Comparative analysis of differentially expressed miRNAs in leaves of three sugarcanes (*Saacharum officinarum* L.) cultivars during salinity stress

Tofigh Mazalmazraei1 · Leila Nejadsadeghi1 · Khosro Mehdi Khanlou1 · Daryoosh Nabati Ahmadi1

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Abstract

Background  Sugarcane is an important industrial plant cultivated mostly in the arid and semi-arid regions. Due to climate change and anthropogenic activities, the sugarcane fields are prone to be damaged as a result of salt deposition. The consequence of such phenomena is turning to become a major thread in sugarcane cultivation. To address this issue, the identification of salinity tolerant cultivars would be a suitable strategy to minimize yield loss in the area. It is well known that the expression of abiotic stress-responsive genes including noncoding microRNAs (miRNAs) and their coding targets could lead to enhancement of stress tolerance in crops. Therefore, the expression study of those noncoding and coding genes under stress conditions is an appropriate approach to screen the tolerant cultivars. In addition, the examination of the expression of miRNA’s target genes could provide deeper insight into the molecular stress mechanism and facilitate the identification of tolerant cultivars.

Methods and results  We aimed to assess the expression of nine candidate miRNAs and their corresponding targeted genes among the studied sugarcane cultivars under high salinity conditions, leading to the identification of 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. The result indicated significant differences in expression with miRNAs and also their target genes. The highest reduction of miRNAs expression occurred in miR160 while the lowest one appeared in miR1432. The data also indicated that the higher and the lowest expression of targeted genes occurred in miR160 and miR393 respectively. Among studied cultivars, the CP-57 showed poor performance while CP-69 expresses a superior tolerance to salt stress.

Conclusions  Taken together, these results suggested that the monitoring of microRNA expression could provide a new approach for the screening of well-adapted cultivars under salt conditions. Such an approach would be the appropriate solution to combat plant stress in high salinity regions/soil. Our result indicated that the miR160 generates sugarcane tolerant to salt stress, can be potentially be used as a biomarker to salt stress.

Introduction

Sugarcane (*Saacharum* sp.) is an important industrial crop. It is considered the main source of sugar production in the worldwide and an 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 the southern part of Iran and the Karun river as the main source of water that irrigated the 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 faulty water management, the Karun river has been heavily contaminated with
untreated sewage which adds huge quantity of salt ions. Nowadays, because of all the above-mentioned problems, salinity is an ever-increasing in the sugarcane field resulting in a reduction of sugarcane production in respect of both dry matter and sucrose content in the region. The desalination of farming soils required a significant amount of time, labor and energy inputs which might create serious economic and social damage in the region [3]. It is known from extensive studies that there is ample variation for tolerance to salinity among cultivars of the same species [4]. Therefore, the development of high salinity tolerant cultivars is an efficient way to tackle the salinity problems in such regions. Such salt-tolerant plants are capable of changing morphological, physiological, biochemical and anatomical mechanisms, in order to adapt to a high salinity environment [5].

Recent studies have indicated that many genes are 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 genes network, which consists of 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 keeping plant cell homeostasis during and after stress [6]. 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 function in RNA silencing and post-transcriptional regulation of gene expression [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 to respond to environmental changes [9]. Notably, several miRNAs have been reported to have higher expression rates in the samples treated with salt treatment. In sugarcane miR166III, 168II, 396II, 398II, 528I, 156 V, 167 V, 169III, 397II, 398I, and 159XVI have been found with different expression in response to moderate salt stress [10]. The most of identified mRNA, targeted by the miRNAs, 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 expression profile of candidate miRNAs in the studied sugarcane cultivars under high salinity conditions and also to recognize the best miRNAs for screening of salt-tolerant cultivar in sugarcane.

Materials and methods

Plant materials, growth conditions, and salt stress treatments

Three Iranian commercial cultivars of Saccharum 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 Sugarcane and By-products Development Company (Khuzestan, Ahvaz, Iran). The seedswere cultivated at the Experimental Research Station of College of Agriculture, Shahid Chamran University of Ahwaz, Iran, in 50 × 50 m² pots with 2/3 soil with low EC and 1/3 sand in the glasshouse. A two-factorial experiment with three sugarcane cultivars (CP-48, CP-57, CP-69) and two salinity levels (0 and 8 ds/m) based on completed block design with three replications was conducted. Total Dissolved Solid (mg/L) ≈ Electrical Conductivity (EC) (deciSiemens / meter) x 800 was applied to calculate the amount of NaCl required to reach desired EC. An amount of 6.4 g NaCl (Sigma-Aldrich, USA) was dissolved in a liter of distilled water. Six true leaf seedlings were subjected to salt stress by adding NaCl solution gradually to achieve final EC value of 8ds/m. After 24 h treatment, leaves with or without NaCl treatment were collected from CP-48, CP-57, CP-69 seedlings and stored at -80 ℃ until RNA extraction.

RNA extraction

Total RNA was extracted from the leaves of the three varieties of sugarcane (two independent biological replicates) using RNasy Plus Mini kit (Qiagen, Germany), followed by DNase (Pars tous Company, Iran) treatment to remove the genomic DNA. RNA concentration was quantified using Nano Drop equipment (Nano Drop Technologies Inc., Wilmington, DE) and the quality was examined using 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 nine miRNAs (miR160, miR164, miR172, miR390, miR393, miR408, miR529, miR827, miR1432) was determined using the SYBR Green PCR Master Mix. 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 °C for 10 min, then 32 cycles of 95 °C for 15 s, 58 to 60 °C for 30 s and 72 °C for 30 s. To validate the absence of primer dimer, the melting curve analysis was added in the range of 60 to 95 °C, after the amplification step. The designed primers were 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 (http://www.premierbiosoft.com/primerdesign/) and listed in Table S3. Reactions were performed at 95 °C for 10 min, then 32 cycles of 95 °C for 15 s, 55 to 60 °C for 30 s and 72 °C for 30 s. GAPDH was used as a reference gene for normalizing the target gene expression. The data of qRT-PCR was calculated using \(2^{-\Delta\Delta CT}\) method. qPCR was carried out by using an AB Step One Plus real-time PCR thermal cycler machine and the obtained data were analyzed using the associated AB Step One Plus Software v2.3 (Applied Biosystems, Carlsbad, California, USA).

Experimental designs and statistical analysis

The experiment was conducted as a 3×2 factorial design using 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).

Result

The results showed that the transcripts of the nine miRNAs and their target genes in the salt stress were significantly dependent on genotype (Pvalue < 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. (Pvalue < 0.01) (Fig. 1). According
to previous reports, miR160 is involved in the auxin response by targeting auxin response factors (ARF) genes [15]. In this study, the greatest degree of down-regulation (9-fold change) in response to salt stress was shown by miR160 in CP-57. In addition, the miR164 and miR1432 that encode transcription factors and transporters were 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 the expression levels of target genes of miRNAs levels varied in sugarcane during salt stress treatments. Among the targets, the lowest expression was shown by the miR393 target gene EB_F box. The 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 ARF18 showed a significant upregulation (7-fold change) following three sugarcane cultivars. However, the increase in the miR160 target gene of CP-48 was lower than the expression level of the CP-69 cultivar (Fig. 3). It should be mentioned here that, plus, the above-mentioned target genes, the evaluated miRNAs can regulate several target genes and are involved in various biological processes. In this study, the most acknowledged target genes of studied miRNAs including ARF17, NAC080, AP2, EBF1, LAC3 and SPL9 genes were selected for co-expression network analysis (Fig. 4). In addition, a co-expression network of NAC080 with LAC3 and SPL9 genes was generated (Figure S1 and Table S5).
of miRNAs showed significant difference profiles depending on the cultivar [18].

The miR390 targeting ARF transcription factors [19] has been found with different expressions in sugarcane. ARF is one of the targeted TFs involved in rooting, responding to drought and salinity stress, plant development, 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 the current study, the expression of miR160 is extremely decreased while the expression of the ARF gene is increased. 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 increase 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]. It is now clear that the NAC TF family involves in response to abiotic stresses including salinity and drought stresses [22]. The increase in NAC TF expression significantly contributes

Discussion

High salinity is an increasingly important agricultural problem. The metabolism of plants is 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 that miR160, miR164, miR172, miR390, miR393, miR408, miR529, miR827, and 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 indicated that there were significant differences in the 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].

Fig. 4 Co-expression network for the ARF17, NAC080, EBF1, AP2, LAC3 and SPL9 gene targets response to salinity treatment. 21 genes with the highest weight are in dark purple.
to tolerance towards abiotic stresses such as salinity and drought. The studies have shown that the NAC TF family is part of plants signal transduction which can induce many physiological mechanisms under stress. The TF can directly bind to promoters of genes that are involved in salinity and drought stress and induce their expressions [23]. Recent studies have proved the interaction between NACTF and miR164. Under salinity stress the expression of miR164 is decreased while the expression of NAC is increased. This expression pattern has represented the effect of plant signaling network and ionic adjustment of homeostasis leading to stabilize of plant growth under salinity stress [24].

The miR172 targeting AP2-like ethylene-responsive transcription factors [25] have been found with different expressions in sugarcane. TFAP is involved in many cellular aspects such as controlling growth factors, development and apoptosis [26]. Key approaches to respond to abiotic stress, such as salinity are to 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.

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 [27]. In this study, as in previous studies, the expression of miR390 decreased while the expression of PLPRK increased. This cause activation of many protein kinases and proteins that are involved in salinity stress.

In plants, there are many proteins that cause sensitivity under certain conditions like abiotic stress. Tolerant plants have mechanisms that induce the degradation of these kinds of proteins. EBF gene is one of the genes that involves proteins degradations. This gene is a potential target of miR393 [28]. In this study, the expression of miR393 strongly is induced by NaCl treatment, while EB-Fbox gene has increased expression under this stress. One can say that this increase in EB-Fbox is for degrading the proteins that cause sensitivity in sugarcane cultivars under salinity stress.

Many studies generally have shown that under salinity stress, absorb of micro element, such as Copper ions in plants is increased. Since the presence of Copper ions is poison for the plants’ cells, this ion is extremely controlled by molecular mechanisms. BBP gene (Basic Blue Protein) is part of this molecular mechanism that is involved in Copper ion control. This gene has an important enzymatic role that especially controls the level of Copper ions under salinity stress. On other hand, when plants are under abiotic stress like high salinity stress, oxidative stress is induced. In this condition, enzyme-like superoxide dismutase is active and control oxidative stress. The activation of the SOD enzyme is regulated by the BBP gene [28].

The 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 the BBP gene is increased. Squamosa Binding Protein (SBP) has roles in plant leaf development, vegetative to a reproductive phase transition, fruit development and gibberellin signal transduction. When plants 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 changes in genes expression like the SBP gene. The expression of the SBP gene is regulated by miR529 [29]. 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, the nutrition of plants is disrupted and cause extreme damage to plant growth. The phosphorus is very important for plant growth and when salinity stresses its absorption is disrupted. The plant has developed many physiological and molecular mechanisms to absorb P ions 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 [30]. 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 match with previous studies in other plants.

Plants are sessile and when they confront the stresses, they adapt to changes within their cells and organelles. Identification of stresses and responses to them is regulated by many signal pathways where numerous proteins are involved. One known protein that plays a role in signal transduction under stress is CBP (Calcium Binding Protein) where 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 involved in regulating and responding to stresses such as salinity stress [31]. MiR1432 by decreasing its expression and increasing CBP expression, play a role in salinity response. In this study, the expression of miR1432 is decreased while CBP expression is increased.

**Conclusions**

The results have shown significant differences between nine miRNAs expression and their targets genes under salinity stress compared to control conditions. The results
also indicated significant differences 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. However, due to the distinguishable expression level of miR160 across the studied varieties, the miR160 can be potentially be used for generating sugarcane tolerant to salt stress. 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.

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**Compliance with Ethical Standards**

**Conflict of interest** Authors declare no conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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