Computational prediction and experimental validation of a novel miRNA in *Suaeda maritima*, a halophyte

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ABSTRACT. The lack of available transcriptome data for plants of no economic or agronomic importance limits the identification of miRNAs in many species. Considering the possible similarity of the transcriptome between related species, the present study used expressed sequence tags (ESTs) of *Suaeda salsa* and *Suaeda glauca* to identify conserved miRNAs, which were validated in a halophyte, *Suaeda maritima*, with the aim of identifying salt-responsive miRNAs from naturally salt-tolerant plants, information on which is limited. In this study, computational analysis predicted three miRNA sequences by mapping non-redundant miRNA sequences from miRBase 16.0 on 1534 ESTs of *S. salsa* and *S. glauca*. The expression of one could be validated in *S. maritima*, and was named sma-miR1867. This miRNA was downregulated in response to NaCl treatment. It was predicted to target ferredoxin-thioredoxin reductase (FTR), cell division control protein 6 (CDC6), and ubiquitin-protein ligase (UPL) in *S. salsa* and/or *S. glauca*. However, only UPL could be amplified in *S. maritima*, and RT-qPCR showed that it was upregulated in response to NaCl treatment. These results indicate that, in halophytes, FTR and CDC6 may promote carbon metabolism and cell division, respectively, in the presence of salt, while UPL may regulate the abundance of proteins that are important for salt tolerance in halophytes.

Thus, sma-miR1867 could be an essential component of salt resistance in halophytes.

Key words: NaCl salinity; *Suaeda salsa*; *Suaeda glauca*; sma-miR1867; Abiotic stress; Halophyte

INTRODUCTION

Biochemical and physiological processes in plants and animals are strictly regulated by various mechanisms at different levels. In this regard, micro RNAs (miRNAs) function as repressors of gene expression through post-transcriptional regulation in all known animal and plant genomes. In plants, miRNAs have been reported to regulate plant growth and development, including leaf morphogenesis, lateral root formation, growth stage transition from juvenile to adult and vegetative to flowering, floral organ development, and reproduction (Mallory and Vaucheret, 2006). Furthermore, a regulatory role of miRNAs in biotic and abiotic responses in plants has also been indicated (Sunkar and Zhu, 2004; Ding et al., 2009; Pandey et al., 2013).

Among abiotic factors, high salinity is a severe and increasing constraint on global crop production, as most of the major crops are highly susceptible to salinity; therefore, there is an urgent need to address this problem. However, our understanding of the mechanism of salt tolerance is far from clear, and discovery of salt-responsiveness miRNAs has added complexity to this already complex mechanism (Sunkar and Zhu, 2004;Ding et al., 2009; De Paola et al., 2012). Sunkar and Zhu (2004) showed, for the first time, that upregulation of miR393, miR402, miR397b, and miR319c occurred in response to at least one of the following stresses; drought, cold, and salt. However, with regard to salt-responsiveness, research has mostly been carried out on test species that are either sensitive or naturally not tolerant to salt (Ding et al., 2009; De Paola et al., 2012), with the exclusion of studies by Khraiwesh et al. (2013) and Feng et al. (2015).

Identification of miRNAs in a species requires prior information on their sequence from other species or information on the small-RNA (sRNA) sequence from the same species obtained through cloning and sequencing. Second, knowledge of the genome or transcript sequences of the species permits the unique secondary structure of the pri-miRNA to be established computationally (Jones-Rhoades and Bartel, 2004; Zhang et al., 2005). A lack of genome/transcriptome/expressed sequence tag (EST) data restricts the identification of miRNAs in a species despite the availability of miRNA sequences from many sources. This is particularly true for plants that are of no economic or agronomic importance, such as halophytes, which have been paid little attention in terms of their genomes. However, it has been increasingly realized that many miRNAs are conserved evolutionarily in the plant kingdom and that conservation occurs from lower plants, including mosses and ferns, to higher flowering plants, and hence it is very likely that miRNAs identified through a computational approach considering ESTs of a plant species will be present in a closely related species for which an EST database is not available. Computational approaches have been very effective, especially in plants in which miRNA and target mRNA have often nearly perfect complementarity (Jones-Rhoades and Bartel, 2004; Zhang et al., 2005). Therefore, the present study takes advantage of the availability of ESTs for *Suaeda salsa* and *Suaeda glauca* in order to identify miRNAs and their precursors in a closely related species, *Suaeda maritima*, which is a halophyte, considering the availability of scant information on salt-responsive miRNAs from a naturally salt-tolerant plant.

MATERIAL AND METHODS

Prediction of potential miRNAs in *Suaeda* ESTs

To identify potential salt-responsive miRNAs in a halophyte, all plant miRNAs were downloaded from the miRNA Registry Database (miRBase 16.0, http://www.sanger.ac.uk/). The repetitive sequences were removed and the remaining non-redundant sequences were used as reference miRNAs. A total of 1534 EST sequences of *S. salsa* and *S. glauca* were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). All non-redundant plant miRNAs were subjected to a BLASTn search of the retrieved ESTs sequences of the *Suaeda* species. Considering that miRBase is not rich in miRNAs from mangrove and/or halophytes, the ESTs sequences with 0-5 mismatches against the plant miRNAs were selected, instead of 0-4 mismatches, which is preferred (Meyers et al., 2008). These ESTs were subjected to a BLASTx search to remove the ESTs coding for known proteins. The remaining ESTs were used to predict the hairpin structure by the mfold software with default parameters (http://mfold.rna.albany.edu/). The length of the precursor was reduced until the minimal folding free energy index (MFEI) was maximal. Furthermore, it was also ensured that the precursors were at least 60 nt long, the secondary structure displayed an appropriate stem loop hairpin, the miRNA sequence occupied only one arm of the hairpin, the maximum number of bases were present in the stem of the stem-loop hairpin structure, and the mature miRNA sequence and its opposing miRNA strand (miRNA*) did not contain more than six nt mismatches (Zhang et al., 2005). The MFEI value was calculated as per the equation: MFEI = [(MFE/length of the precursor miRNA sequence) X 100] / (G + C)% (Zhang et al., 2006a).

Test plant and NaCl application

Seeds of *S. maritima* L. were collected from adult plants growing along the mangrove coastal belt in Bhadrak (21.13°N, 86.76°E), Odisha, India. The seeds were spread on autoclaved soil mixed with sand in plastic pots with holes in the bottom and were watered every day, alternating between 1/10th Hoagland's solution and Milli-Q water. The seedlings were grown in a greenhouse maintained at 24° ± 3°C and 70-75% relative humidity under a natural day/night cycle with provision of additional light (intensity 200 µmol x m^{-2} x s^{-1}) from cool white fluorescent bulbs. After 3-4 weeks, the seedlings were approximately 2 cm in height. At this stage, groups of three seedlings were transferred to separate plastic pots and were set to acclimatize and grow for ~3 months in the greenhouse. The individual pots were watered every day, alternating between 1/10th Hoagland's solution and Milli-Q water except on the penultimate day when NaCl was applied. Initially 500 mL 0.5% NaCl prepared in 1/10th strength Hoagland's solution was poured into the individual pots early in the morning. The control pots received only 1/10th Hoagland's solution. After 30 min, 100 mL 2.0% NaCl prepared in 1/10th strength Hoagland's solution was poured into the treated pots at 30 min intervals. After 9-h exposure, the aerial portions of the test plants were excised, preserved in liquid N_2, and stored at -80°C until analysis.

Expression study of the predicted miRNAs

Total RNA was isolated from preserved shoot samples of the control and 2.0% NaCl-treated (9 h) *S. maritima* plants using a miRNeasy mini kit (Qiagen) following the manufacturer

protocol. For northern blot analysis, 10 µg total RNA was resolved on a 15% urea-polyacrylamide gel electrophoresis and transferred on to nylon membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The blot was air dried and UV-crossed linked at 150 mJ using a UV cross linker (HoeferTM UVC 500 Cross linker). The sense and anti-sense DNA oligonucleotide probes were end-labeled individually with [^{32}P]γ-dATP using T4 Polynucleotide Kinase (Fermentas) according to the manufacturer instructions. Individual membrane blots were pre-hybridized for 1 h with hybridization buffer (Sigma). The radiolabelled probe was added individually to each membrane and hybridization continued for 16 h at 37°C. After hybridization, the membrane was washed with 2X SSC (saline sodium citrate), 0.1% SDS (sodium dodecyl sulfate) at 32°C for 15 min and finally with 1X SSC and 0.1% SDS for 15 min at 37°C. The membrane was air dried and then exposed to X-ray film. When needed, membranes were stripped, re-exposed to X-ray film to ensure complete signal removal, and reused for a second hybridization. A DNA oligonucleotide complementary to U6 small nuclear RNA (snRNA) was used as a probe to detect the U6 snRNA as a reference control for normalization.

Expression study of miRNA precursor by RT-PCR

RT-PCR was carried out to determine changes in the expression of the sma-miR1867 precursor in response to NaCl application. Total RNA from the preserved control and 2.0% NaCl-treated (9 h) *S. maritima* shoot samples was isolated as described above to prepare cDNA using a SuperScript®III First-Strand Synthesis kit from Invitrogen following the manufacturer protocol. Briefly, 1 µg DNase treated total RNA, 1 µL dNTP mix (10 mM each), and 1 µL random hexamer were added to a 2-mL PCR tube and incubated at 65°C for 5 min followed by incubation on ice for ~5 min. In the same tube, 4 µL 5X First Strand Buffer, 1 µL 0.1M DTT, 1 µL RNaseOUT (40 U/1 µL), and 1 µL (200 U) SuperScript III reverse transcriptase was added to a final volume of 20 µL. Next, the contents of the PCR tube were incubated at 50°C for 1 h followed by 70°C for 15 min, and finally at 4°C for 5 min. The prepared 1st strand cDNA was used as a template for amplification of the sma-miR1867 precursor using primers flanking the miRNA1867 region. PCR amplification was carried out on a DNA engine (Bio-Rad) thermo cycler taking cDNA synthesized as a template and using GoTaq® DNA polymerase (Promega) and PCR nucleotide mix (Promega) following the protocol detailed in the manual. The PCR program was set as 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s, and finally, 72°C for 5 min. The amplified products (7 µL) were resolved on 2.0% agarose gel and visualized by ethidium bromide staining and UV illumination. Actin served as a loading control.

Cloning and sequencing of precursor amplified by RT-PCR

The precursor amplified by RT-PCR was cloned in pGEMT-Easy vector and transformed into JM109 competent cells. The transformed bacteria were plated on LB/agar plates containing ampicillin. For blue-white colony screening, 100 µL 100 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and 20 µL 50 µg/mL X-Gal were spread on each plate. Several white colonies were isolated and the insert was amplified by colony PCR. The reaction was performed with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The amplicons were sequenced using SP6 and T7 vector specific primers on an Applied Biosystem DNA sequencer (Genetic analyzer 3500).

Expression analysis of miRNA target by RT-qPCR

Total RNA was extracted and first-strand cDNA was synthesized from the control and NaCl treated *S. maritima* samples as described. A QuantiTect SYBR Green PCR Kit (Qiagen) was used for the RT-qPCR on a LightCycler® 480 Real-Time PCR System (Roche). The reactions were individually run in 20-µL volumes in 96-well plates as detailed in the manual. Actin expression in each cDNA preparation served a reference. The relative expression/abundance of individual target genes in NaCl-treated samples compared with the control sample was calculated using the $2^{-\Delta\Delta Ct}$ method considering actin templates in the sample as the reference level.

sma-miR1867 target prediction

Target prediction was carried out using psRNATarget tools (http://plantgrn.noble.org/psRNATarget/) considering the EST sequences of the halophytes *S. salsa* and *S. glauca* that were available at the NCBI database with default parameters. Considering the availability of only 1534 ESTs from these two species in the database, the target prediction was performed with less stringency (E values ≤ 4) than usually followed (E ≤ 3). The predicted target transcript sequences were BLASTx searched through the NCBI site for annotation of their probable function.

RESULTS AND DISCUSSION

The identification and characterization of miRNAs are widely pursued research fields for multiple reasons. First, the number of miRNAs identified is still relatively low; second, the number of species covered so far is low, particularly in plants, considering their wide taxonomical and ecological diversity; and third, this subject can be applied to all fields of research in modern biology. Furthermore, the discovery of miRNAs has added another dimension to molecular biology research concerned with understanding the regulatory aspects of molecular events that maintain life under extreme environmental conditions. Being sessile, plants have evolved to adapt to harsh environmental conditions, and recently, a number of miRNAs have been shown to be sensitive to abiotic stresses (Sunkar and Zhu, 2004) indicating their importance in the adaptive process (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). However, all such miRNAs have been identified by deep sequencing of sRNA followed by mapping of the sequences with the ESTs of the species under investigation (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). Recently, Pandey et al., (2013) used a computational approach to identify drought stress-responsive miRNAs in wheat using abiotic stressed cDNA libraries for this plant. However, the results of the current study show that ESTs of related species can be used to successfully identify miRNAs using a computational approach.

Three potential miRNAs were predicted using 1534 ESTs of *S. salsa* and *S. glauca* and non-redundant miRNA sequences from miRBase (Table 1) showing mismatches at a maximum five nucleotide positions (Figure 1). The predicted conserved miRNA sequences belonged to the miR529, miR1867, and miR2657 families. The negative MFE of precursor miRNAs varied significantly within the range -28.20 to -40.10 kcal/mol, the length of the precursors was greater than the desired length of 60 nt, the miRNAs occupied only one arm of the concerned secondary structure, and there were no more than six mismatches between the miRNA/miRNA* duplex (Table 1, Figure 2), suggesting that the precursors could be diced to form mature miRNAs (Zhang

et al., 2005). The A+U contents of the precursors varied from 57 to 71.63% (Table 2), which is consistent with previous reports (Zhang et al., 2006b). The MFEI values of the miR529, miR2657, and miR1867 precursors were 0.46, 0.62, and 0.72, respectively (Table 1). Among these, the MFEI of the miR1867 precursor was higher than that of other non-coding RNAs indicating that this is the most likely candidate miRNA precursor (Zhang et al., 2006a).

Table 1. Chemical features of predicted miRNAs and their precursors.

Sl. No.	miRNA predicted	Gene ID	Loc	LM (nt)	LP (nt)	G+C (%)	MFE (-ΔG)	MFEI
1	miR529	gi|9057119	5'	21	213	28.63	28.20	0.46
2	miR1867	gi|9057160	3'	23	128	42.96	40.10	0.72
3	miR2657	gi|314999170	3'	22	208	28.36	36.80	0.62

Loc: location of mature miRNA in the stem-loop, LM: length of the mature miRNA, LP: length of the precursor, MFE: minimal folding free energy, MFEI: minimal folding free energy index.

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gi|9057119      120 cuuaacccucucuuuucuuc 139
                    ||   ||||||||| ||||||
osa-miR529a       1 cuguacccucucucuucuuc 20

gi|9057160      273 uguuugauguaggaaagagggag 295
                    | |||   | ||||| ||||||||
osa-miR1867       1 uuuuuuuucuaggacagagggag 23

gi|314999170    859 uuuuauuuuaugaauuuuguug 880
                    | |||||| ||  |||||||||
mtr-miR2657a      1 uguuauuucaucgauuuuguug 22
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Figure 1. Nucleotide base pairing of the miRNAs predicted with those available in miRBase.

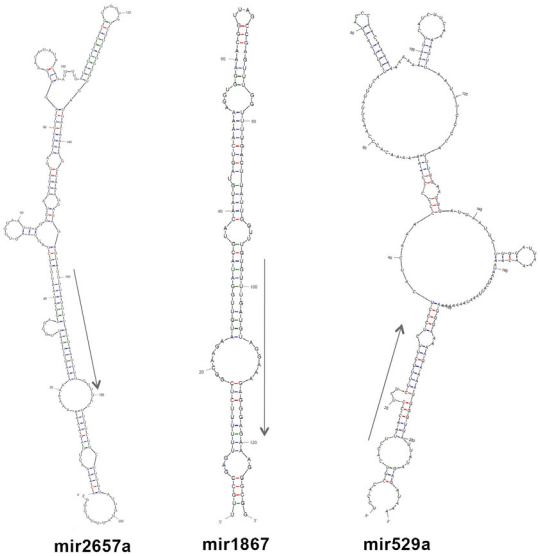

Figure 2. Stem-loop secondary structure of predicted miRNA precursors. The arrow in each secondary structure indicates the miRNA sequence.

Table 2. Nucleotide composition of the predicted miRNA precursors.

Sr. No.	miRNAs predicted	A%	C%	G%	U%	A/U ratio	G/C ratio	A+U (%)
1	mir529	42.25	21.12	7.51	29.10	1.45	0.35	71.36
2	mir1867	25.78	10.15	32.81	31.25	0.82	3.23	57.00
3	mir2657	31.73	6.25	22.11	39.90	0.79	3.53	71.63

Of the three antisense oligo-probes, only that for miR1867 generated a signal in northern blot hybridization (Figure 3-I), indicating that it is expressed in the plant (*S. maritima*) fulfilling the miRNA annotation criteria (Ambros et al., 2003). Its expression was detected in both control and NaCl-treated plants and was named sma-miR1867 as per the nomenclature criteria set forth by Ambros et al. (2003). When predicted miRNAs were not detected by northern blot hybridization in control or NaCl-treated plants, they were considered to be absent in *S. maritima*. Northern blot hybridization performed with DNA oligonucleotides identical to sma-miR1867 did not produce any signals, confirming that miRNA sma-miR1867 is expressed in *S. maritima* (Figure 3-II); since siRNAs are derived from sense and antisense RNA duplexes, a hybridization signal with the identical oligonucleotide would be expected if sma-miR1867 was a siRNA (Jian et al., 2010). Densitometric analysis of the sma-miR1867 northern blot hybridization signal revealed that the expression of this miRNA was downregulated in NaCl-treated plants (Figure 3-III) suggesting its importance in the salt tolerance process.

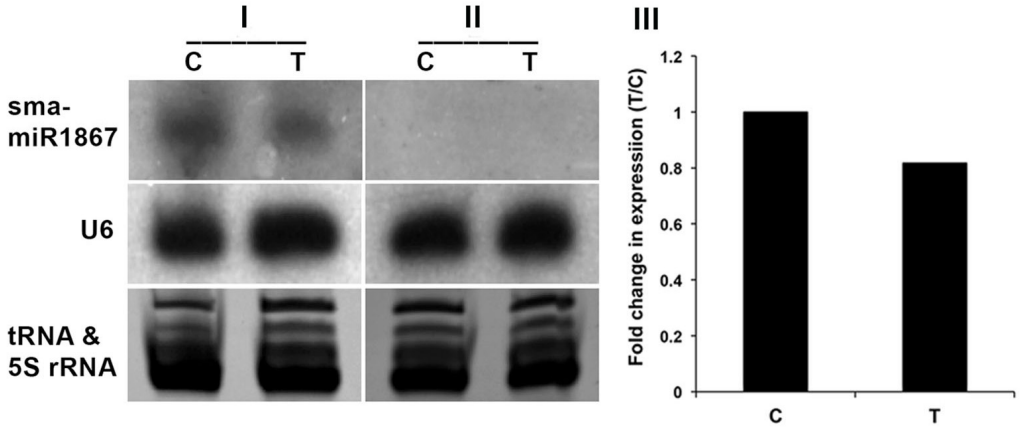

Figure 3. Northern blot hybridization confirming the expression of sma-miR1867 in *Suaeda maritima*. The RNA blots were probed either with a [^{32}P] end-labeled oligo complimentary to sma-miR1867 (I), or with a [^{32}P] end-labeled oligo identical to sma-miR1867 (II). In each blot, lanes C and T represent RNA isolated from control and 2.0% NaCl-treated plants, respectively. Only blot-I showed a hybridization signal of ~20-24 nt in size suggesting that the predicted miRNA sequence, sma-miR1867, is a miRNA. Both blots were probed with a radio-labeled oligonucleotide complementary to U6 snRNA for use as a loading control. tRNA/5S rRNA bands were also visualized by ethidium bromide staining of polyacrylamide gels before blotting. (III) Densitometric representation of the hybridization signal in blot-I showing downregulation of the miRNA upon NaCl-treatment.

RT-PCR analysis using *S. maritima* cDNA and the primer pairs designed to amplify the sma-miR1867 precursor revealed an amplified product of the desired length (Figure 4-I). Sequencing of the cloned RT-PCR product of the sma-miR1867 precursor and alignment of the sequence to the EST of *S. salsa* revealed complete homology, with the two sequences differing at only two base positions (Figure 4-II). Amplification of this precursor from *S. maritima* confirmed that sma-miR1867 is expressed in *S. maritima* and also suggests that miR1867 is well conserved in plants. Deep sequencing of rice spikelets has also revealed the presence of miR1867 (Jian et al., 2010). Furthermore, expression of miR1867 has also been confirmed in *Triticum dicoccoides* (Kantar et al., 2011) and *Brachypodium distachyon* (Budak and Akpinar, 2011) by qRT-PCR (stem-loop PCR) and microarray analysis, respectively, providing further evidence of its conservation in plant species across taxonomic groups.

The expression of sma-miR1867 was downregulated following treatment with 2.0% NaCl, which is the concentration of NaCl present in seawater (Figure 3-III). RT-qPCR also revealed a decrease in the level of sma-miR1867 precursor expression following NaCl treatment (Figure 5), confirming that the expression of this miRNA is regulated by salt stress. In addition, miR1867 has also been reported to be responsive to dehydration stress (Budak and Akpinar, 2011; Kantar et al., 2011). However, in contrast to salt stress, dehydration stress upregulated the expression of miR1867. Moreover, miR1867 was also upregulated in rice spikelets towards the late stage of grain filling, i.e., when the moisture content of the grains started to decrease (Peng et al., 2014), simulating dehydration stress. Hence, miR1867 seems to respond differently depending on the abiotic stress encountered to fulfill the requirement for altered metabolism under given conditions.

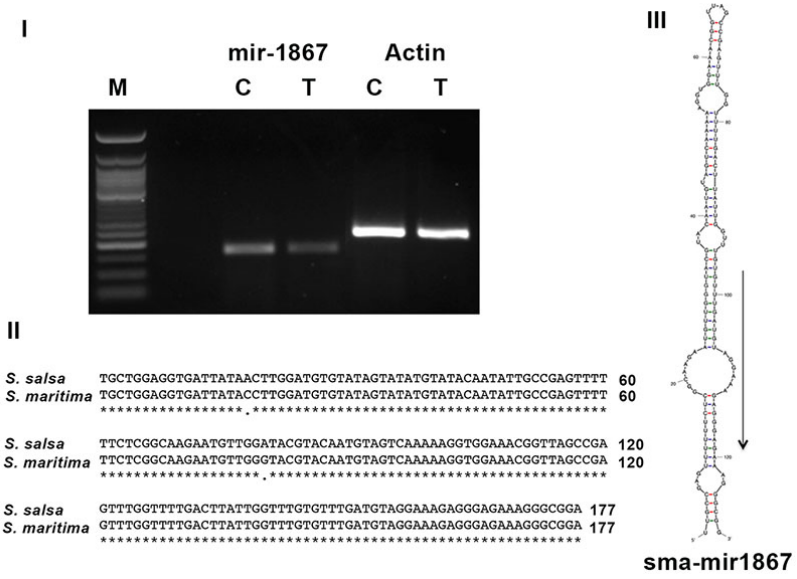

Figure 4. Expression analysis of the sma-miR1867 precursor by RT-PCR in *Suaeda maritima* and its secondary structure. (I). 1st strand cDNA was synthesized by reverse transcription of the total RNA and used in PCR. PCR was carried out for 30 cycles and the amplified products (7 µL each) were resolved on 2% agarose gel and visualized by ethidium bromide staining and UV illumination. Actin was used as a loading control. M: 50-bp DNA ladder, C: RNA from control plant, T: RNA from the plant treated with 2.0% NaCl for 9 h. (II). The amplified sma-mir1867 fragment was sequenced and aligned with that of the *Suaeda salsa* EST. (III). Secondary structure of sma-mir1867.

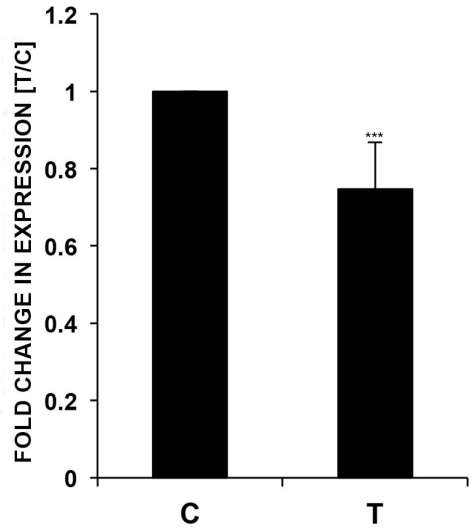

Figure 5. RT-qPCR of the sma-miR1867 precursor in *Suaeda maritima* showing its downregulation in response to NaCl application. Data are reported as means ± SD of three replicate studies. ***Significant difference at $P \leq 0.001$.

Three targets of known function were predicted for sma-miR1867 considering the ESTs of the halophytes (Table 3). These were the ferredoxin-thioredoxin reductase catalytic chain, the cell division control protein CDC6 and ubiquitin protein ligase (Table 3). Ferredoxin-thioredoxin reductase catalyzes the electron transfer between ferredoxin, the first soluble electron acceptor of the photosynthetic electron transfer chain, and thioredoxin, a ubiquitous protein, which is efficient at exchanging thiol-disulfide with various protein disulfides because of its active site dithiol. Reduced thioredoxin can reduce disulfides of several target enzymes, most of which are weakly active or inactive in the oxidized form. Regulatory enzymes of a number of fundamental chloroplast processes have been reported to be linked to thioredoxin, including glucose 6-phosphate dehydrogenase of the oxidative pentose phosphate cycle, ATP synthase of ATP synthesis, NADP-malate dehydrogenase of C4 photosynthesis, fructose 1, 6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, NADP-glyceraldehyde 3-phosphate dehydrogenase, and Rubisco of the Calvin-Benson cycle (Buchanan et al., 2002). Thus, NaCl application seems to stimulate photosynthetic responses in halophytes by enhancing the formation of reduced thioredoxin through downregulation of sma-miR1867 expression. Similarly, cell division also seems to be promoted in halophytes following NaCl application, as sma-miR1867 also targets the cell division control protein CDC6, which is essential for the initiation of DNA replication (Masuda et al., 2004).

Table 3. Characterization of sma-miR1867 expression in *Suaeda maritima*.

Target accesstion No.	Target description	Inhibition type	miRNA/mRNA pair
>gi|314999457	Ferredoxin-thioredoxin reductase catalytic chain	Cleavage	.:...:.:::..:::::::..:
>gi|9057103	Cell division control protein CDC6	Translation	::::.::::::::.:.:::
>gi|9057157	Ubiquitin-protein ligase	Cleavage	::::::::.:::::::.

Among the three targets identified, it was possible to amplify the target gene ubiquitin-protein ligase from *S. maritima* by designing primers based on the EST of *S. salsa*. Sequencing of the amplified fragment revealed nearly perfect identity with *S. salsa* ESTs (Figure 6-I). Furthermore, RT-qPCR revealed significant upregulation of the target transcript (Figure 6-II) providing indirect evidence that the ubiquitin-protein ligase gene product is the target of sma-miR1867. Ubiquitin-protein ligase serves as a unit of the ubiquitin/26S proteasome system, which is involved in poly-ubiquitination-mediated degradation of soluble proteins and influences their activity and abundance and thus affecting a variety of cellular functions. Suppression of the miRNA (seu-miR396h/l) that targets ubiquitin-specific protease, which is involved in protein degradation, has also been reported in the euhalophyte *S. europaea* during long-term salt treatment (Feng et al., 2015). The biological role of the ubiquitin/26S proteasome system has been investigated in relation to abiotic stress tolerance in plants, which can be positively or negatively regulated (Stone, 2014). Therefore, regulation of the activity and abundance of both membrane and cytosolic proteins appears to be important for enabling plants to grow in saline environments.

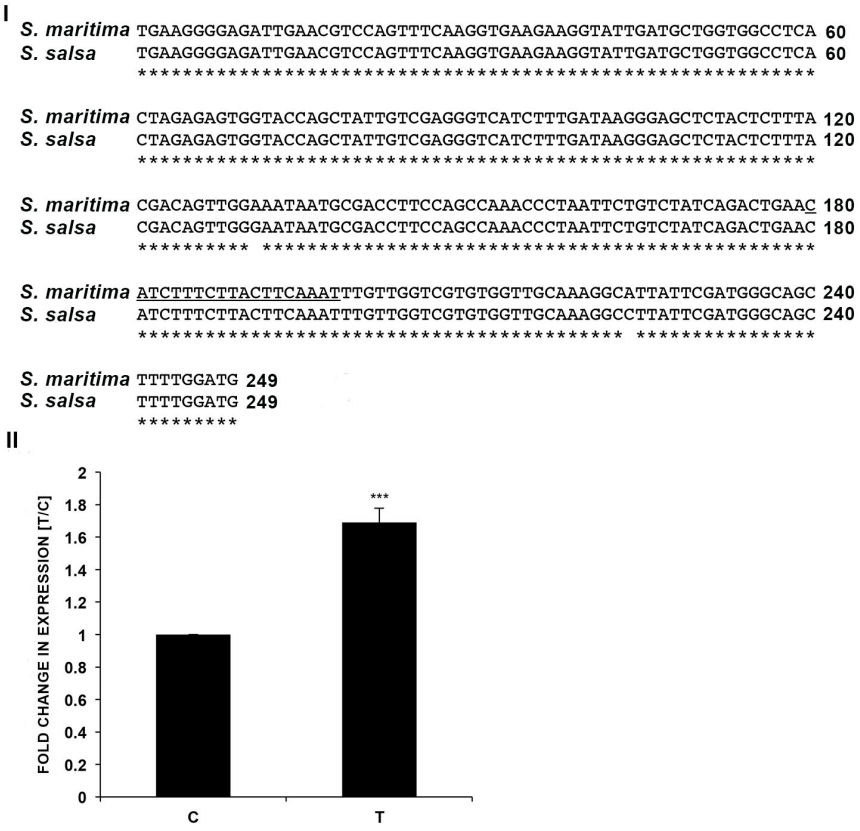

Figure 6. PCR amplification and RT-qPCR of the sma-miR1867 target ubiquitin-protein ligase. (I). RT-PCR amplified target fragment in *Suaeda maritima* was sequenced and aligned with the EST of *S. salsa* using Clustal Omega. (II). RT-qPCR of ubiquitin-protein ligase in *S. maritima* showing its upregulation in response to NaCl application. Data are reported as means ± SD of 3 replicate studies. ***Significant difference at $P \leq 0.001$.

In conclusion, this study predicted three miRNAs from 1534 ESTs of *S. salsa* and *S. glauca* present in the NCBI database through a computational approach, and experimentally validated the presence of one, sma-miR1867, in *S. maritima* suggesting that EST information of related species can be used for this purpose. This miRNA was found to be salt-responsive and was downregulated in response to NaCl application. Although three targets for sma-miR1867 were predicted using ESTs of the halophytes, only one, ubiquitin-protein ligase, could be amplified in *S. maritima*. A review of the literature revealed that among the three targets, ferredoxin-thioredoxin reductase and CDC6 were responsible for promoting carbon metabolism and cell division, respectively, suggesting that NaCl promotes these processes in halophytes. Negative regulation of the third target, ubiquitin-protein ligase, by sma-miR1867, nevertheless, indicated that ubiquitin-mediated changes in the abundance of membrane and cytosolic proteins could be important biochemical processes for salt tolerance in halophytes. Downregulation of sma-miR1867 in *S. maritima* as a response to the use of NaCl in this study indicates that this miRNA might be important to enable the plant to survive and grow in saline environments.

Conflicts of interest

The authors declare no conflict of interest.

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