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ORIGINAL PAPER

Characterization of miRNA160/164 and Their Targets Expression of Beet (*Beta vulgaris*) Seedlings Under the Salt Tolerance

Jie Cui[1] · Zongyan Sun[1] · Junliang Li[1] · Dayou Cheng[1] · Chengfei Luo[1] · Cuihong Dai[1]

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Abstract

MicroRNAs (miRNAs) are non-coding endogenous small RNAs that play important roles in plant growth, development, and stress response. Soil salinization leads to environmental and ecological problems, which greatly restricts agricultural production. MiRNAs are activated in various plants in response to salinity stress, although data from beet (*Beta vulgaris*) is still lacking. We addressed this in the present study by investigating the mechanisms of salt tolerance in the seedlings of two different varieties of beet. We examined the involvement of the miR160 and miR164 and their targets *auxin response factor* (*ARF*) and *no apical meristem* (*NAM*)–*Arabidopsis transcription* activation *factor* (*ATAF*)–*cup-shaped cotyledon* (*CUC*) (collectively referred to as *NAC*), respectively. Seedlings from different leaf stages were treated with 300 mM NaCl for 0, 12, 24, 48, or 72 h, and *miR160-ARF17/18* and *miR164-NAC(21/22)/100* expression in roots and leaves was analyzed by quantitative real-time PCR. *MiR160/164* expression differed markedly between the two varieties of beet and according to stress duration, organ, and growth stage. Meanwhile, changes in the expression of *ARF17/18* and *NAC(21/22)/100* were the opposite of those observed for their regulatory miRNAs. These results provide insight into the mechanisms of salt tolerance in this economically valuable crop. The signal pathways of *miR160/164* and its target genes *ARF17/18* and *NAC(21/22)/100* were analyzed by bioinformatics technique using KEGG pathway and Interpro. The results showed that *miR160* and its target genes were involved in plant hormone signaling (map04075) and mitogen-activated protein kinase (MAPK) signaling (map04016), through the indirect regulation of the two metabolic pathways, to deal with salt stress.

Keywords *Beta vulgaris* · Salt stress · *miR160/164* · Auxin response factor (ARF) · NAM–ATAF–CUC (NAC) transcription factors

Introduction

Crops are subjected to a variety of biological and abiotic stresses during growth and development that can reduce crop yield or lead to death. With the degradation of the environment, soil salinization has become a serious global problem. A recent survey found that about 1 billion hectares of land worldwide is affected by salinization, accounting for about 7% of the total land area; 58% is in irrigated agricultural areas, and nearly 20% percent of irrigated soil is salinized, with the proportion continuously increasing (Peng 2016; Zhou et al. 2012). Over the past few decades, there has been considerable progress in elucidating the salt stress response mechanisms of plants, with many relevant genes identified that regulate molecular, biochemical, cell, physiological, and morphological adaptations; metabolic pathways; and basic biological processes (Baker et al. 2005; Dong et al. 2009; Jia et al. 2009). Posttranscriptional regulatory mechanisms in the response to high salinity have also been demonstrated (Yin et al. 2012).

✉ Jie Cui
cuijie@hit.edu.cn

Zongyan Sun
904448417@qq.com

Junliang Li
xiaoga1206@163.com

Dayou Cheng
hgdcdy@126.com

Chengfei Luo
cfl7375@sina.com

Cuihong Dai
519757240@qq.com

[1] Department of Chemistry and Chemical Engineering, Harbin Institute of Technology, No. 92, Xidazhi Street, Nangang District, Harbin City, Heilongjiang Province, China

Micro (mi)RNAs are a class of non-coding small RNA molecules with sizes ranging from 19 to 24 nucleotides that are transcribed by RNA polymerase II. The precursor miRNA transcript is processed into mature miRNA by Dicer-like and various protein complexes. MiRNAs mainly regulate their targets at the posttranscriptional level via degradation of target mRNA molecules or translation inhibition (Jonesrhoades and Bartel 2004; Llave and Carrington 2002; Navarro et al. 2006; Zhi-Yong et al. 2016). Studies have shown that miRNAs regulate plant growth and stress responses. The expression levels of some miRNAs in plants are altered under conditions of salt stress, for example, in *G. hirsutum* L., *miR156*, *miR159*, *miR160*, *miR164*, *miR167*, *miR169*, *miR172*, *miR397*, and *miR399* changed their expression. In the three legume crops, *miR156* and *miR160* exhibited differential expression patterns (Abdelrahman et al. 2018). In *Triticum aestivum*, *miR396* was reported associated with salt stress. In *Arabidopsis thaliana*, the expressions of *miR156*, *miR159*, *miR160*, *miR164*, *miR167*, *miR169*, *miR172*, *miR397*, and *miR399* were changed (Kurtoglu et al. 2013; Chun-He et al. 2009; Khan and Rizwanul 2015; Verma et al. 2014), while *miR160* also has been linked to the regulation of plant hormone signaling. Auxins control all aspects of plant growth and development via regulation of auxin response factor (ARF) genes (Lin et al. 2015; Liu et al. 2016). To date, 23 ARF family members have been identified in *A. thaliana*, of which at least three members, *ARF10*, *ARF16*, and *ARF17*, are *miR160* targets (Qiao et al. 2012). No apical meristem (NAM)–*Arabidopsis* transcription activation factor (ATAF)–cup-shaped cotyledon (CUC) (collectively referred to as NAC) transcription factors also play important roles in plant seed germination, organ boundary formation, flowering senescence, and the abiotic biological stress response (Ernst et al. 2004; Jian et al. 2016b). A total of 102 NAC family members have been predicted in *Arabidopsis*, including more than 10 that have been identified as miR164 target genes (e.g., CUC1 (cup-shaped cotyledon 1), *CUC2* (cup-shaped cotyledon 2), *NAC1* (NAM–ATAF–CUC 1), *NAC100* (NAM–ATAF–CUC 100)) (Ooka et al. 2003).

Beet (*Beta vulgaris*) is a high-value cash crop that accounts for about 35% of global sugar production (Zhang et al. 2016). Bioinformatics-based predictions indicate that the beet genome encodes 13 mature miRNAs from 12 miRNA families in 29,857 expressed sequence tags and 279,223 genome survey sequences (Li et al. 2015). Target genes of these miRNAs encode transcription factors, signal transduction components, and factors associated with stress response, growth, and development. However, there have been no studies to date investigating miRNA regulation of the response to salt stress in beets. To address this issue, the present study examined changes in the expression of miRNAs and their target genes in response to salt stress in beet seedlings by degradation group sequencing, focusing on *miR160* and *miR164* and their respective targets *ARF17/18* and *NAC(21/22)/100*.

Results

Prediction of *miR160/164* Target Genes

In this study, we used bioinformatics combined with degradome sequencing to accurately predict *miR160/164* target genes. We identified *ARF17* and *ARF18* as target genes of *miR160*, and the degradome cleavage sites are 2196 and 1407 bp at the full length of their genes, respectively. *NAC21/22* and *NAC100* are target genes of *miR164*, and the degradome cleavage sites are 1027 and 723/717 bp at the full length of their genes, respectively. The details are shown in Table 1. The raw data has been uploaded to the NCBI Short Read Archive (SRA) database under the accession numbers SRR5957154 and SRR5957155. The details are shown in Table 1.

Spatial and Temporal Expression Patterns of *miR160/164* Under Salt Stress

We compared the expression of *miR160/164* in two beet varieties with different salt tolerance responses, growth stages, and plant organs following treatment with salt solution for 72 h. We observed significant differences in the expression of *miR160/164* between the two varieties ($P < 0.01$), although there was little difference in the expression of miR164 in four-leaf stage seedling roots and six-leaf stage seedling leaves

Table 1 Degradome sequencing data of *miR160/164*

Small RNA	Transcript	Transcript annotation	Symbol	Alignment score	Degradome cleavage site
mdm-miR160a	Bv7_161970_eqms.t1	Hypothetical protein	ARF18	0.5	2196
mdm-miR160a	Bv1_000540_ghtj.t1	Hypothetical protein	ARF17	0.5	1407
mtr-miR164a	Bv5_112840_pjnp.t1	NAC domain-containing protein 89-like	NAC100	2	1027
mtr-miR164a	Bv_03220_dyzk.t1	NAC domain-containing protein 89-like	NAC021	2	723
mtr-miR164a	Bv_03220_dyzk.t2	NAC domain-containing protein 89-like	NAC021	2	717

(Fig. 1a). In four-leaf stage seedlings, miR160 level was lower in "O"68 than in Shuang 6, whereas the converse was observed in six-leaf stage seedlings. MiR164 expression was lower in "O"68 than in Shuang 6 in both four-leaf stage leaves and six-leaf stage roots (Fig. 1a).

MiR160/164 was more lowly expressed in roots than in leaves ($P < 0.01$) (Fig. 1b); in addition, significant differences were observed in the leaves between seedlings at the two different growth stages ($P < 0.01$) (Fig. 1c). In "O"68 seedlings, miR160 expression was lower at the four-leaf than at the six-leaf stage, whereas the opposite was true for Shuang 6. In the roots, expression was higher at the four-leaf than at the six-leaf stage, while the converse was observed in leaves.

In summary, *miRNA160/164* expression differed according to beet variety, growth stage, and organ. The greatest differences were observed between the two organs, with more complex trends observed for growth stage and beet variety.

Expression of *miR160* and Its Target Genes *ARF17/18*

We investigated changes in the expression of *miR160* and its target genes *ARF17/18* under conditions of salt stress by qRT-PCR analysis of four- and six-leaf stage seedlings of two beet varieties (Fig. 2). A downregulation tendency of *miR160* under salt stress was detected in OR4, OR6, SR4, and SR6 at different time points. In general, *miR160* expression was decreased after 12 h; at later time points, the level increased and then decreased, except in the case of OR4. The level at 72 h was lower than that in the control group ($P < 0.01$). Whereas, OL4, OL6, SL4, and SL6 exhibit an upregulation expression at 48 h post-treatment (Fig. 2a). Conversely, *ARF17/18* expression showed a trend of growth although upregulation is not obvious at some time points after treatment ($P < 0.01$) (Fig. 2b, c).

Expression of *miR164* and Its Target Genes *NAC(21/22)/100*

We also investigated changes in the expression of *miR164* and its target genes *NAC(21/22)/100* under conditions of salt stress by qRT-PCR analysis of four- and six-leaf stage seedlings of the two beet varieties (Fig. 3). Compared to expression levels at 0 h, *miR164* level was downregulated in most treatment groups at high salt concentration ($P < 0.01$). However, OL4, OL6, and SL4 showed the opposite trend ($P < 0.01$) whereas no change was observed in the SL6 group. *MiR164* expression decreased rapidly after 12 h of salt stress, while at longer treatment times, all treated samples except for those in the SR6 group showed an initial increase followed by a decrease, with lower levels relative to the control at 72 h ($P < 0.01$) (Fig. 3a). Conversely, *NAC(21/22)/100* levels were upregulated in most treatment groups ($P < 0.01$), although no differences were observed at 24 and 48 h in some groups. However, except for *NAC21/22* in the OR6 and SR6 and *NAC100* in the OR4 and OL4 groups, expression levels in the treatment groups were higher than those in the control group at 72 h ($P < 0.01$) (Fig. 3b, c).

MiR160 and *miR164* were downregulated in both roots and leaves at two different growth stages, except for a temporary increase in certain groups. In contrast, the corresponding target genes *ARF17*, *ARF18*, *NAC21/22*, and *NAC100* were upregulated in a high-salt environment. These results indicate that *ARF17/18* and *NAC(21/22)/100* are negatively regulated by *miR160* and *miR164*, respectively.

Preliminary Study on Resistance Mechanism of Beet Seedlings

When beet was subjected to salt stress and drought stress, the expression of *miR160* was significantly downregulated, and its target genes *ARF17* and *ARF18* were overexpressed. The conserved DNA domain on ARF protein can specifically recognize the expression of Gretchen Hagen3 (GH3) gene on the GH3 gene activator and inhibit the expression of GH3 gene. In addition, as indicated by the bioinformatics analysis, ARF can inhibit plant growth by affecting the plant hormone AUX/IAA (auxin/indole-3-acetic acid) synthesis pathway (Xie et al. 2015).

miR160 and its targets *ARF17* and *ARF18* improve the plant's ability to resist salt stress in three ways: First mechanism is through indirect increase in the free proline content in plants; because free proline is recognized as osmotic stress, it plays an important role in stress response. Second, increasing the IAA content indirectly increases the expression of some stress response genes, in turn improving the stress tolerance of plants. Third, increased content of ABA involved in the MAPK signaling pathway in stress response improves the ability of plants to cope with stress, as shown in Fig. 4.

The NAC family is large, and only a few proteins of this family are currently fully explored. There is little research on the involvement of *NAC21/22* and *NAC100* in salt stress response, and based on the KEGG pathway database, the signal pathways involved have not been predicted so far. To our knowledge, this study is the first to report that *NAC21/22* and *NAC100* are involved in salt stress and that the same family members of NAC are similar in terms of both functional and structural aspects. Therefore, we speculate that their stress resistance mechanism is similar to that of other NAC family proteins and may involve regulation of downstream gene expression in response to stress.

Discussion

Seedlings are susceptible to various biological and abiotic stresses including salinity, making this a critical stage in plant

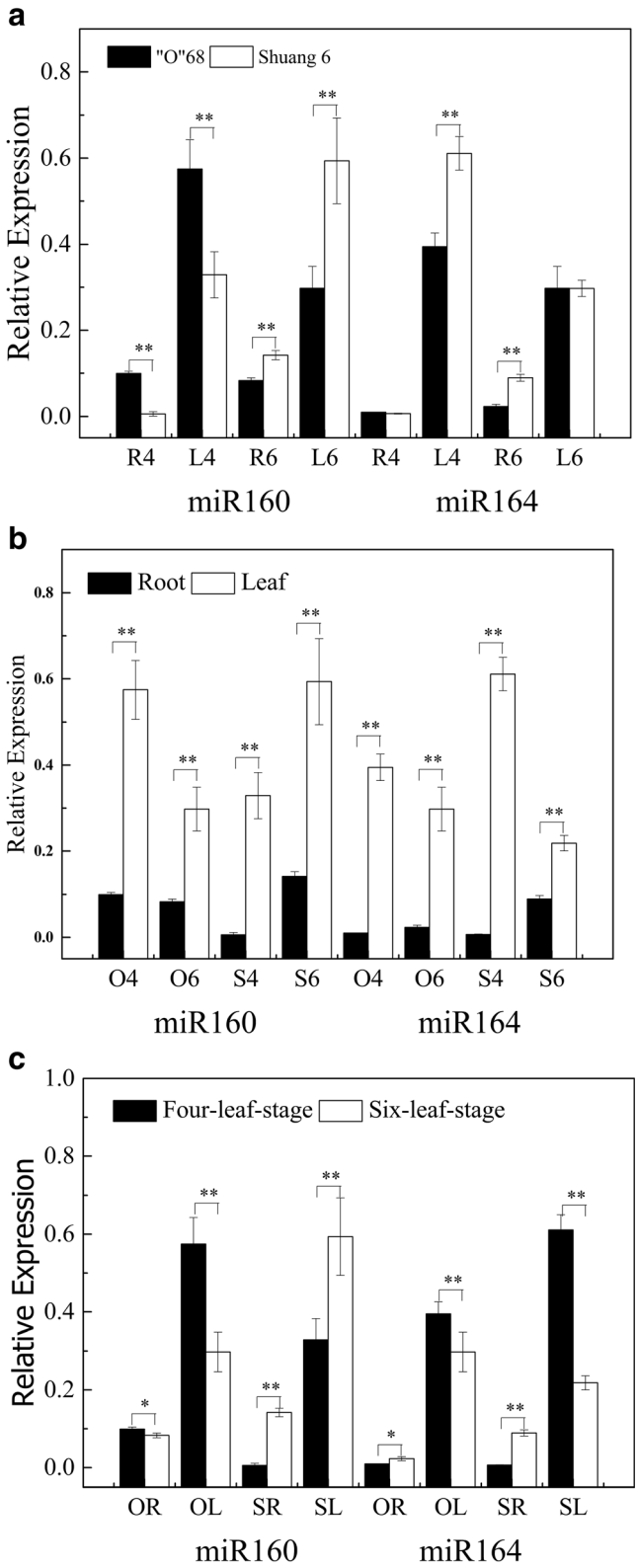

Fig. 1 Relative expression levels of miR160/164 in beet seedlings after 72 h of salt stress. Expression levels **a** in two different beet varieties, **b** in root and leaf, and **C** at two developmental stages are shown. Expression levels of the control group treated for 0 h was taken as 1. $*P < 0.05$, $**P < 0.01$ (Tukey's test), In the abbreviations, "O" represents "O68," "S" represents "Shuang 6," "L" represents "leaf," "R" represents "root," "4" represents "four-leaf stage," and "6" represents "six-leaf stage." All individual reactions were done in triplicate with three biological replicates. Panel **a** presents the same data in panels **b** and **c**; three panels are used to visualize the effect of different factors on miRNA expression

growth and development (Zhang et al. 2007). Elucidating molecular changes in plants under stress can help to identify suitable germplasm resources and generate more productive and high-quality crop varieties.

Many miRNAs related to the response to salinity have been identified by combining bioinformatics and high-throughput sequencing technology (Deng et al. 2015). Degradome and miRNA sequencing of six-leaf stage seedlings treated with

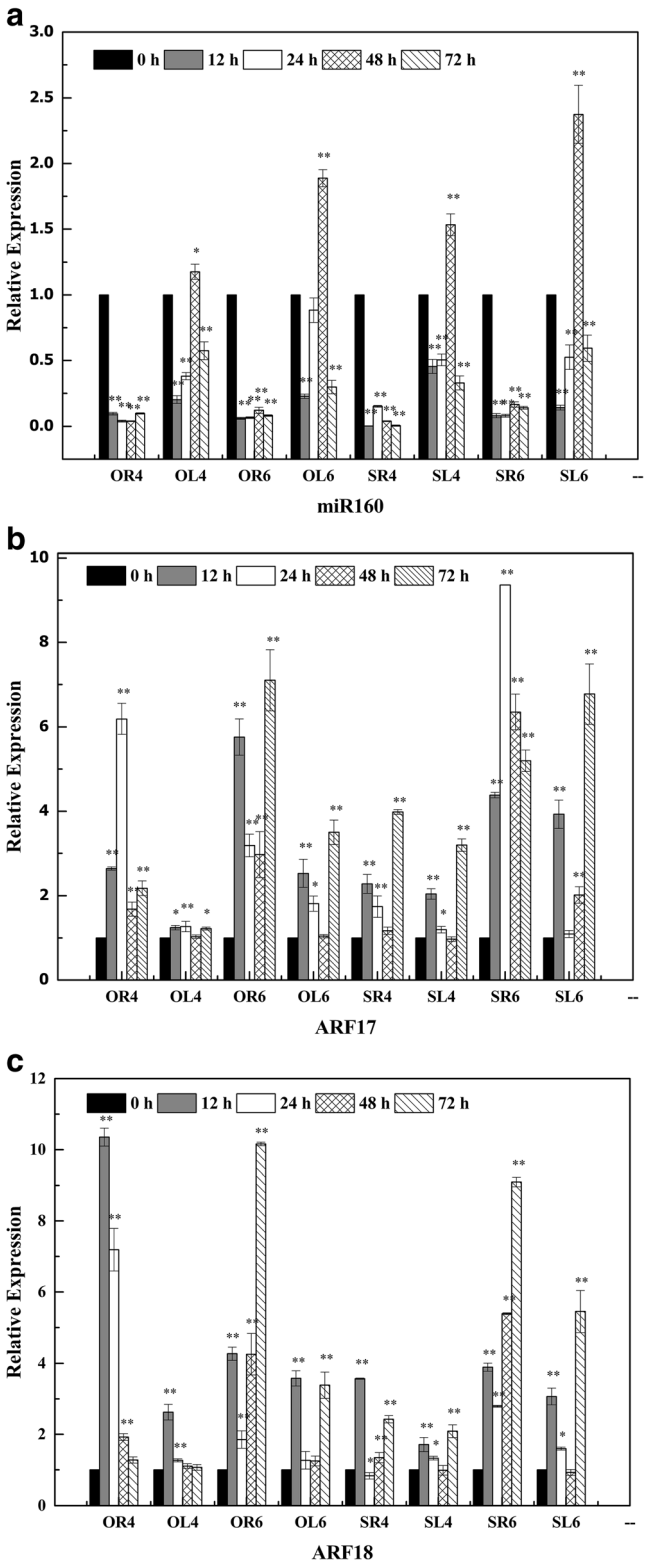

Fig. 2 Relative expression levels of miR160 and its target genes. Expression levels of **a** miR160, **b** ARF17, and **c** ARF18 are shown. *$P < 0.05$, **$P < 0.01$ (Tukey's test), In the abbreviations, "O" represents "O68," "S" represents "Shuang 6," "L" represents "leaf," "R" represents "root," "4" represents "four-leaf stage," and "6" represents "six-leaf stage." All individual reactions were done in triplicate with three biological replicates

300 mM NaCl revealed that *miR160* and *miR164* expressions were markedly downregulated under salt stress, with corresponding increases in the levels of their respective targets *ARF17/18* and *NAC21/22/100*. To identify internal and external factors influencing the expression of miR160/164 and their target genes in beet seedlings, we examined four variables including beet variety, salt stress duration, seedling growth stage, and organs. A previous study also reported a significant change in the levels of 68 miRNAs from 43 miRNA families (*miR160* and *miR164* families) in radish, as

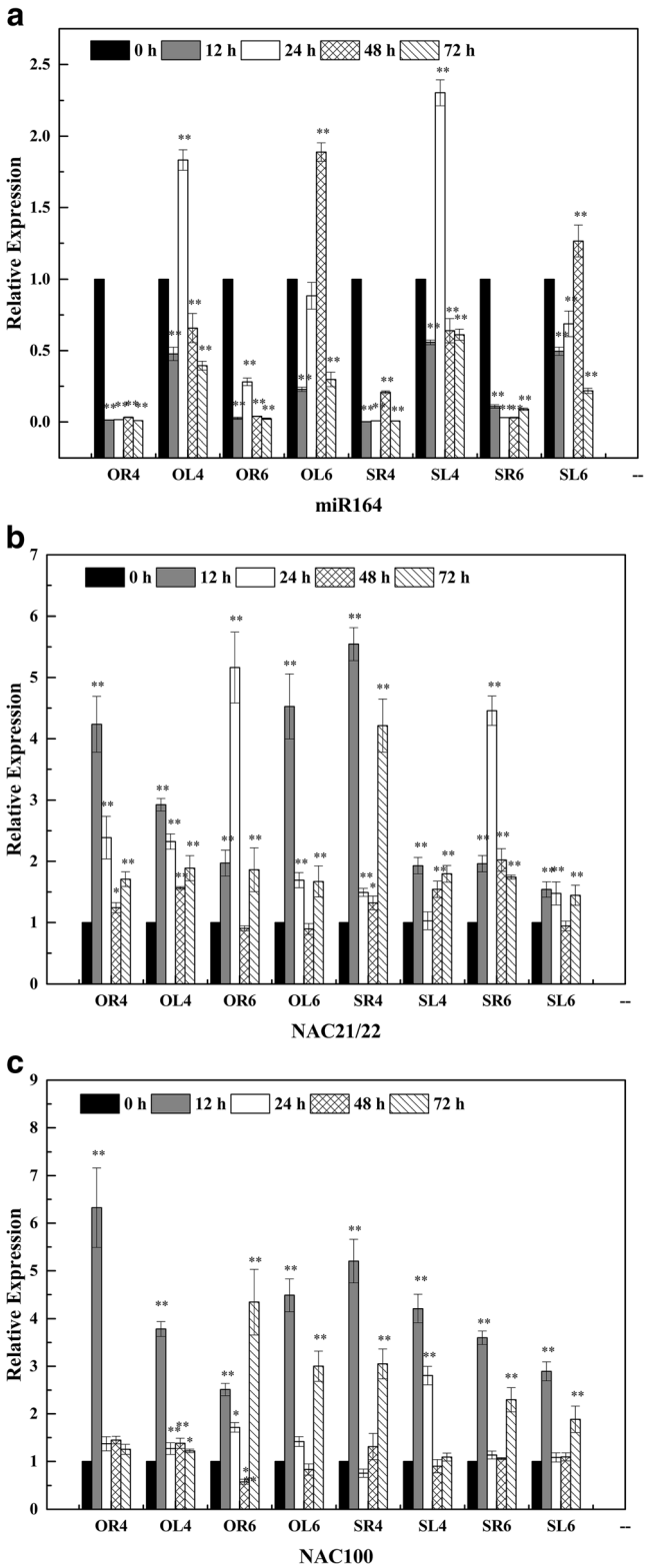

Fig. 3 Relative expression levels of miR164 and its target genes. Expression levels of **a** *miR164*, **b** *NAC21/22*, and **c** *NAC100* are shown. $*P < 0.05$, $**P < 0.01$ (Tukey's test). In the abbreviations, "O" represents "O68," "S" represents "Shuang 6," "L" represents "leaf," "R" represents "root," "4" represents "four-leaf stage," and "6" represents "six-leaf stage." All individual reactions were done in triplicate with three biological replicates

determined by high-throughput sequencing (Sun et al. 2015). In addition, *miR398* was found to be downregulated in both *A. thaliana* and *Populus euphratica* under salt stress (Jia et al. 2009). On the other hand, *miR528* expression was upregulated at high salt concentrations (Yuan et al. 2015).

ARFs are transcription factors that activate or inhibit auxin-related gene expression by specifically binding to the TGTCTC of the auxin response element (Finet et al. 2013; Liu et al. 2015; Wang et al. 2012). The interaction of signal transduction, hormone signaling, and miRNA regulation has been demonstrated

in many studies. In *Arabidopsis*, 23 ARFs have been identified, of which eight (*ARF2*, *ARF3*, *ARF4*, *ARF6*, *ARF8*, *ARF10*, *ARF16*, and *ARF17*) are miRNA target genes (Liu et al. 2010). In rice, 11 of 25 identified ARFs are target genes of miRNAs (Jian et al. 2016a). MiR160 is a highly conserved miRNA in plants that has three known target genes (*ARF10*, *ARF16*, and *ARF17*) (Bustossanmamed et al. 2013; Subramanian 2016), which play key roles in normal development, including seed germination and embryo, root, stem, leaf, fruit, and floral organ formation (Lin et al. 2015). In this study, we identified *ARF17* and *ARF18* as *miR160* target genes in beet that are associated with salt tolerance. *ARF18* modulates rice growth and development as a target gene of *miR160* (Jian et al. 2016a), but it is the first time to find *ARF18* can respond to salt stress as the target of *miR160*. In the present study, *miR160* expression was downregulated by salt stress while that of *ARF17* and *ARF18* was upregulated (Fig. 1). This inverse correlation provides evidence for the negative regulatory relationship between *miR160* and *ARF17/18*; moreover, these results suggest that plants adapt to high salinity by inhibiting *miR160* and promoting the rapid release of auxin regulators. With prolonged salt stress, the seedlings showed adaptation, as evidenced by the recovery of *miR160* expression; however, the subsequent downregulation indicated that the capacity for adaptation was limited.

NAC (NAM, ATAF1, ATAF2, and CUC2) family proteins are plant-specific transcription factors expressed in a variety of land plants. NAC proteins share the same general structure, which includes a highly conserved N-terminal NAC domain and a variable C-terminal activation domain (Jensen et al. 2008; Ernst et al. 2004; Kikuchi et al. 2000; Ooka et al. 2003). NAC proteins control plant development, response to stress, and hormone signaling (Xie and Chua 2005), and many are regulated by miR164 in response to biological and abiotic stresses. *CUC1* (At5g53950), *NAC1* (At1g56010), At5g07680, and At5g61430 were identified as target genes of *miR164*; *CUC1* and *CUC2* are involved in organ boundary formation, whereas *NAC1* has been implicated in the generation of lateral roots (Rhoades et al. 2002). In rice, six of the nine target genes predicted for *miR164* encode NAC proteins (Fang et al. 2014). Of the two *miR164* target genes identified in the present study, *NAC21/22* belongs to the NAM subspecies that is associated with resistance to stripe rust in wheat (Feng et al. 2014). Meanwhile, *Vitis vinifera* NAC21/22-like plays an important role in the formation of the upper lateral sinus in grapevine leaves (Takato et al. 2014), which can potentially be bred to enhance adaptation to environmental conditions. For the first time, *NAC100* was identified as the target gene of miR164.

The expression of *miR160* and *ARF17/18* and that of *miR164* and *NAC(21/22)/100* were not correlated in some treatment groups (Qiu et al. 2016). There are several possible reasons for this observation. Firstly, the abiotic stress response is complex and target genes may be regulated by multiple miRNAs. Secondly, components of other signaling pathways may influence target gene expression independent of miRNAs. Thirdly, miRNAs can have specific temporal and spatial expression patterns. Further study of gene regulation by miRNAs can clarify the mechanisms underlying salt stress response in plants.

It was previously reported that *miR164* and *miR167* induced the expression of *NAC1* and *ARF8*, respectively, in the roots of salt-tolerant varieties of maize (Ding et al. 2009). In this study, we found that miRNA expression varied between seedling leaves and roots. Under conditions of salt stress, a greater reduction in miRNA level was observed in roots than in leaves. This is reasonable, since roots are directly exposed to the salt solution and are sites of ion absorption. *MiR160/164* expression also varied as a function of beet

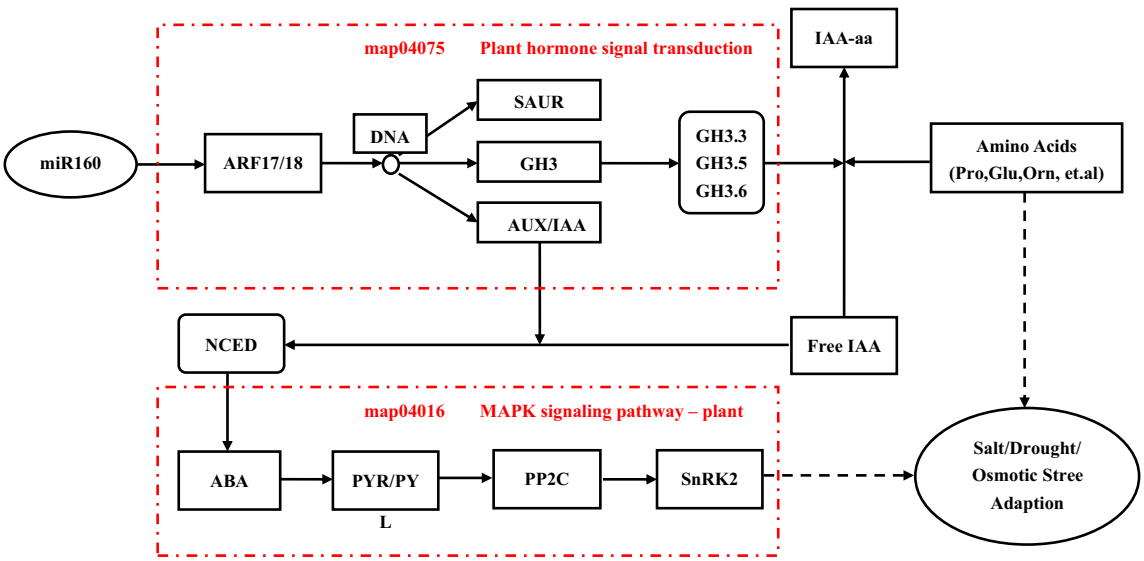

Fig. 4 Predicting the salt tolerance/drought mechanism of *miR160* and its target gene *ARF17/18*. aa, amino acids; NCED, 9-cis-epoxycarotenoid dioxygenase; PYR/PY, ABA receptor PYR/PY protein; PP2C, 2C protein phosphatase; SnRK2, SNF1-related protein kinase 2

Table 2 Sample designations for "O"68 and Shuang 6 varieties of *Beta vulgaris* seedling

Treatment	"O"68		Shuang 6	
	Four-leaf stage	Six-leaf stage	Four-leaf stage	Six-leaf stage
Root	OR4	OR6	SR4	SR6
Leaf	OL4	OL6	SL4	SL6

cultivar and at different growth stages. Comparative transcriptome analyses have revealed that genes and miRNAs associated with salt tolerance were expressed only weakly or not at all in salt-sensitive cotton under high-salt conditions (Peng et al. 2014). In our study, beet seedlings resisted stress caused by high salinity by overexpressing specific genes, with greater induction indicating a higher resistance. We therefore speculated that there would be a greater difference in the expression of miR160/164 in "O"68 and six-leaf stage seedlings. However, this was not supported by our results, suggesting that the regulation of miR160/164 expression is complex and warrants more detailed study.

The signal pathways of *miR160/164* and its target were analyzed by bioinformatics using KEGG pathway. It was found that the downregulation of *miR160* regulation of its target gene *ARF17* and *ARF18* under drought and salt stress improved the plant's ability to resist stress in three ways. Currently, the antagonistic pathways of *miR164* regulating its target genes are unclear. The mechanism of antagonism is similar to that observed in other members, which may involve regulation of expression of downstream genes in response to stress.

Table 3 Primer sequences

Primer name	Sequence 5′→3′
BvICDH-F	CACACCAGATGAAGGCCGT
BvICDH-R	CCCTGAAGACCGTGCCAT
ARF17-F	AGCATGGCTGCTACTAAACCA
ARF17-R	ACGAACTTCAAACGTACCATGC
ARF18-F	TGTTCGTTGGGATTCGGAGG
ARF18-R	ATTCCGCTCGCACTTTCTCA
NAC21/22-F	ATGGTGGAGGCTAAACTGCC
NAC21/22-R	TCCGGTGGCTTTCCAGTAAC
NAC100-F	TTCCGATTTCACCCTACC
NAC100-R	AACCTGCCACAGTTGCTC
U6- Probe	CCTGCGCAAGGATGACACCGCAT
miR160/164- Probe	Provided by the ABI

ARF, auxin response factor; *BvICDH*, *B. vulgaris* isocitrate dehydrogenase; *NAC*, no apical meristem (*N*AM)–*Arabidopsis* transcription activation factor (*A*TAF)–cup-shaped cotyledon (*C*UC)

Conclusions

In conclusion, our results demonstrate that *miR160-ARF17/18* and *miR164-NAC(21/22)/100* expression was altered in response to salt stress. We also found that changes in the expression of these miRNAs and their target genes differed according to the organ, beet variety, and growth stage. These findings provide a basis for future investigations on the mechanism of salt tolerance in beets so that hardier cultivars can be developed.

Materials and Methods

Plant Materials and Treatments

Beet varieties "O"68 and Shuang 6 were used in this study. "O"68 is an elite cultivar with higher tolerance to salinity than the sensitive variety Shuang 6. Seeds were soaked in flowing water for 12 h at 25 °C, then sown in plastic pots filled with 1/3 vermiculite, 1/3 sand, and 1/3 soil and cultured in a greenhouse under conditions of 16-h light (25 °C)/8-h dark (16 °C). At the four- and six-leaf stages, seedlings were transferred from the pots to a modified Hoagland broth and left overnight; they were then transferred to a modified Hoagland broth containing 300 mM NaCl to induce a salt stress response and incubated for 12, 24, 48, or 72 h. Control groups were treated in the same manner but without NaCl. After treatment, the whole root and leaf tissues were harvested and immediately frozen in liquid nitrogen and stored at − 80 °C until analysis. The data presented are the means of three biological replicates. The various sample groups are shown in Table 2.

Prediction of *miR160/164* Target Genes and Search of the Signal Pathways in Which They Participate

The plant small RNA target analysis server psRNATarget (http://plantgrn.noble.org/psRNATarget/) was used to predict potential targets of *miR160* (UGCCUGGCUCCCUGUAUGCCA) and *miR164* (UGGAGAAGCAGGGCAC GUGCA). The complete genome sequence of *Beta vulgaris* has not been published; therefore, the homologous alignment method was used for target gene prediction, with the maximum expectation set as 4.0. The roots and leaves of six-leaf stage seedlings treated with 300 mM NaCl were used to construct small RNA and RNA libraries for high-throughput sequencing. More accurate target identification was achieved by combining psRNATarget outputs and high-throughput sequencing miRNA and transcriptome data.

The signal pathways of miR160/164 and its target genes *ARF17* (*Beta vulgaris*; XM_010674257.2), *ARF18* (*Beta vulgaris*; XM_010695094), *NAC(21/22)* (*Beta vulgaris*;

XM_010667880.2), and *NAC 100* (*Beta vulgaris*; XM_010680181.2) were analyzed by bioinformatics technique using KEGG (http://www.kegg.jp/kegg/pathway.html) and Interpro (http://www.ebi.ac.uk/interpro/). We searched the KEGG for *ARF17/18* or *NAC21/100* to find out the signaling pathways they participated in, predicted their possible biological functions through Interpro's prediction of domain and gene ontology information, combined with the existing reports to find their final regulatory pathway to abiotic stress.

Isolation of Total RNA and miRNAs from Beet Root and Leaf

Total RNA was isolated from beet roots and leaves using the MiniBEST Plant RNA Extraction kit (Takara Bio, Dalian, China) according to the manufacturer's instructions. MiRNAs were isolated using the mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA, USA). RNA quality was evaluated by electrophoresis on a 1% (*w*/*v*) denaturing agarose gel. Total RNA and miRNA concentrations were determined on an ultraviolet-visible light spectrophotometer (Unic, Shanghai, China), and samples were then reverse transcribed into cDNAs using the PrimeScript RT Reagent kit (Takara Bio) and TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems), respectively. The 5× Taqman probes for reverse transcription of miRNA samples were designed by Applied Biosystems. cDNA samples were stored at − 20 °C.

Quantitative Real-time PCR Analysis of miRNA and Target Gene Expression

The TaqMan Gene Expression Master Mix (Applied Biosystems) and SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) were used to detect miRNA and target gene expression levels, respectively, on a 7300 Real-Time PCR system (Applied Biosystems), and made three technical repeats. The 10× Taqman probes for miR160/164 quantitative real-time (qRT-)PCR were designed by Applied Biosystems, catalog numbers are 000341 and 000344, with *U6* (GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT) serving as the reference gene. For candidate target genes, primers were designed using Primer Premier v.5.0 software (Premier Biosoft, Palo Alto, CA, USA), with *B. vulgaris isocitrate dehydrogenase* serving as the reference gene. Primer sequences are shown in Table 3.

Authors' Contributions CJ and ZS conceived and designed the study; ZS performed the experiments and wrote the manuscript; CJ, ZS, and JL analyzed the data; CD reviewed and edited the manuscript, and DC and CL provided the technical directing of experiments. All the authors agreed on the content of this manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict interest.

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