4 weeks after fungal inoculation. Bars represent the standard error of the mean. Statistically significant differences ($P < 0.05$) between are indicated by different letters.

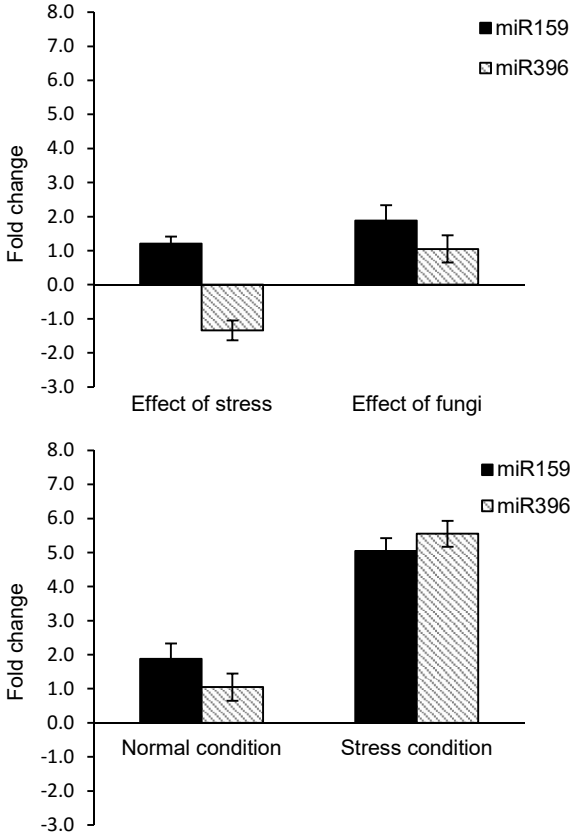

Figure 3. Left figures show the treatments that used in this experiment and right figures present differential expression of miRNAs under different conditions. **a)** Bar chart indicates differential expression of miRNAs when drought stress (no 1) and fungi treatment (no 2) are the sole source of differential expression, respectively. **b)** miRNAs expression pattern under drought stress with simultaneous effect of fungi (no 3) are compared with conditions that fungus is only the sole source of differential expression (no 2).

Under drought stress (25% F.C.), the shoot fresh and dry weight of *P. indica*-colonized plants were up to 1.69 and 1.52 times higher than non-inoculated plants. The relative water content (RWC) of *P. indica*-colonized and non-inoculated plants were decreased by 15% and 24%, respectively under drought condition; however, *P. indica* promoted RWC by 1.18 times higher compared to that of non-inoculated plants under drought stress (Figure 2).

Effects of fungi treatment and drought stress on the expression of miR159 and miR396

In this study, two important miRNAs including miR159 and miR396 were studied against drought stress. These miRNAs showed contrary expression pattern under drought stress condition. The results showed up-regulation and down-regulation of miR159 and miR396 under drought stress, respectively (figure 3a).

Effects of *P. indica* in miR159 and miR396 expression patterns in well-watered plants has been evaluated and similar expression pattern for both miRNAs observed. Up-regulation of both miR159 and miR396 under the influence of *P. indica* is presented in figure 3a.

In order to identify the effect of *P. indica* on the expression pattern of miR159 and miR396 in rice plant under normal and drought stress conditions, seedlings were affected by combined effect of drought stress and *P. indica* induction simultaneously. As shown in figure 3b more than five times up-regulation of these two miRNAs was observed by effect of *P. indica* under drought stress. While, non-significant up-regulation and down-regulation of miR159 and miR396 have been observed in normal condition, respectively (figure 3b).

DISCUSSION

The expression of miR159 and miR396 under drought stress has been studied in many reports. In the other hands, there are also several reports indicated drought tolerance induction by inoculation of *P. indica*. Thus, it is important to answer how this fungus could affect rice plant miRNAs such as miR159 and miR396 under drought stress condition. In this study, physiological aspects and expression pattern of miR159 and miR396 were evaluated in rice plants with and without *P. indica* inoculation under drought stress. It would be expected that *P. indica* could serve as inducer of these miRNAs that can be followed by physiological changes led to improve drought tolerance under drought stress condition.

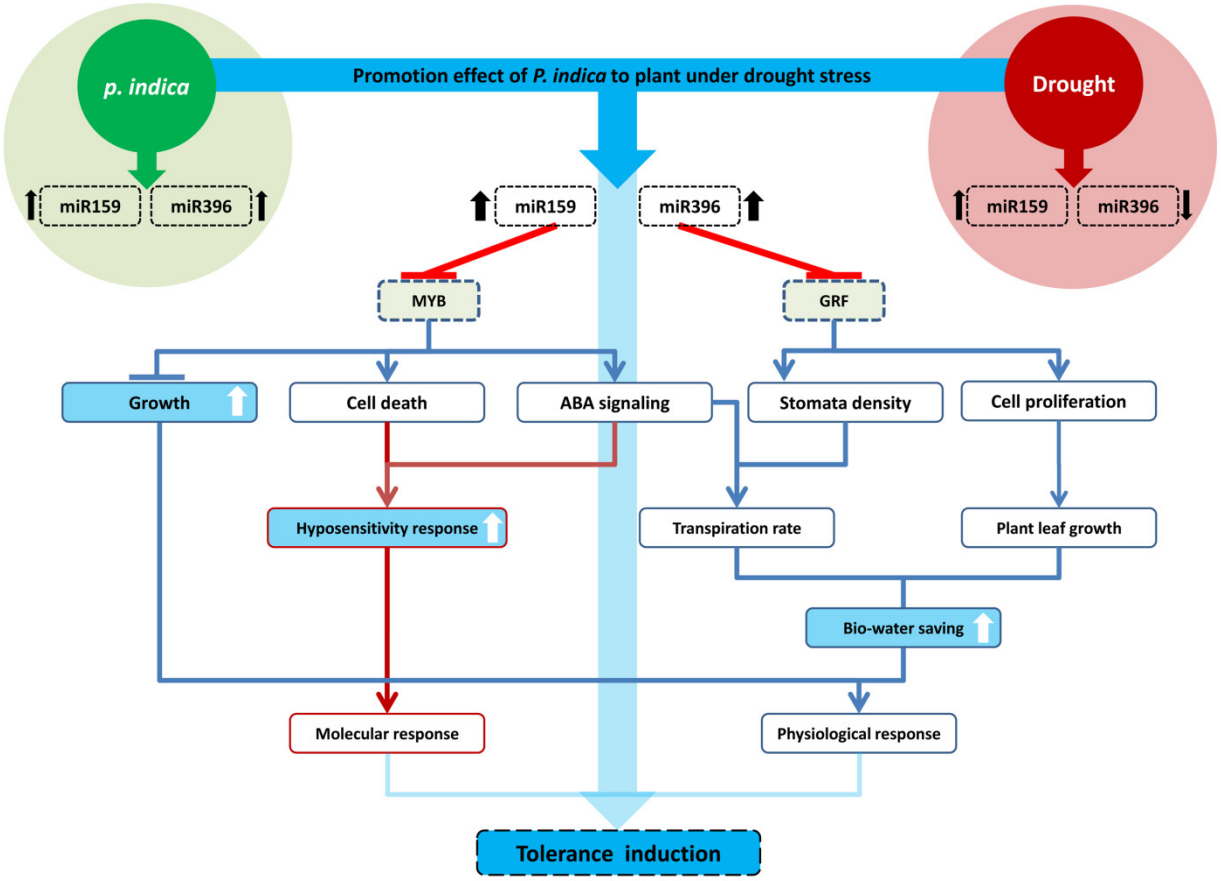

Figure 4. Pathways involved in rice plant tolerant induction under drought stress using *P. indica* fungi

Our target prediction revealed MYB transcription factor as one of the most important targets of miR159. It has been shown previously that miR159 up-regulation could decrease MYB transcription factor [38]. It was also reported that MYB play a negative role in growth promotion [46]. Thus, up-regulation of miR159 in our study could be resulted to MYB down-regulation followed by growth promotion. Furthermore it has been also reported that MYB transcription factor could play a positive role in programming cell death [46]. Thus, probable down-regulation of MYB transcription factor as result of miR159 up-regulation might reduce programming cell death. Recently essential role of miR159 in ABA signaling has been reported in several studies [20, 38]. ABA signaling positively regulated by MYB transcription factor [38]. Thus, up-regulation of miR159 could play important role in inhibition of ABA signaling through down-regulation of MYB transcription factor. This mechanism has been named as hyposensitivity in contrast to hypersensitivity mechanism that cause by MYB up-regulation. It has been reported that drought stress resulted to enhance in ABA in rice [47, 48] and recently its regulation by miR159 is explained as well [20]. Thus, *P. indica* could induce hyposensitivity in plant to moderate drought stress signaling followed by increasing growth induction through miR159 regulation.

We have also observed miR396 could regulate GRFs (Growth Regulating Factors). GRFs play important role in growth and development of leaves [49, 50]. Repressed expression of GRF has been observed by miR396 over-expression [51]. In this context, 26.7 and 36.2 reduction in stomata density has been observed in 35S:MIR396a and 35S:MIR396b transgenic Arabidopsis plants [51]. The narrow leaf and reduction in cell number along the leaf-width axis has been reported as result of *grf1/2/3* triple mutant [50]. Thus, up-regulation of miR396 or down-regulation of GRF could be introduced as a mechanism that plants use to reduce stomata density. Reduction in stomata density led to decrease transpiration rate [52] followed by reduce water loss and increase in relative water content during drought stress [51] that could be happened in drought tolerant plants. It has been reported that miR396 predominantly expressed in leaf and seedling[51]. In the current study, we have observed down-regulation of miR396 under drought stress condition, but interestingly after using *P. indica* its

expression pattern changed to a strong significant up-regulation. It has been reported that over-expression of miR396 could decrease cell proliferation, plant leaf growth and stomata densities followed by reduce in transpiration that resulted to tolerance to drought stress [21, 51, 39]. In this study, we have observed that relative water content is higher in inoculated plants with *P. indica* than those of not inoculated plants under drought stress. Therefore, up-regulation of miR396 by *P. indica* inoculation led to a bio-water saving followed by drought tolerance. This could be happened through GRF down-regulation that could be resulted to leaf proliferation reduction, stomata density and transpiration.

CONCLUSION

We have observed increased drought tolerance in rice plants under drought stress when plants inoculated by *P. indica*. This tolerance could achieve by *P. indica* inoculation through miRNA regulation such as miR159 and miR396. We could conclude that miRNAs are affected by *P. indica* inoculation and play a vital role in promotion of growth and drought tolerance through regulation of ABA signaling, Growth and development, bio-water saving and many other pathways (Figure 4).

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القای مقاومت به خشکی در برنج توسط *Piriformospora indica* از طریق تنظیم بیان miR159 و miR396

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چکیده

تنش خشکی یکی از مهم‌ترین فاکتورهای تعیین کننده در کشاورزی بوده و نقش عمده‌ای در محدودیت تولیدات زراعی دارد که این محدودیت با تغییرات آب و هوایی افزایش می‌یابد. با اینحال، گیاهان سیستم دفاعی خاص خود را برای تعدیل اثرات مضر تغییرات آب و هوایی دارند. تغییرات پس از ترجمه اعمال شده توسط میکرو آر.ان.آ ها (miRNAs)، یکی از این راهکارهای دفاعی است. قارچ اندوفیت *Piriformospora indica* مقاومت گیاهان در مقابل تنش‌های محیطی را از طریق راهکارهای عمومی و غیراختصاصی بهبود می‌دهد. در این پژوهش، اثرات خشکی و تلقیح با قارچ *P. indica* بر بیان دو میکرو آر. ان. آ مهم، miR159 و miR396، در گیاه برنج بررسی شد. برای این منظور، نمونه‌های برگی از گیاهان تلقیح شده و گیاهان تلقیح نشده با قارچ در شرایط نرمال (ظرفیت زراعی) و تنش خشکی شدید (٪۲۵ ظرفیت زراعی)، چهار هفته پس از تلقیح برداشت شدند. در شرایط تنش خشکی الگوی بیان متضاد miR396 (کاهش بیان) و miR159 (افزایش بیان) مشاهده شد. با این وجود بیان هر دو میکرو آر. ان. آ توسط تلقیح با قارچ افزایش یافت. همچنین تیمار با قارچ موجب افزایش بیان معنی دار miR396 و miR159 در شرایط تنش خشکی شد. تنظیم رشد، پاسخ فوق‌حساسیت و مسیرهای بیولوژیکی موثر در حفظ آب، مستقیما توسط فاکتورهای نسخه برداری MYB و GRF تحت تاثیر قرار می‌گیرند. از اینرو، تغییر قابل توجه بیان miR156 و miR396 به ترتیب از طریق تنظیم دقیق MYB و GRF، می تواند منجر به ایجاد مقاومت در گیاهان در شرایط تنش خشکی گردد.

کلمات کلیدی: اندوفیت، مقاومت به تنش خشکی، miRNAs، تنظیم پس از نسخه برداری ژن