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ORIGINAL ARTICLE

# Sequencing and expression analysis of salt-responsive miRNAs and target genes in the halophyte smooth cordgrass (*Spartina alternifolia* Loisel)

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**Abstract** MicroRNAs have been shown to be involved in regulating plant's response to environmental stresses, including salinity. There is no report yet on the miRNA-mediated posttranscriptional regulation of salt stress response of a grass halophyte by miRNAs. Here we report on the deep-sequencing followed by expression validation through (s)qRT-PCR of a selected set of salt-responsive miRNAs and their targets of the salt marsh monocot halophyte smooth cordgrass (*Spartina alterniflora* Loisel). Expression kinetics study of 12 miRNAs showed differential up/down-regulation in leaf and root tissues under salinity. Induction of expression of six putative novel microRNAs with high read counts in the sequence library suggested that the halophyte grass may possess different/novel gene posttranscriptional regulation of its salinity adaptation. Similarly, expression analysis of target genes of four selected miRNAs showed temporal and spatial variation in the up/down-regulation of their transcript accumulation under salt stress. The expression levels of miRNAs and their respective targets were coherent, non-coherent, or semi-coherent type. Understanding the gene regulation mechanism(s) at the miRNA level will broaden our fundamental understanding of the biology of the salt stress tolerance of the halophyte and provide novel positive regulators of salt stress tolerance for downstream research.

**Keywords** MicroRNA · Halophyte · Salinity · *Spartina alterniflora* · Target gene

## Introduction

Salinity is one of the most important problems in arid and semi-arid areas causing crop losses worldwide. Salt-affected areas account for about 6 % of the world's agricultural land [1]. Salt stress has negative effect on plant growth and development due to water stress caused by reduced osmotic potential in the soil solution and ionic stress caused by excessive salt absorption by the cells [2, 3]. These effects result in inhibition of many physiological, biochemical and molecular processes, such as nutrient uptake and assimilation, and cell signaling pathway including those that lead to synthesis of osmotically active metabolites, specific proteins and certain free radical scavenging enzymes [4]. Salt stress tolerance in plants is a complex phenomenon that may involve developmental changes as well as physiological and biochemical processes [5, 6]. Glycophytes cannot tolerate high salt concentration and therefore cannot be grown on a salt affected land. Halophytes, on the other hand, can tolerate very high level of salinity. Halophyte plants represent only 2 % of terrestrial plant species, but they represent a wide diversity of plant forms [7]. Understanding the mechanisms of salinity adaptation in halophytes at the molecular, cellular and physiological levels will help us devise strategies to improve salt tolerance in crop plants through genetic modification and plant selection programs. Halophytes have been used as a source of genes for engineering salt tolerance in both dicots and monocots. Because of the differences in adaptation strategies of the monocot and dicot halophytes, plant scientists are on a look out for

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monocot halophytes to exploit their resources in plant breeding and biotechnology programs for improvement of important food crops, such as wheat, rice, and maize that are mostly grasses [8].

*Spartina alterniflora*, a perennial monocot deciduous grass, which is found in marsh areas with an environment that is characterized by high salinity, waterlogging and nutrient limitation, has long drawn interests among plant stress biologists, especially those investigating on salt stress tolerance [9–11]. *S. alterniflora* naturally exists along the Eastern and Gulf Coasts of the United States and acts as a natural coastal engineer providing protection from storm, capturing sediment, and filtering pollutants [12, 13]. *S. alterniflora*, an aneupolyploid with 2n = 62 and a genome size of 1.8 Gbp, shares 80–90 % similarity with rice at the DNA and protein levels [14]. It is known to possess all possible mechanisms, such as excretion of salt onto the leaf surface through salt glands, selective uptake of $K^+$ over $Na^+$ through exclusion mechanism, tissue compartmentalization of toxic ions, vacuolar sequestration of toxic $Na^+$, and protection of cells through production of compatible solutes and enzymes for scavenging free oxygen radicals to survive and grow under salt [15, 16]. The high level of salinity tolerance and similarity with rice makes this species an important source of genes for their utilization in improving salinity tolerance in rice.

Small-scale investigation at the molecular level has led to the identification of candidate genes that possibly play an important role in the salinity tolerance mechanism of *S. alterniflora* [14, 17]. Genetic engineering of plants, including *Arabidopsis*, tobacco and rice, with a few candidate genes showed that transgenic plants had enhanced tolerance to salt stress than the wild-type plants, mainly through adjustments in early stage and preparatory physiological responses, such as higher relative water content and closure of the leaf stoma and reduced stomata density, increased biosynthesis of antioxidative enzymes and osmolytes, and maintenance of photosynthesis under salt stress [18]. While regulation of gene expression under salinity has been studied in *S. alterniflora* [14, 17, 19], there is no report so far, to our knowledge, about the posttranscriptional regulation of its genes expression by miRNA under salt stress.

Mature microRNAs (miRNAs) are 18–22 nt long endogenous nucleotides that belong to the family of small noncoding RNAs, which mediate posttranscriptional gene regulation. The miRNAs interact with their target coding mRNAs to inhibit translation by degradation of target mRNA or blocking translation [20, 21]. While core components of the miRNA-target mRNA interaction are conserved between plants and animals, the interaction in plants occurs when the 5′ end of the miRNA has near-perfect pairing with the coding sequence (CDS) or untranslated regions (UTR) of target mRNAs [22, 23]. In animals, miRNAs take effect with target mRNA only if the 5′ end seed (6–8 nt) of the miRNA is complementary to the 3′ UTR site of the target mRNA [24–26]. Therefore, a miRNA might regulate several different mRNA targets, and conversely a target mRNA might be regulated by multiple miRNAs, especially in plants because of the more flexibility in the interaction between miRNA and their target genes [27, 28]. The miRNAs in plants are species specific but majority of them are highly evolutionarily conserved among many distantly related species [29]. Research evidences strongly suggest that miRNAs play an important role in the response of plants to environmental stresses, including salinity stress [30–36]. The expressions level of a number of miRNAs is changed in response to salt stress. Increased transcript abundance of miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR396, miR319, miR393, miR394, miR397 and miR169 was observed in *Arabidopsis thaliana* in response to salt stress [16, 37]. In *Populus trichocarpa*, miR530a, miR1445, miR1446a-e, miR1447 and miR171 l-n were down-regulated, whereas miR482.2 and miR1450 were increased in abundance under salinity [38]. Recent studies showed that miR156c, miR166i, miR167a and miR5300 showed a decrease in abundance while miR397a was up-regulated and miR403a showed no change in abundance in the root tissues of *Solanum linnaeanum* [39]. Microarray analysis revealed differential expression pattern of miRNAs in salt-sensitive and salt-tolerant lines of *Zea mays* [40]. Other studies found that miR393, miR394, miR396, miR169 g, miR169n, and miR156 were specifically responsive to salt stress in *A. thaliana*, *Zea mays*, *Populus tremula*, rice and soybean [37, 38, 40–44]. All these studies suggested that miRNAs play vital roles in plant's adaptation to salt stress, and different plant species or organs of a single species may cope with salt stress using different miRNA-mediated regulatory strategies. In the present study, we report the temporal and spatial expression profiles of miRNAs and their target genes in both leaf and root tissues of the halophyte *S. alternifolia* after salinity treatment.

## Materials and methods

### Plant materials and salt stress treatment

Young rhizomes containing small shoots of *S. alterniflora* were hand-split and grown for a month in sand-filled 4 inch pots kept in a plastic tub filled with Hoagland's nutrient solution. The pots with the plants were then transferred to fresh Hoagland solution with 500 mM sea salt, the level beyond which *S. alterniflora* has been reported to show

inhibition in its growth [17]. Leaf and root tissues were collected at 6, 12, 24, and 72 h after imposing salt stress in order to determine the early (6, 12 h)- as well as late (24, 72 h) stage salt response regulation of the miRNAs, and control plants were not subjected to salt stress.

## Preparation of small RNA library and sequencing

For deep sequencing, total RNA with small RNA was extracted using miRNeasy kit (Qiagen Inc, Valencia, CA). After isolation of small RNA by denaturing PAGE gel, small RNA library was prepared following Illumina's sample preparation instructions. Samples from different time points were barcoded, and before sequencing equal amounts of the barcoded samples were mixed as described below.

### *Small RNA isolation by denaturing PAGE gel*

For each sample, 10 µg of RNA were size-fractionated on a 15 % tris–borate-EDTA (TBE) urea polyacrylamide gel, and the gel slice at 15–50 nt fraction was excised. Small RNA fraction was eluted from the polyacrylamide gel slice by ethanol precipitation in 500 µl of 0.3 M NaCl.

### *Adapter ligation to the isolated small RNA*

Following Illumina/Solexa's manual, the 5′ and 3′ RNA adapters were ligated to the precipitated RNA with T4 RNA ligase (Promega, Madison, WI). Ligated RNA was size fractionated on a 15 % TBE urea polyacrylamide gel and 65–100 nt fraction was excised and eluted from the gel.

### *Reverse transcription and PCR-amplification*

The small RNA was converted to single-stranded cDNA using M-MLV (Invitrogen, Carlsbad, CA) with the Illumina/Solexa's RT-primer. The cDNA was PCR amplified with Platinum® pfx DNA polymerase (Invitrogen) in 20 cycles using Illumina/Solexa's small RNA primers set.

### *Purification of amplified cDNA and sequencing*

PCR products were purified on a 12 % TBE polyacrylamide gel and gel slice at 80–120 nt fraction was excised and eluted. The purified PCR products were quantified on a TBS-380 mini-fluorometer (Promega, Madison, WI) using Quant-iT™ Picogreen® dsDNA reagent (Invitrogen, Carlsbad, CA) and diluted to 10 nM. 10 µl of barcoded samples at each time points were mixed and sequenced in a single lane on the Illumina/Solexa GAII sequencing platform (LC Sciences, Houston, TX).

## Sequence data analysis

Raw sequence reads were cleaned initially by filtering out adaptor-only sequences, sequences less than 15 nt long and with more than two Ns. Low copy filtering was performed to filter out sequences with frequencies less than three. Data analysis was carried out using an in-house Perl-based pipeline [19]. The filtered sequences were blasted against mRNA, RFam & repbase, and any sequence with hits (0 or 1 mismatch) was removed from subsequent analysis. Only hit sequences between 19 and 24 nt were considered as putative miRNAs. The sequencing data is provided in the supplemental file (Suppl 1).

The cleaned unique sequences were blasted against available miRNA sequences in miRbase (www.miRBase.org; release 21) and PMRD database (http://bioinformatics.cau.edu.cn/PMRD) using a matching criteria of at least 18 nt in length with more than 90 % identity. BLAST 2.2.14 and customized tools were used to parse the blast output data. The number of small RNAs per miRNA family and the frequency of each putative miRNA were estimated in the library.

## Expression profiling of miRNAs and targets

Small RNA was extracted from the leaf and root tissues at different time points (same as that was used for small RNA library preparation) using the miRNesay kit as described earlier. PCR ready cDNA was synthesized using the QuantimiR RT kit (SBI, Mountain view, CA). Poly A was added to the 3′ end of all miRNAs by poly-A polymerase followed by cDNA synthesis using a poly-T primer, which was linked to a special adaptor. The cDNA was diluted 10 times with nuclease-free water and two µl of the diluted cDNA was used for (semi)quantitative reverse transcription PCR [(s)qRT-PCR)] with the miRNA-specific primer and the adaptor-specific universal reverse primer (Table 1) in a total volume of 25 µl as per the manual of QuantimiR RT kit. Twelve miRNAs (six matching to known miRNAs and six putative novel microRNAs) with high copy numbers under salinity were selected for expression analysis (Table 1).

Putative target genes with a matching score of 0.5 for four miRNAs (Table 2) were identified from the PMRD database and primers were designed using the primer 3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Total RNA was extracted from the tissue samples (same as mentioned for miRNA) by RNesay plant minikit (Qiagen, Valencia, CA) and cDNA synthesis carried out by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as per its instructions. The (s)qRT-PCR was performed for each target gene following the method described earlier [14]. U6 and GAPDH3 (Glyceraldehyde-3-

**Table 1** Selected microRNAs of *Spartina alterniflora* used for analysis of their expression under salinity stress

| MicroRNA | # copies in the library | Primer sequence (5′–3′) |
|---|---|---|
| Sal-miR397a | 2111 | TCATTCAGTGCAGCGTTGATG |
| Sal-miR397b | – | TTATTGAGTGCAGCGTTGATG |
| Sal-miR528 | 4184 | TGGAAGGGGCATGCAGAGGAG |
| Sal-miR740 | 362 | GGTGTCGTGGTGTAGTTGGTT |
| Sal-miR827 | 328 | GGTGGCTGTAGTTTAGTGGTG |
| Sal-miR990 | 286 | TTCTTGACCTTGCAAGACTTT |
| *Sal-miR1788** | 175 | TCTTGATCTTGTAGATGATGA |
| *Sal-miR2084* | 156 | AACGGGACGCCGTAGAGGGTG |
| *Sal-miR2585* | 131 | CTCTGTGGAACCGCATGCCCT |
| *Sal-miR2786* | 144 | AGCTGGTTAGGATACTCGGCT |
| *Sal-miR3389* | 104 | AGGAGGTTGGCTTAGAAGCAG |
| *Sal-miR3426* | 103 | AGGAGACGGAGGATTGTCCCG |
| U6_F | | TCGGGGACATCCGATAAAATTG |
| Universal reverse primer | – | TTGGACCATTTCTCGATTTGTGC |

* The miRNAs in italics did not show significant match (≥15 nt) with known miRNAs

**Table 2** Target genes of selected microRNAs (in parenthesis) used for expression analysis in *Spartina alterniflroa* under salt stress

| Target gene ID (miRNA) | Annotation | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|
| Os01g62490.1 (Sal-miR397) | L-ascorbate oxidase | CTGTCGCCGTTCAACTACAC | AAGAAGTTGAAGCCGTGCAG |
| Os05g38390 (Sal-miR397) | Laccase 11 | CGAAGCCAGAGAGACAAAGC | TCGATGTCAAGTGAGCCCTT |
| Os11g48060.1 (Sal-miR397) | Laccase 22 | GAAAGGGGTCGGGAACTACA | TGGGTGGTGGAATCAGAGTC |
| Os01g09740 (Sal-miR528) | F-box/LRR repeat protein | CGGCTACAACTTCTTCGTCG | CTCAAGGTGGCAATGCAAGA |
| Os07g38290.1 (Sal-miR528) | Copper ion binding protein | TTGGGAACTAAACTCGGCCT | TCGCAGTTCTTGTAGTCCGA |
| Os08g04310.1 (Sal-miR528) | Uclacyanin | CAACAACATCTCCGCCTTCC | ACAAGAGCACGAGGACTAGG |
| Os01g48060.1 (Sal-miR990) | Auxin response factor 3 | CCAATGTGGCATGGTTCGAA | GGAAGCATGATTTCAGCCGT |
| Os05g43920 (miR990) | Auxin response factor 14 | TACCACCAAGAAGGGTCAGG | ACCACCAGCAGAAAAACCAC |
| Os05g48870.6 (Sal-miR990) | Auxin response factor 15 | GAAGTCTCACTCCCCTGGTC | CAATCACCCTGCCAACAACA |
| Os06g49660.1 (Sal-miR2585) | Axadien 5-apha O-acetyl transferase | CTGGTGAAGAAGGCCAAGTC | GAAGTCGACGCTCTTGAACC |
| SaGA3PDH | Glyceraldehyde 3-phosphate dehydrogenase | GAAGGGCGGTGCTAAGAAAG | CATTGAAGGGCCATCGACAG |

phosphate dehydrogenase) were used as internal reference genes for miRNA and target genes, respectively. The qRT-PCR was conducted with RNA from three independent plants per treatment (biological replicates) and two technical replicates.

## Results

### Small RNA library sequence analysis

Out of 186,757 filtered small RNA sequences (18–24 nt) generated through deep sequencing of *S. alterniflora* small RNA library, 902 matched to known miRNAs unique to other plants species. 113,214 and 69,191 small RNAs had higher and lower read counts under salinity compared to the non-stress control and the rest did not show any difference. All matched miRNAs belonged to 32 families, and miR156 was the most frequent in the library. There were 129 and 103 miRNAs that showed 100 % sequence identity with miRNAs of other plants with an alignment length of 21 and 20 nt, respectively. Sequences 21 nt long were the most dominant among the reads. A total of 120,983 sequences (>18 nt) showed no hit, which could be considered putative novel candidate microRNAs that need further analysis for validation. Targets identified from the EST/transcriptome database of *S. alterniflora* [14, 19] annotated as transcription factors, genes of metabolic pathways and others that have been reported to have implications in salt tolerance mechanism in addition to those involved in plant development and flowering, and hypothetical genes or genes with unknown functions.

### Expression of known miRNAs and putative novel microRNAs under salt stress

For all 12 miRNAs studied, sqRT-PCR (Fig. 1) and qRT-PCR results (Fig. 2) were in agreement with each other. Melt curve analysis showed multiple amplicon peaks for Sal-miR990 and hence its qRT-PCR was not included in analysis (Fig. 2). RT-PCR results showed differential expression pattern of the miRNAs over time and tissue. The expression pattern of two members of miR397 (Sal-miR397a and Sal-miR397b) was roller-coaster type in the leaf tissue. Sal-miR397a was up-regulated at 6, 12 and 24 h of stress with slight reduction at 12 and 24 h, but the level was still higher compared to the control, while the expression was drastically reduced at 72 h in the root. On the other hand, the expression of Sal-miR397b was down-

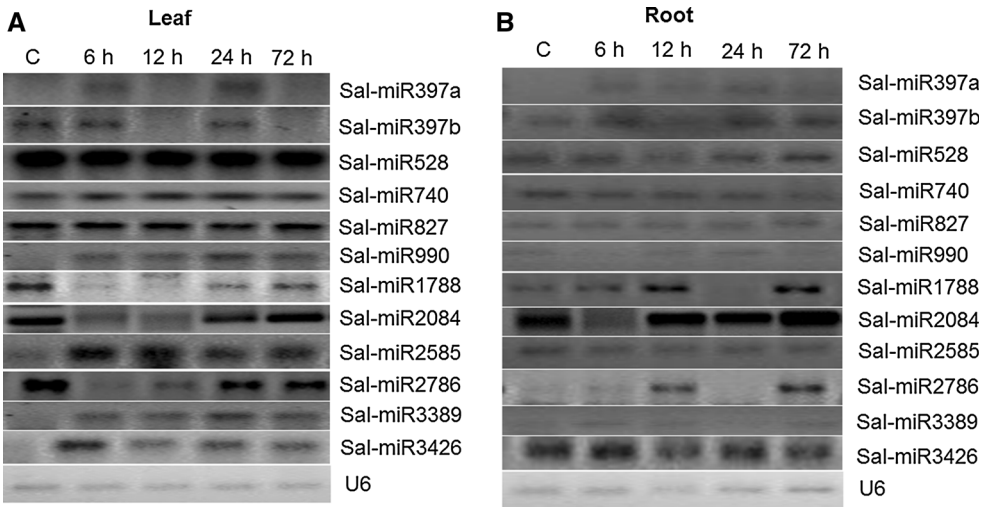


**Fig. 1** Semiquantitative RT-PCR analysis showing expression of miRNAs of *Spartina alterniflora* at different time points in leaf (**a**) and root (**b**) under salinity stress

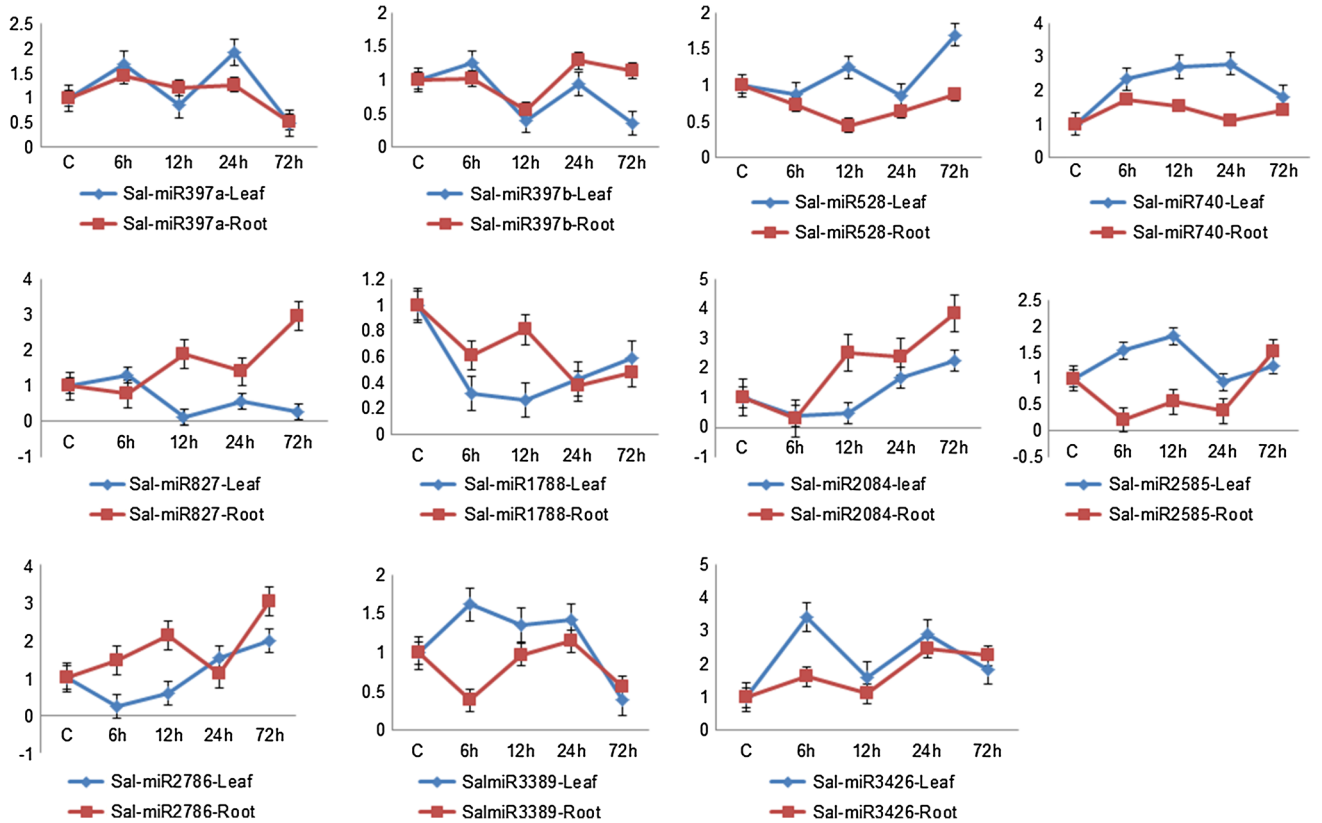


**Fig. 2** Quantitative RT-PCR analysis showing expression of miRNAs of *Spartina alterniflora* at different time points in leaf and root tissues under salinity stress

regulated at 6 and 12 h, but up-regulated subsequently compared to the control. In the leaf, Sal-miR397a showed up-regulation at 6 and 24 h and down-regulation at 12 and 72 h, whereas Sal-miR397b showed down-regulation after an initial up-regulation at 6 h after salt stress. The highest peak was observed at 24 h (leaf) and 6 h (root) for Sal-miR397a, while it was 6 h (leaf) and 24 h (root) for Sal-miR397b.

Sal-miR528 expression was very high in the leaf tissue compared to root. Its expression was highly up-regulated at 12 and 72 h of salt stress, each preceding a slight down-regulation at 6 and 24 h. However, in the root tissue, Sal-miR528 showed down-regulation at all the time points although the expression level showed an increasing trend at 72 h of salt stress. Sal-miR740 showed up-regulation in both leaf and root tissues at all the time points compared with the control. However, at 72 h of stress, the expression of the miRNA showed a slight reduction in the leaf, while in root, its expression was on the rise after a slight decline at 12 and 24 h.

The miRNAs, Sal-miR827 and Sal-miR2084 showed similar trend in their expression pattern under salinity stress in the root tissue. Their expression was down-regulated at an early stage, i.e., at 6 h of salt stress and was up-regulated at subsequent time points compared to the control with their highest expression at 72 h of salt stress. The trend was, however, different in the leaf tissue. Sal-miR827 was down-regulated at all the time points with a slight up-regulation at the early stage (6 h) salt stress, whereas, Sal-miR2084 was up-regulated at the later stage of stress (12 and 24 h) following their down-regulation at both 6 and 12 h of stress. Except at 6 h in the root tissue, Sal-miR2084 and Sal-miR2786 showed similar expression patterns under salt stress. Their expression was down-regulated at 6 and 12 h but subsequently up-regulated in the leaf tissue. In the root tissue, Sal-miR2084 showed down-regulation at 6 h and up-regulation at all other time points, whereas Sal-miR2786 showed consistent up-regulation with a slight down-regulation at 24 h.

The expression of Sal-miR1788 was significantly down-regulated under salinity in both leaf and root tissues. Although there was a slight increase in its expression at 12 and 72 h in the leaf and at 72 h in the root, the expression level was still lower as compared to the control. Sal-miR2585 was up-regulated in the early stage salt stress (6 and 12 h) after which the level declined sharply to near-basal level at 24 h and then was again up-regulated at 72 h in comparison to the control in the leaf tissue. On the other hand, its expression in the root tissue was up-regulated only at 72 h and down-regulated otherwise.

Sal-miR3389 showed consistently low expression in the root tissue where the expression was completely shut down at 6 h of salt stress and returned to basal level subsequently. However, in the leaf tissue, its expression was up-regulated with temporal variation where it was significantly down-regulated to almost undetectable level at 72 h of salt stress. Sal-miR3426 expression was somewhat similar to Sal-miR740. Its expression was of similar trend in both leaf and root tissue although the highest expression for leaf was at 6 h and for root it was at 24 h after exposure to salinity stress. Although there was a decrease in their expression at 12 and 72 h, the levels were still higher than the control.

## Expression of target genes under salt stress

Ten target mRNAs for five microRNAs [Sal-miR397a, b (3), Sal-miR528 (3), Sal-miR990 (3), Sal-miR2585 (1)] were identified from the PMRD database using both *S. alterniflora* (19) and rice gene models (http://rice.plantbiology.msu.edu; release 7) for their time-scale expression profile under salt stress. The results (Figs. 3, 4) showed that all of the putative targets of Sal-miR397 (Os01g62490, Os05g38390, and Os11g48060) were highly induced only at 24 and 72 h of salt stress in the leaf tissue, while their expression in the root was induced only after the plants were subjected to 72 h of salt stress. But, the candidate targets of Sal-miR528 behaved differently with their expression pattern. Os08g04310 was induced only at 12 and 24 h after salt stress in the leaf, while in the root it was induced only after 24 h of stress. Os01g09740 was highly accumulated at all stages upon imposition of salt stress with its peak of expression at 24 and 72 h. On the other hand, its expression was down-regulated at 6, 12 and 24 h, but was significantly up-regulated at 72 h of salt stress. However, the other probable target Os07g38290 of Sal-miR528 showed similar expression pattern in both leaf and root tissue where its mRNA level was significantly up-regulated at 24 and 72 h with its peak at 72 h of salt stress.

The targets of Sal-miR990 also showed variation in its expression over tissue and time points. The transcript of Os01g48060 was highly accumulated after 24 and 72 h in the leaf while it was induced only after 72 h of salt stress in the root tissue. The mRNA of Os05g48870 was induced to a detectable level only at 12 h of stress in the leaf. But in the root, its level increased at 24 and 72 h after its initial induction at 12 h after exposure to salt stress. Os05g43920 showed its induction only at 72 h of salt stress in leaf tissue, whereas its expression in root was induced at 24 h and then increased slightly at 72 h of salt stress. Melt curve analysis of Os05g43920 showed multiple amplicon peaks and hence its qRT-PCR was not included in the analysis (Fig. 4).

Os06g49660, the only putative target of Sal-miR2585, showed up-regulation of its transcript accumulation at all the time points under salinity with a slight down-regulation

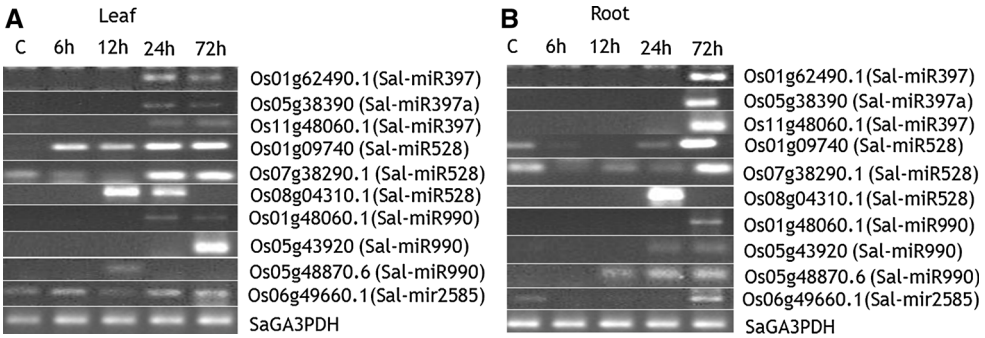


**Fig. 3** Semiquantitative RT-PCR analysis showing expression of miRNA targets of *Spartina alterniflora* at different time points in leaf (**a**) and root (**b**) under salinity stress

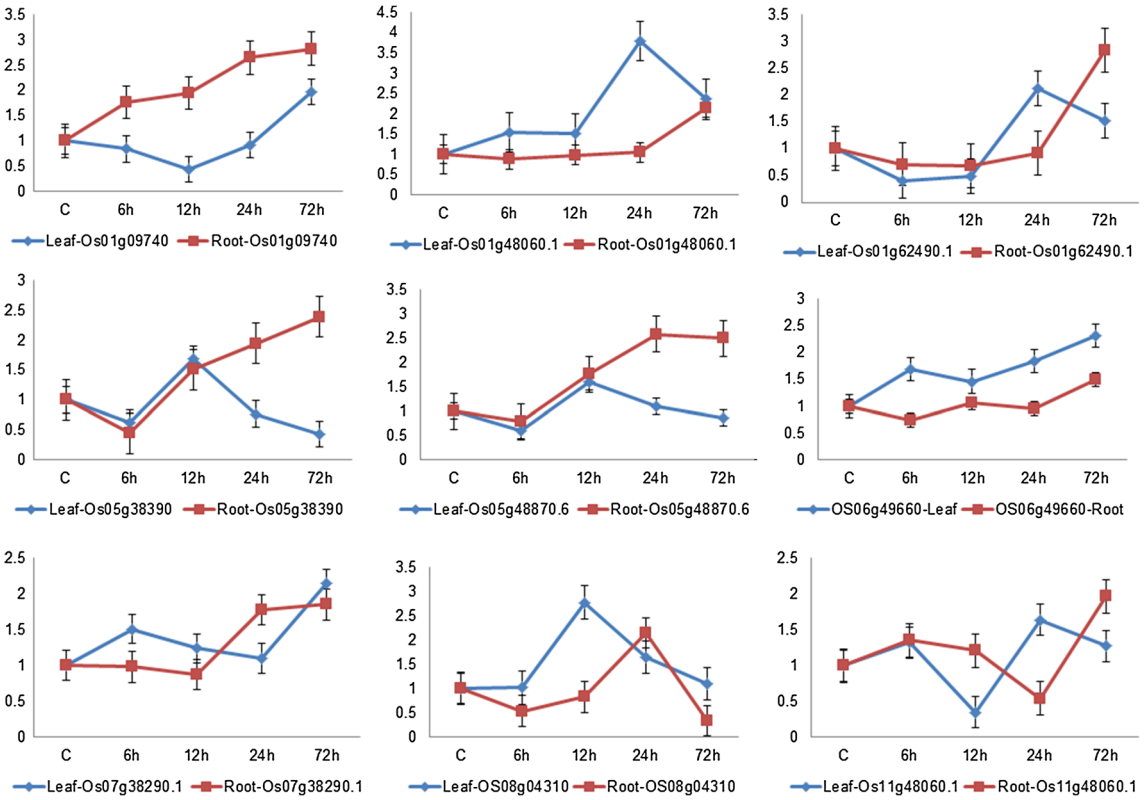


**Fig. 4** Quantitative RT-PCR analysis showing expression of miRNA targets of *Spartina alterniflora* at different time points in leaf and root tissues under salinity stress

at 12 h in the leaf tissue. But in the root tissue, its expression was down-regulated up to 24 h after exposure to salt stress but was significantly up-regulated at 72 h of salinity. In general, with minor deviation, most targets were highly up-regulated at 24 or 72 h of salt stress depending on the tissue.

## Discussion

The role of miRNAs as critical regulators of plant development and hormone homeostasis is well known [26, 45]. Contemporary functional studies strongly suggest that miRNAs act as key regulators of plant's physiological adaptation responses to abiotic stresses, such as oxidative stress, nutrient stress, UV-B stress, salinity stress, drought stress, and heavy metal stress [37, 40–42, 46–49].

In the present study, both Sal-miRNA397a and Sal-miR397b were regulated under salt stress in *S. alterniflora*. Sal-miR397a was not detectable under control condition, whereas Sal-miR397b had basal expression in both leaf and root tissues under control (Fig. 1). Further, the present observation that miRNA397a was highly up-regulated under salt stress compared to Sal-miR397b in the leaf (Fig. 1) is in agreement with previous reports [32, 41] that miR393, miR397a, miR-169 g and miR-169n (o) were strongly up-

regulated, whereas miR397b and miR402 were slightly up-regulated by salt stress in *Arabidopsis*. It was interesting that no sequence read from the small RNA library of *S. alterniflora* had 100 % identity to known miR397b sequence. Such observation has also been reported in rice where no conclusive correspondence was observed with sequencing and expression profiling data of the miRNA under salt [50, 51]. In the present study, Sal-miR528, Sal-miR740, Sal-miR1788, Sal-miR2094, Sal-miR2585, Sal-miR2796, Sal-miR3389, and Sal-miR3426 showed temporal and tissue-dependent expression under salt stress in *S. alterniflora*. A number of other miRNAs were reported to be up-regulated under salt in *Populus* [38] and *Vigna radiata* [52]. Temporal up- or down-regulation of miRNAs in maize under salinity was dependent upon the concentration of salt [40]. Sal-miRNAs, such as Sal-miR740, Sal-miR1788, Sal-miR2094, Sal-miR2585, Sal-miR2796, Sal-miR3389, and Sal-miR3426 that did not have any match with the known miRNAs in the database suggested the possible role of unidentified and novel microRNAs in regulation of salt stress adaptation of halophyte, especially in *S. alterniflora*.

(Semi)quantitative expression of 12 miRNAs and target genes of four of the miRNAs, showed that the pattern of their expression was, in general, semi-coherent in nature. However, coherent (as expected) and non-coherent patterns were also observed. For example, three possible targets of Sal-miR397, such as laccase 11, laccase 22 and L-ascorbate oxidase were all highly expressed at 24 and 72 h of stress where the miR397 showed down-regulation. Laccase gene, the target of miR397 is known to catalyze the oxidative polymerization of monolignols in lignin biosynthesis [38]. Several studies showed an increase in laccase transcript abundance under high concentrations of NaCl in tomato, maize, *Arabidopsis*, and artichoke [53–56]. We observed up-regulation of miR397a in root samples at all the time points except 72 h of salt stress that is coincident with Zhuang et al. [39] that reported up-regulation of miR397a in salt-treated roots of *Solanum linnaeanum*. It has been reported in several plants that miR397 was up-regulated under different abiotic stresses such as drought, cold, oxidative stress and salt stress [39, 41, 50, 57, 58]. Reduced expression of miR397a at 72 h of salt stress in root and leaf might possibly lead to enhanced expression of laccase. Cai et al. [54] evaluated some laccase mutants and showed compromised root elongation and early flowering under PEG-induced dehydration conditions for laccase mutant, which indicated that miR397 up-regulation in the root may prevent root elongation under salt through regulation of the laccase genes [54]. Similar was the case with the expression of Sal-miR990 and its possible target Os05g48870 in the root and Os01g48060 in the leaf tissue where the up-regulation of miRNA indicated down-regulation of its target and vice versa. On the other hand, there was no definitive correspondence between the expression of Sal-miR528 and its three putative targets. Sal-miR528 target genes coding for copper binding proteins, thus suggesting their roles in maintaining the copper homeostasis and reactive oxygen species levels in plant [41, 48, 59, 60]. Sal-miR528 is also predicted to target L-ascorbate oxidase, an apoplastic enzyme in plants that catalyzes the oxidation of ascorbate to monodehydroascorbate. Plants with defective ascorbate oxidase gene expression showed positive effect on the seed germination, photosynthetic activity and seed yield under salinity stress [61].

The miR827 was observed to be up-regulated under phosphorus (P) limitation in *Arabidopsis*, which suggested its involvement in P-specific regulation events [31]. The present observation that Sal-miR827 was regulated under salt stress could be explained by its response to nutrient stress, including P, which is also associated with salinity. The stress-induced miRNAs target negative regulators of stress tolerance, whereas miRNAs decreased under stress conditions may target positive regulators. However, mRNA expression is not always decreased by the induction of miRNA expression, which implies that the post-transcriptional regulation of target mRNA levels by miRNA-directed cleavage is not always rate-limiting for mRNA accumulation under stress [62]. Thus, regulation of miRNA expression alters target gene expression in response to salinity and other stresses, and this is important for plant's acclimation to stress conditions [63]. Our ongoing efforts to overexpress these positive regulators of salt stress adaptation in glycophytes, such as rice, may lead to the development of salt tolerant crops in addition to expanding our understanding of the interaction network of these miRNAs and their targets and associated proteins.

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