Comparative Analysis of Differentially Expressed miRNAs in Leaves of Three Sugarcane (*Saacharum Officinarum L.*) Cultivars During Salinity Stress

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Abstract

Sugarcane is an important industrial plant which cultivated in the most arid and semiarid regions. Due to climate change and anthropogenic activities, the sugarcane field damage due to salt deposition and the cultivation of sugarcane has been posed a major threat in the region. To address this issue, the identification of salinity tolerant cultivars would be a suitable strategy to minimize yield loss in the area. MicroRNAs (miRNAs) play important roles in regulating gene expression. The monitoring of the expression of miRNAs and their targeted genes could provide deeper insight into the molecular stress mechanism and screen tolerant cultivars. Our aim was to assess the expression of nine candidate miRNAs and their corresponding targeted genes among the studied sugarcane cultivars under salinity condition, leading to identify the salt-tolerant cultivar. To achieve our goal, a two-factorial experiment with three sugarcane cultivars (CP-48, CP-57, CP-69) and two salinity levels (0 and 8 ds/m) was conducted. One-way ANOVA indicated that there was a significant difference between miRNAs and targeted gene expression. The highest reduction of miRNAs expression was occurred in miR160 while the lower one was happening in miR1432. The data also indicated that the higher and the lowest of targeted genes were in miR160 and miR393 respectively. Among studied cultivars, the CP-57 showed poor performance while CP-69 expresses a superior tolerance to salt stress. Taken together, these results suggested that the screening of well adapted cultivars under salt conditions would be appropriate solutions to combat salinity stress in saline lands.

Introduction

Sugarcane (*Saacharum* sp.) is an important industrial crop. It is considered as a main source of sugar production in the world and important crop to produce bioenergy. Sugarcane is cultivated in nearly 100 countries over an area of about 22 million hectares which is 0.5% of the total arable area in the world [1]. Sugarcane has been cultivated in southern part of Iran and Karun River being as the main source of water which irrigated sugarcane plantation [2].

In recent years due to climate change, the temperature and evaporation in the region have been increased, while rainfall has decreased which all results in higher surface salinities. In addition, because of the faulty water management, Karun River has been heavily contaminated with untreated sewage which adds the huge quantity of salt ions. Nowadays, because of all above mentioned problems, salinity is an ever increasing in the sugarcane field resulting in reduction of sugarcane production in respect of both dry matter and sucrose content in the region. The desalination of farming soils required significant amount of times, labors and energy inputs which might create serious economic and social damage in the region [3]. Since, it is well known that there is an ample variation for tolerance to salinity among cultivars of the same species [4]. Therefore, the development of salinity tolerant cultivars is an efficient way to tackle salinity problem in the region. Such salt tolerant plants are capable of changing morphological, physiological, biochemical and anatomical mechanisms, in order to adapt to salinity environment [5].

Recent studies have indicated that many genes involved in the expression and synthesis of proteins related to abiotic stresses [6]. A large body of studies in recent years has proved that plants can trigger regulator genetic network, which consist expression of certain genes involved with transcription and translation regulation to activate the protection mechanisms to defend the plant under harsh environment [7]. In the protection mechanisms, the post-transcriptional is a vital process for recovering and keeps plant cell homeostasis during and after stress [6]. The recent researches have shown that plants implement miRNAs as gene expression regulator at a post-transcriptional level to minimize the growth and development of plant stress conditions [8].

The microRNAs (miRNAs) are small RNAs of 18–25 nucleotides in length that play important roles in responses to stress [6]. In plants, miRNAs are involved in multiple processes including organ development and plant responses to environmental stresses. It has been reported that sugarcane can activate certain complex network mechanisms which enable the plant respond to environmental changes [9]. Notably, several miRNAs have been reported to have higher expression rates in the samples treated with salt treatment. miR166III, 168II, 396II, 398II, 528I, 156V, 167V, 169III, 397II, 398I, and 159XVI have been found with different expression in sugarcane in response to moderate salt stress [10]. The mRNA-targets identified are transcription factors involved in plant development mainly. Previous works have described that GAMyB, HAP12 and GRF transcription factors have been validated as targets of miR159XVI, miR169III and miR396II, respectively [11].

The main aim of this research is to identify the salt-tolerant cultivar for the region by the monitoring of expression profile of candidate miRNAs in the studied sugarcane cultivars under salinity condition and also to recognize the best miRNAs for screening of salt-tolerant cultivar in sugarcane.

Material And Methods

Plant materials, growth conditions, and salt stress treatments

Three cultivars of *Saacharum officinarum* L. (CP-48, CP-57 and CP-69) were used in this study.

Seeds of CP-48 namely the salt-sensitive, CP-57 namely the salt-semi-tolerant sensitive and the salt-tolerant CP-69 were purchased from development sugarcane and related industrial company (Khuzestan, Ahwaz, Iran). They were cultivated at the Experimental Research Station of College of Agriculture, Shahid Chamran University of Ahwaz, Iran, in 50×50 m^2 pots with 2/3 soil with low EC and 1/3 sand in the glasshouse. Leaves with or without NaCl treatment (24 h, 8 ds/m; Sigma-Aldrich, USA) were collected at the six trueleaf stage of CP-48, CP-57, CP-69 plantlets and stored at -80 °C until RNA extraction.

RNA extraction

Total RNA was extracted from the leaves of the three varieties of sugarcane (two independent biological replicates) using RNeasy Plus Mini kit (Qiagen, Germany), followed by DNase (Pars tous Company, Iran)

treatment to remove the genomic DNA. RNA concentration was quantified using NanoDrop equipment (NanoDrop Technologies Inc., Wilmington, DE) and was qualified by 1% agarose gel electrophoresis.

In silico analysis

Co-expression networks were constructed using the GeneMANIA prediction web server, with default parameters (https://genemania.org/) [12]. The expression values of nine salt responsive miRNAs of three cultivars of *S. officinarum* were illustrated as a heatmap using HemI software (Heatmap Illustrator (v1.0)) [13].

Analysis of miRNA expression by Stem-looped qRT-PCR

For the miRNA, cDNA was synthesized according to the protocol developed by Varkonyi (2007) [14] and RT primers (long stem-loop extension primers) (Table S1), according to the instructions of the manufacturers. The expression level of miRNAs was determined using the SYBR Green PCR Master Mix (Pars tous Company, Iran). The qRT-PCR was performed in three biological and two technical replicates. The thermal conditions for miRNAs were included an initial denaturation step at 95 ^{0}C for 10 minutes, then 32 cycles of 95 ^{0}C for 15 s, 58 to 60 ^{0}C for 30 s and 72 ^{0}C for 30 s. For each miRNA, specific primers listed in Table S2.

Analysis of target gene expression by quantitative RT-PCR

For the potential target genes, the cDNA synthesis was performed according to the instruction of the cDNA synthesis kit (Pars tous Company, Iran) according to the manufacturer's instructions. Expression of nine target genes was assayed with qRT-PCR according to the protocols mentioned above gene-specific primers for qRT-PCR. The primers were designed using Primer premier6 software and listed in Table S3. Reactions were performed at 95 ^{0}C for 10 minutes, then 32 cycles of 95 ^{0}C for 15 s, 55 to 60 ^{0}C for 30 s and 72 ^{0}C for 30 s. GAPDH was used as reference gene for normalizing the target gene expression. The data of qRT-PCR was calculating using $2^{-\Delta\Delta CT}$ method. qPCR was carried out using ABI PRISM 7500Real-Time PCR System (ABI, USA).

Experimental designs and statistical analysis

The experiment was arranged as 3*2 factorials involving three cultivars of *S. officinarum* (Factor A) and two levels of stress (Factor B) in a completely randomized design (CRD) with three replicates. The qRT-PCR results were compared by one-way analysis of variance (ANOVA). The mean values were compared by Duncan's New Multiple Range Test (DMRT).

Results

In order to identify the miRNAs involved in salt tolerance, three sugarcane cultivars including CP-48 (salt sensitivity), CP-57 (relative salt tolerance) and CP-69 (salt tolerance) were used and nine sugarcane miRNAs were selected.

To validate the salt treatment, the expression of nine salt responsive miRNAs (miR160, miR164, miR172, miR390, miR393, miR408, miR529, miR827, miR1432) was examined by RT-qPCR. The ANOVA was done for nine miRNAs and their target genes based on completed block design in factorial arrangement with three replications. The results showed that the transcripts of the nine miRNAs and their target genes in the salt stressed were significantly depending on genotype ($p\_value < 0.01$) (Table S4 A and B).

A comparison of miRNA expressions showed that a total of nine miRNA were differentially expressed during salinity stress in three cultivars. ($p\_value < 0.01$) (Fig. 1). According to previous reports, miR160 is involved in the auxin response by targeting of auxin response factors (ARF) genes [15]. In this study, the greatest degree of down-regulation in response to salt stress was shown by miR160 in CP-57. In addition, the miR164 and miR1432 that encode transcription factors and transporters, was shown the lowest degree of down-regulation in response to salt stress (Figs. 1 and 2). Furthermore, it was found that the miR390, miR393 and miR408 was found in CP-48, CP-57 and CP-69 with a similar response of down regulation. qRT-PCR revealed that target genes of miRNAs levels increased in sugarcane during salt stress treatments. The expression of miR160 target gene was significantly increased by NaCl treatment. Among the targets, the lowest expression was shown by miR393 target genes. miR393 is involved in the transport inhibitor response (TIR) and EIN3-binding F-box protein (EBF) genes [16]. The expression of the miR160 target gene showed a significant upregulation following three sugarcane cultivars. However, increase in the miR160 target gene of CP-48 was lower than expression level of CP-69 cultivar (Fig. 3). The evaluated miRNAs can regulate several target genes and involved in various biological processes. ARF17, NAC080, AP2, EBF1, LAC3 and SPL9 genes were selected for co-expression network analysis, which includes miR160, miR164, miR172, miR393, miR408 and miR529 targets (Fig. 4). The ARF17; Auxin response factors 17 that is co-expressed directly with AP2, EBF1. A co-expression network of NAC080 with LAC3 and SPL9 genes was generated (Figure S1 and Table S5).

Discussion

Salinity is an increasingly important agricultural problem. The metabolism of plants are affected by salt stress and in recent years many studies have been devoted to understanding the molecular mechanisms of plant salt tolerance. Sugarcane cultivars differ in their responses to salt stress. There are several miRNAs that have been identified in different species, but only a few studies have been performed to analyze their expression in response to salt stress in sugarcane.

In the present study, the results indicated miR160, miR164, miR172, miR390, miR393, miR408, miR529, miR827, miR1432, have been implicated in stress caused by salt [15, 17]. Analysis of the relative expression of all these miRNAs (Fig. 1) showed that miR160 had the highest expression in NaCl treatment. Others showed small differences in expression compared with the control. The result shown that there are significant differences in expression of miRNAs and their targets. However, some miRNAs had significant expression. Low expression of miRNAs showed significant difference profiles depending on the cultivar [18].

miR390 targeting ARF transcription factors [19] has been found with different expression in sugarcane. ARF is one of the targeted TFs which involved in rooting, responder to drought and salinity stress, plant develops, response to auxin and auxin signaling [20]. This microRNA is preparing the background for Aux/IAA protein degradation by regulating the activity of SCF E3 Ubiquitin ligase. In current study the expression of miR160 is extremely decrease while the expression of ARF gene is increase. The possible reason for this expression pattern is providing an appropriate condition for keep on the growth of plants under salinity stress. This expression pattern show increasing in the length of lateral roots which make absorption of water more easily in these limitation conditions.

In sugarcane, NAC TFs and ARFs TFs have been validated as targets of miR164, miR160, respectively [21]. NAC TF family involved in response to abiotic stresses such as salinity and drought stresses [22]. The increasing in NAC expression can make plants to be tolerant to abiotic stresses like salinity and drought. The studies have shown that this TF can directly bind to promoter of genes are involved in salinity and drought stress and induce their expression [23]. The recent studies have proofed the interaction between this TF and miR164. This TF is part of plants signal transduction which can induce many physiological mechanisms under salinity stress. Under salinity stress the expression of miR164 is decrease while the expression of NAC is increased. This expression pattern has represented the effect of plant signaling network and ionic adjustment of homeostasis and finally stabilize of plant growth under salinity stress [24].

miR172 targeting AP2-like ethylene-responsive transcription factors [25] have been found with different expression in sugarcane. TFAP is involved in many cellular aspects such as controlling growth factor, development and apoptosis [26]. Key approaches to response abiotic stress, such as salinity is decrease growth and development and activate the apoptosis mechanism. In this study the expression of miR172 is decreased and the expression of TFAP is increased. This expression pattern is happening in order to limit the growth and development and the activate apoptosis mechanism. Phosphorylation is a mechanism which is phosphate can be added or remove by protein kinases and activate functional proteins [27].

Protein kinase genes (PLPRKs) are potential targets for miR390. Previous studies have shown that the expression of miR390 is decreased under salinity stress and its target has increased in expression [28]. In this study as previous studies the expression of miR390 decrease whiles the expression of PLPRK increase. This cause activation many protein kinases and proteins that is involved in salinity stress.

In plants there is many proteins cause sensitivity under certain conditions like abiotic stress. Tolerant plants have mechanisms that inducing degradation of these kind of proteins. EBF gene is one of the genes that involves in proteins degradations. This gene is potentially target of miR393 [29].

In this study the expression of miR393 strongly induced by NaCl treatment, while EB-Fbox gene has increased in expression under this stress. It can say that this increasing in EB-Fbox is for degrading the proteins that cause sensitivity in sugarcane cultivars under salinity stress.

Many of studies generally have shown that under salinity stress, absorb of micro element, such as Copper ion in plants is increased. Since the presence of Copper ion is poisonous for the plants cell, this ion is extremely controlled by molecular mechanisms. BBP gene (Basic Blue Protein) is part of this molecular mechanism that involved in Copper ion control. This gene has an important enzymatic role that especially controls the level of Copper ion under salinity stress. On other hand, when plants are under abiotic stress like salinity stress, the oxidative stress is induced. In this condition, enzyme like superoxide dismutase is active and control the oxidative stress. The activation of SOD enzyme is regulated by BBP gene [29].

miR408 targets the mRNAs of the BBP gene and Laccase (LAC). The results had shown that expression of miR408 under salinity stress is decreased and the expression of BBP gene is increased. Squamosa Binding Protein (SBP) has roles in plant leaf development, vegetative to reproductive phase transition, fruit development and gibberellin signal transduction. When plants are encounter a harsh environment such as abiotic stress in their life cycle, they are accelerating the development of leaves and vegetative to reproductive transition. This event is controlled by molecular mechanisms and changing in genes expression like SBP gene. The expression of SBP gene is regulated by miR529 [30].

We also found opposite expression patterns of these three miRNAs (miR529, 827 and 1432) and their target gene (SPL9, SDP and CBP).

Under salinity stress, nutrition of plants is disrupted and causing extreme damage to plant growth. The phosphorus is very important for plant growth and salinity stress its absorption is disrupted. The plant has developed many physiological and molecular mechanisms to absorb P ion under salinity stress. One mechanism is activated SPX-domain containing protein (SDP) under this condition. This protein is involved in the adjustment of P homeostasis and plants by activating these proteins in salinity stress confront with P starvation [31]. It has shown that this gene is regulated by miR827 in plants. The result of this study showed decreasing in miR827 expression and increasing SDP expression under salinity stress, which they match with previous studies in other plants.

Plants are sessile and when they confront with the stresses, they make changes within their cells and organelles. Identification of stresses and response to them is regulated many signal pathways. In this pathway there is many proteins are involved. One known protein that play role in signal transduction under stresses is CBP (Calcium Binding Protein). MiR1432 is targeting this protein in plants. Under stress the cytosol filled by Ca^{2+} and then CBP bind to Ca^{2+} and provides conditions to trigger many signal pathways involving in regulating and responding to stresses such as salinity stress [32]. MiR1432 by decreasing its expression and increasing CBP expression, play its role in salinity response. In this study expression of miR1432 decrease and CBP expression is increased.

Conclusion

The results have shown a significant difference between nine miRNAs expression and their targets gens under salinity stress compared to control conditions. The results also have shown a significant difference between three cultivars under salinity stress compared to control conditions. All miRNAs are down regulated under salinity stress while all target genes are up-regulated. The present results obtained from this study may be used for manipulating the pathways that are related to salinity tolerance and generating tolerant plants for environments with saline soils.

Declarations

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Author contributions Writing–original draft and investigation: Tofigh Mazalmazraei; Supervision and project administration: Leila Nejhadsadeghi; Data curation and formal analysis: Khosro Mehdikhanlou; Validation: Daryoosh Nabati Ahmadi

Consent to Participate Authors declare no conflicts of interest.

Consent to Publish this article does not contain any studies with human or animal subjects.

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Figures

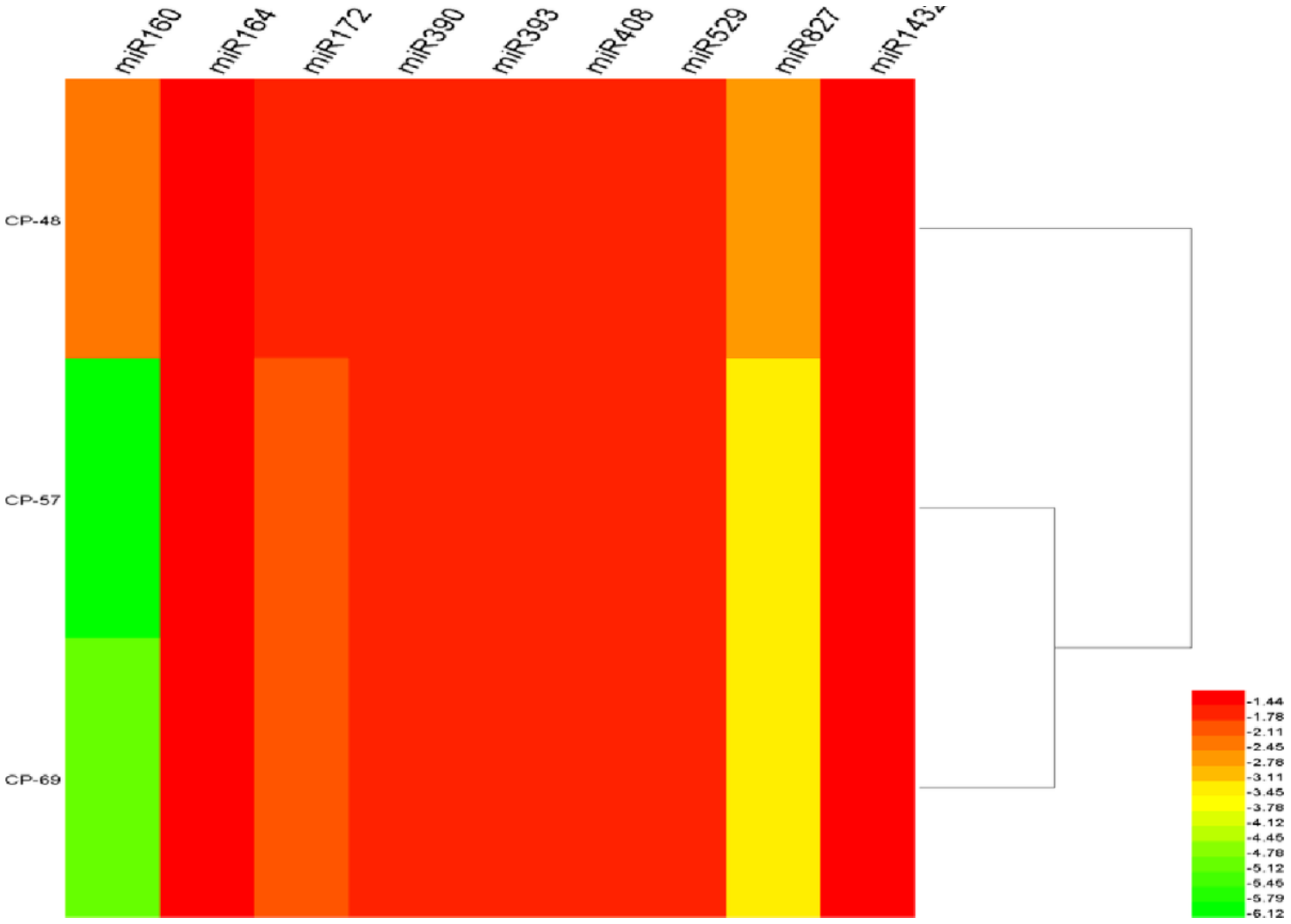

Figure 1

A heatmap of expression values for the nine miRNAs of three cultivars of S. officinarum validated by qRT-PCR in the present study.

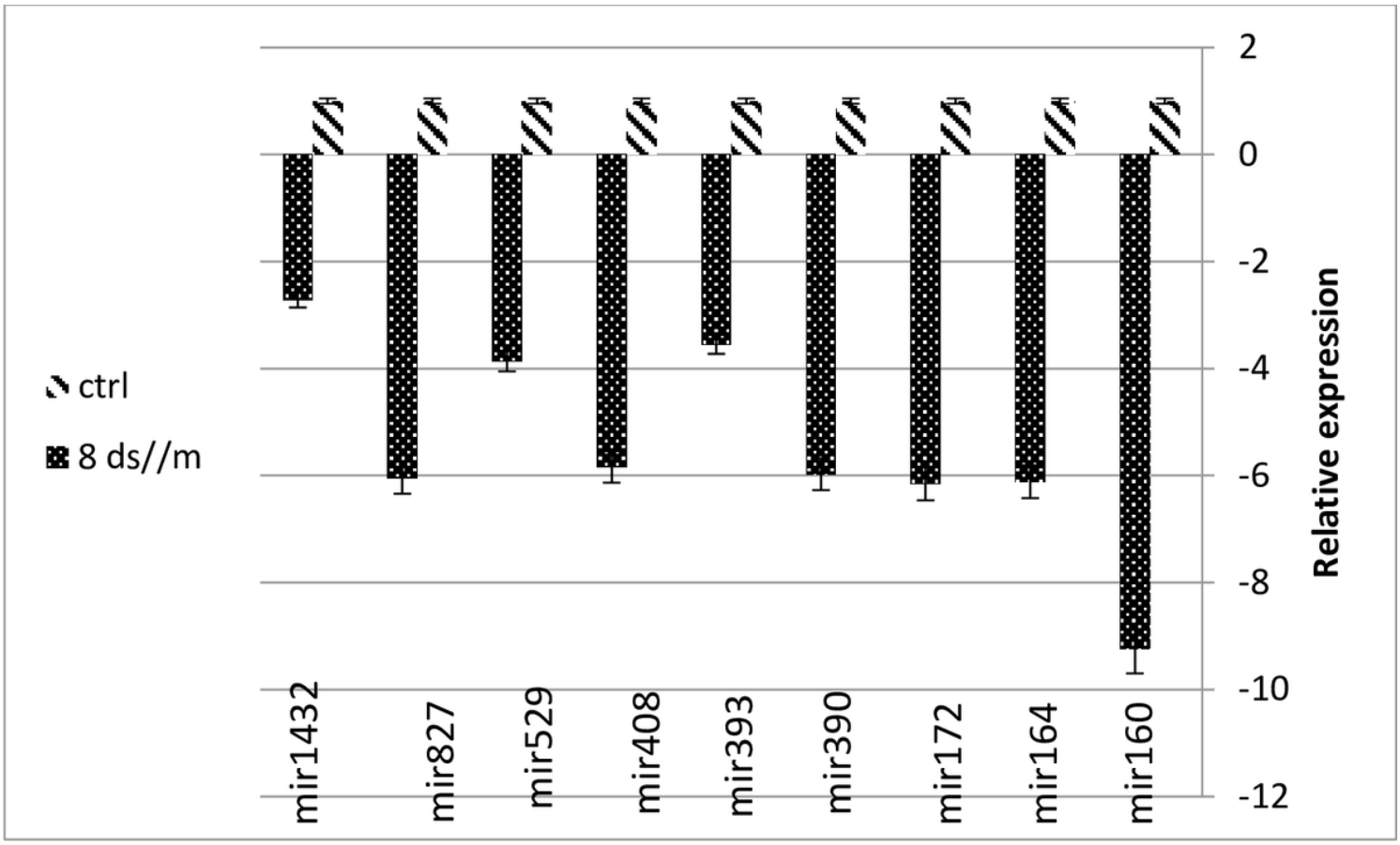

Figure 2

Relative expression of nine miRNAs under salinity stress compares to control conditions.

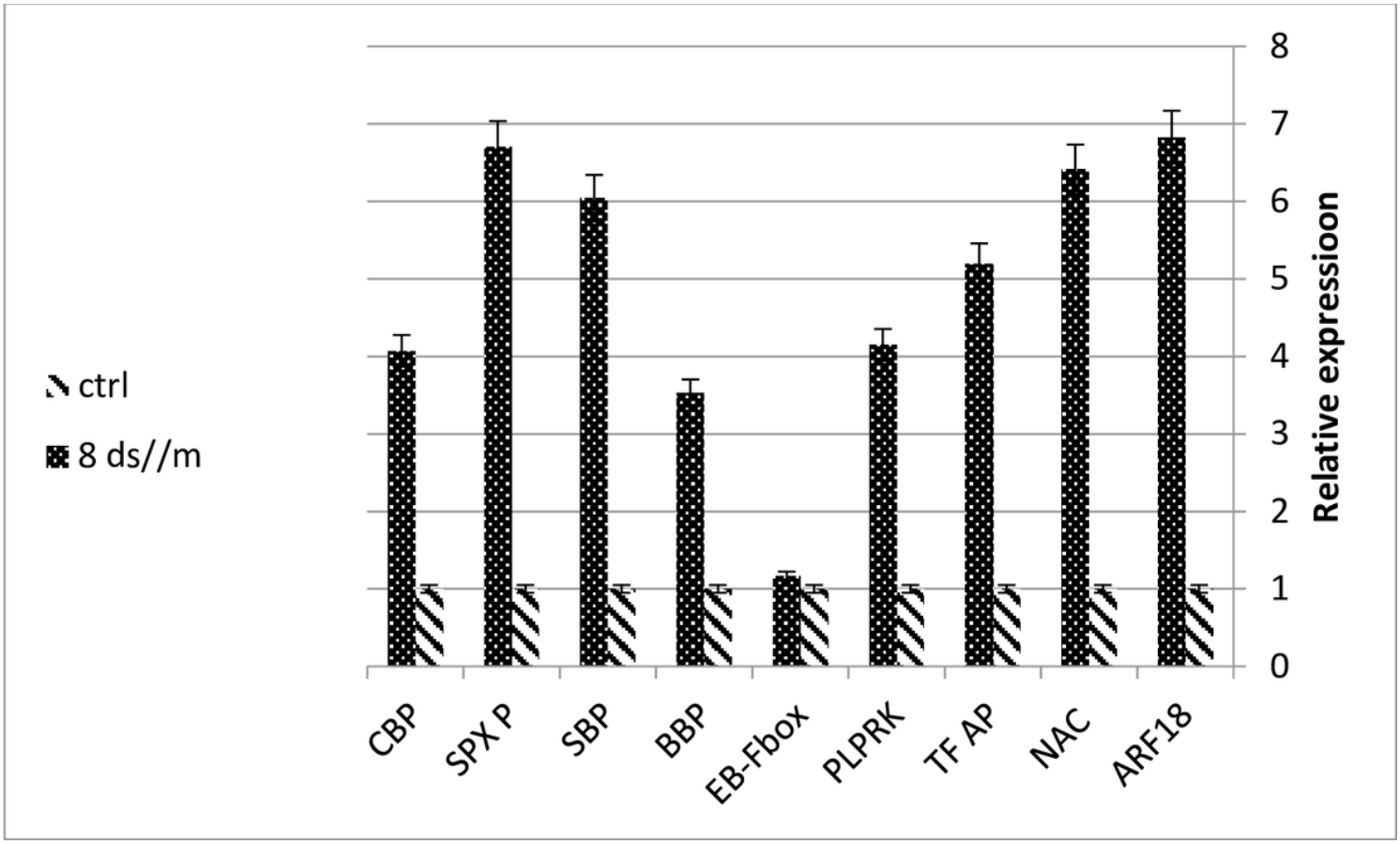

Figure 3

Relative expression of nine target genes under salinity stress compares to control conditions.

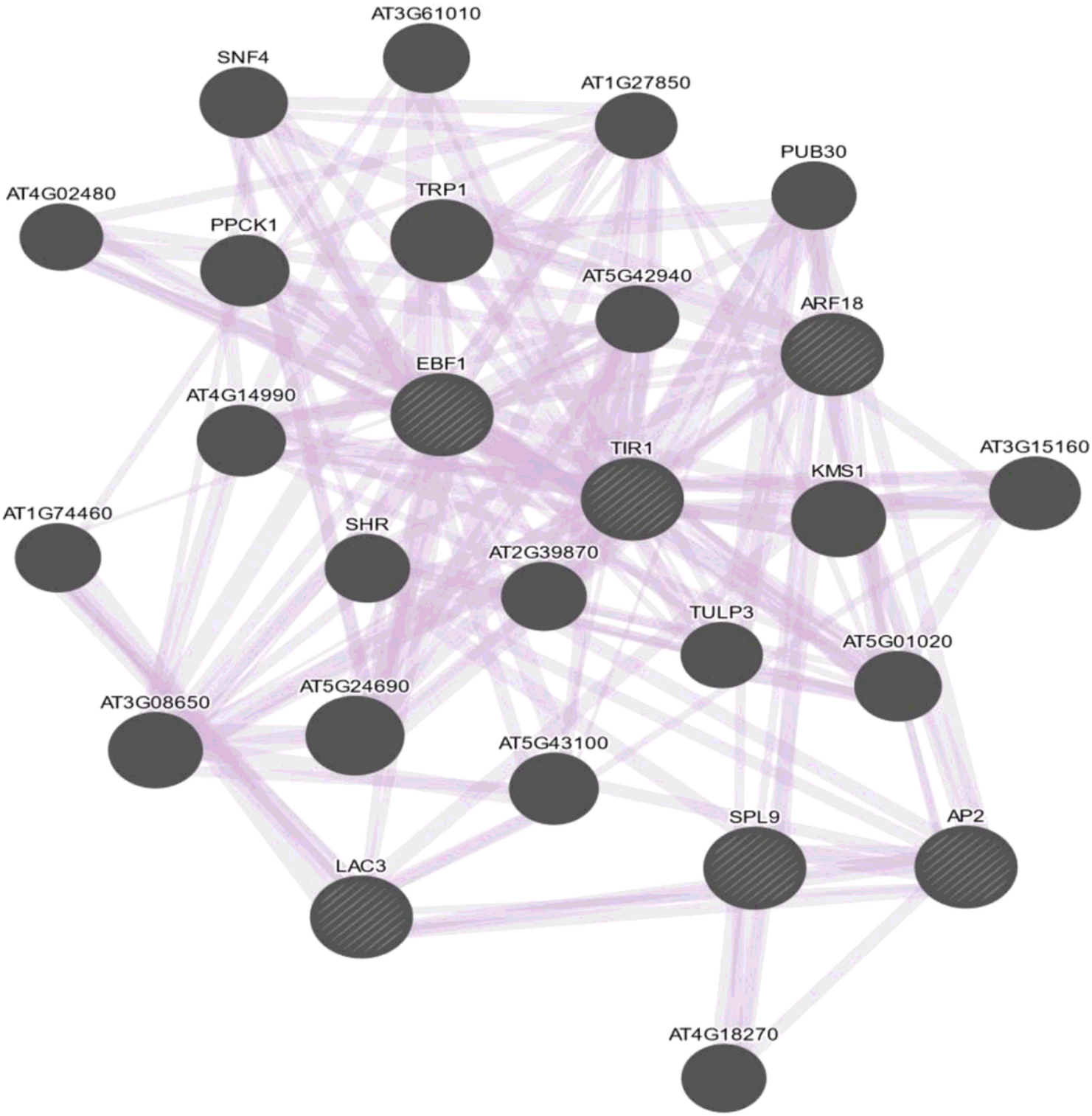

Figure 4

Co-expression network for the ARF17, NAC080, EBF1, AP2, LAC3 and SPL9 targets.

Supplementary Files

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