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Improving Salt-Stress Tolerance of Cultivated Rice by Overexpression of MicroRNA1861c from Dongxiang Wild Rice

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Abstract

Salinity is one of the major impediments in rice cultivation worldwide. MicroRNAs (miRNAs) are 21-24 nucleotide RNAs that function as ubiquitous regulators of gene expression in both plants and animals. Many plant miRNAs, such as miR1861, have vital roles in plant growth, development, and responses to biotic and abiotic stresses. In this study, transgenic rice plants overexpressing miR1861c in Dongxiang wild rice displayed increased salt stress tolerance compared to wild-type plants. We observed that miR1861c expression was significantly up regulated under salt stress. Consequently, the target gene of miR1861c, LOC_Os08g27860, was dramatically reduced by salt treatment. The expression of LOC_Os08g27860 was also significantly down regulated in 35S:miR1861c plants compared to wild-type plants. Our results therefore show that miR1861c in Dongxiang wild rice could serve as a positive regulator of salt stress tolerance.

Keywords: Genetic resources; MicroRNA; miR1861c; Rice; Salt stress tolerance

Introduction

Salinity is one of the major impediments to crop production as it affects crop grain composition, quality, and growth, ultimately leading to grain yield reduction [1]. It is estimated that about 20% of the earth's land and 50% of irrigated land are affected by salinity [2]. Rice (*Oryza sativa* L.) is the world's most important cereal crop and is the main food source for about half of the world's population [3]. Rice production is adversely affected by several biotic and abiotic stresses including pests, diseases, cold, drought, flood, high temperatures, and salinity [4]. Salt stress is the main limiting factor in rice production, as rice is a known salt-sensitive crop [5]. The development of rice cultivars with the capacity to tolerate salt is therefore an important way to maintain rice yield and ensure rice quality under current soil environments.

Dongxiang wild rice (*Oryza rufipogon*, DXWR) is a common wild rice discovered in the northernmost (28°14'N) area of the world [6]. DXWR has various beneficial traits, such as high yield, good quality, cold tolerance, drought tolerance, and salt tolerance [7,8]. As such, it is a valuable germplasm resource for improving rice resistance. MicroRNAs (miRNAs) are small regulatory RNA molecules of about 21-24 nucleotides in length that can negatively regulate target genes by splicing RNA and inhibiting protein translation [9-11]. The miR1861 family regulates the growth and development of rice [12]. Current studies have shown that miR1861 has roles in multiple developmental and signaling pathways related to plant hormone homeostasis and starch accumulation [13]. It is also involved in plant responses to biotic and abiotic stimuli, such as in drought stress during the nutritional period of rice [14,15]. However, there have only been a few studies the involvement of the miR1861 regulatory pathway in plant salt tolerance. Therefore, this study aimed to explore the function of miR1861c and the mechanisms behind miR1861c-mediated DXWR salt tolerance.

Materials and Methods

Plant materials and salt tolerance treatment

Dongxiang wild rice (DXWR) is ex situ conserved in Jiangxi Academy of Agricultural Sciences, Nanchang, China and the seeds of DXWR are freely available for scientific research. The seeds of Zhonghua11 (ZH11) were conserved in our lab. The seeds were submerged in water at 32°C for 48 h. The germinated seeds were sown in a bottomless 96-well plate in a container of IRRI (International Rice Research Institute) nutrient solution. The plants were placed in a growth chamber under 14 h light (26°C)/10 h dark (24°C) photoperiod, with a light intensity of 3000 lux and 80% relative humidity. For salinity stress, the 4-leaf stage seedlings were transferred into the normal solution containing 200 mM NaCl [16].

Vector construction and transformation

The 880-bp DNA fragment containing the miR1861c stem-loop structure was isolated from the genome of DXWR using the forward and reverse primer miR1861c-F/R which contained KpnI and SalI restriction sites, respectively (Table 1). The PCR products were cloned into pCAMBIA1300 vector, which was drived by CaMv 35S promoter [17]. The Hyg gene for hygromycin resistance used as a selectable marker. For subsequent plant transformation, the construct was transferred into Agrobacterium tumefaciens strain EHA105 [18]. The constructed vector was used for the rice transformation through a modified high-efficiency transformation system by co-cultivating rice calli with Agrobacterium on filter papers moistened with enriched

liquid media [19]. The transgenic plants were identified through hygromycin selection and PCR analysis using specific primers HYG-F/R (Table 1). The homozygotes of T_2 generation of transgenic plants (miROE1-3 and miROE3-4) were chosen for further study.

RNA isolation and transcriptome sequencing

Total RNA was extracted from whole plants using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality of RNA was checked on 1% agarose gel, and the quantity of RNA was measured using a Nano Drop 2000c spectrophotometer (Thermo Fisher Scientific, Lenexa, KS, USA). For transcriptome sequencing, leaf and root tissues from the transgenic plants and the wild-type plants were collected and immediately frozen in liquid nitrogen. Next, cDNA libraries were constructed from the RNA of the two samples (miROE1-3 and ZH11) and sequenced, both by Shanghai Personal Biotechnology Co., Ltd.

Reverse transcription (RT) and real-time PCR

For miRNA and mRNA reverse transcriptions, miRNA First-Strand cDNA Kit (Sangon Biotech) and Primescript™ RT reagent kit with gDNA Eraser (Takara Bio Inc) were recruited as per procedures guided by manufacturer's instructions. The qRT-PCR analysis was carry out with TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio Inc). The U6 and OsActin1 genes were used as internal reference genes (Table 1). All reactions were repeated three times for each sample. The relative fold change for each collation was evaluated by $2^{-\Delta\Delta Ct}$ [20,21].

Primers	Sequence (5'-3')
miR1861c-F	ACAGGTACCCTTCTTGCCATCCCCTATGA
miR1861c-R	ACAGTCGACTTCATGTCACCGTTGGTACG
HYG-F	CGAGAGCCTGACCTATTGCAT
HYG-R	CTGCTCCATACAAGCCAACCAC
miR1861c-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACCTCAGTT
miR1861c-qF	GCGCGATCTTGTAGCAAGA
miR1861c-qR	GTGCAGGGTCCGAGGTATT
LOC_Os08g27860-qF	CGATCTCTACGCTAATCTCT
LOC_Os08g27860-qR	ACCGAATCTACCTCCTGTC
U6-F	CGATAAAATTGGAACGATACAGA
U6-R	ATTTGGACCATTTCTCGATTTGT
OsActin-F	GTATCCATGAGACTACATACAACT
OsActin-R	TACTCAGCCTTGGCAATCCACA

Table 1: The primers used in this study.

Putative target genes prediction

Putative targets were predicted using software psRNATarget [22]. *Oryza sativa* (rice) transcript MSU Rice Genome Annotation version 7 was used as the cDNA library for the target search. The parameters were as follows: (1) expectation is less than 7.0; (2) length for complement arity scoring (HSP size) is shorter than 19 nt; (3) number of top target genes for each microRNA is less than 200; (4) target accessibility-allowed maximum energy to un pair the target site (UPE) is shorter than 25.0; (5) flanking length around target site for target accessibility analysis is 17 bp in upstream and 13 bp in downstream; (6) range of central mismatch leading to translational inhibition is 10-11 nt.

Results

Expression of miR1861c was up-regulated in the transgenic plants

To analyze the function of miR1861c, we overexpressed miR1861c in ZH11 under the control of a CaMV35S promoter. The transgenic plants were identified through hygromycin selection and PCR amplification. An expression pattern assay showed that the expression levels of miR1861c in the transgenic lines (miROE1-3 and miROE3-4) were significantly higher than those in wild-type plants (ZH11) (Figure 1).

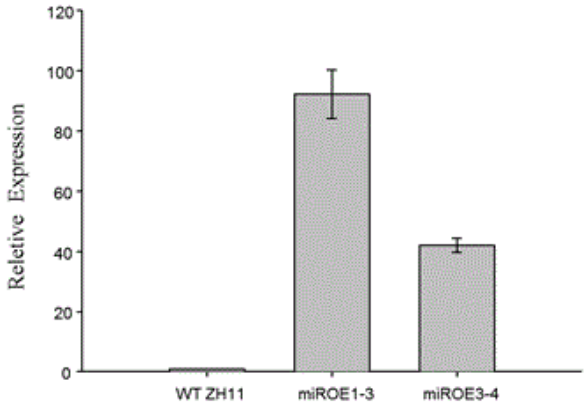

Figure 1: Comparison of miR1861c accumulation between the transgenic lines harboring 35S: miR1861c and wild-type (WT).

Overexpression of miR1861c increased plant salt-stress tolerance

We investigated the major agronomic traits when the transgenic plants matured completely. Plant height, tiller number, seed setting rate, and 1000-grain weight were not significantly affected compared to the wild-type plants (Table 2). Under normal nutrient solution, no obvious changes in morphological or developmental phenotypes were observed in the miROE1-3 and miROE3-4 transgenic lines. Furthermore, the growth of ZH11 and transgenic plants was inhibited to varying degrees when grown in a nutrient solution with 200 mM NaCl. ZH11 (control plants) started to show leaf curl symptoms while the transgenic plants remained healthy without obvious damage. Five days after salt treatment, control plants showed serious tissue damage, whereas miR1861c transgenic plants remained largely green and turgid. Moreover, the survival rate of wild-type plants dropped to below 20%, whereas survival rate of the miR1861c-overexpression (Figure 2). These results suggested that miR1861c could be utilized in rice breeding for improving salt tolerance.

Agronomic trait	ZH11 (WT)	miROE1-3	Compared with WT (%)
Plant height (cm)	103.3 ± 0.6	101.3 ± 1.5	−1.9
Tiller number	9.0 ± 1.0	8.3 ± 1.5	−7.8
Seed setting rate (%)	88.5 ± 2.9	85.2 ± 2.3	−3.7
1000-grain weight (g)	23.5 ± 0.4	22.4 ± 0.5	−4.7

Table 2: Comparison of the major agronomic traits between the transgenic plants (miROE1-3) and its wild-type plants (ZH11).

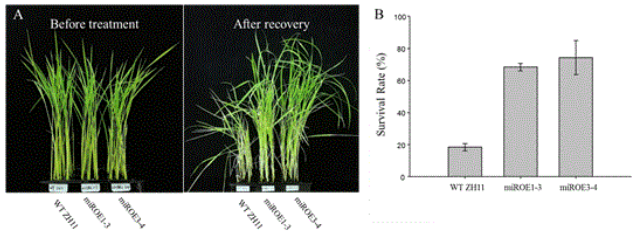

Figure 2: Improved tolerance to the salt stress in the transgenic plants (A) Phenotypic comparison of the transgenic plants and wild-type plants (B) Survival rate comparison of the transgenic lines and wild-type plants.

Putative target genes of miR1861c

To investigate the molecular mechanism of miR1861c in plant responses to salt stress, we searched for the putative target genes of miR1861c using the psRNATarget website; detailed information on the putative target genes was presented in Table S1. Furthermore, to gain a better understanding of the mechanism underlying salt stress tolerance in the transgenic plants, strand-specific RNA sequencing was carried out, and differentially expressed genes (DEGs) between wild-type plants and transgenic plants were analyzed. In total, 2085 DEGs were identified; out of these, 1388 were up regulated and 697 were down regulated in the transgenic plants (Table S2). Transcriptome sequencing analysis and psRNATarget enabled the identification of one putative miR1861c target gene, namely, LOC_Os08g27860. qRT-PCR analyses showed that the expression of LOC_Os08g27860 was down regulated in the miR1861c transgenic plants (Figure 3).

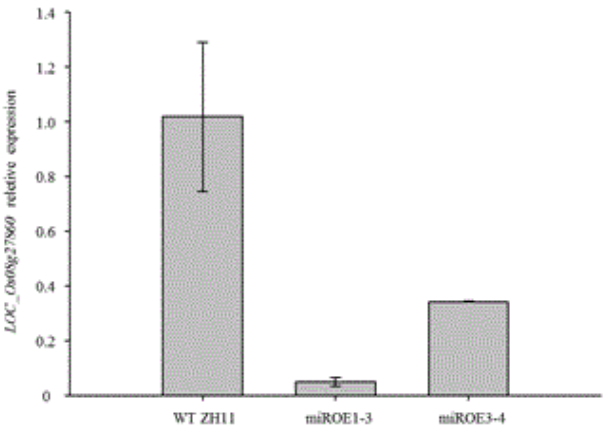

Figure 3: Comparison of the target gene LOC_Os08g27860 accumulation between the transgenic lines and wild-type plants.

Discussion

Rice (*Oryza sativa* L.) is one of the world's major staple crops. However, rice is often exposed to drought, salt, and other stresses, which may severely affect rice yield [23]. Globally, salt stress is one of the most serious abiotic stresses that threaten both agriculture and the environment, as it can reduce nutrient solubility, increase external osmotic pressure, and destroy ion balance [24]. Salinity also affects the stability of cell acidity and alkalinity, thus restricting rice growth and development at all stages [25,26].

DXWR has many genes that are useful in cultivated rice, including stress resistance to phosphorus deficiency, cold, salt, and drought; as such, DXWR material is considered as an important germplasm resource for rice breeding [27]. A previous study revealed that OsHKT1, OsHKT7, and numerous transcription factor genes, including ZFP, NAC, MYB, and AP2/ERF were differentially expressed in DXWR under salt stress, suggesting that multiple genes were responsible for DXWR salt tolerance [28]. Thus, DXWR is an important germplasm resource for breeding salt-tolerant rice varieties.

New studies suggest that miRNAs and their targets may serve as the main governing factors in response to various stresses including drought, salinity, extreme temperatures, nutrient homeostasis, hypoxia, oxidative stress, and mechanical stress [29-31]. For example, miR319 has been shown to target the plant-specific TCP transcription factor gene in order to enhance salt tolerance by increasing the waxy content and water retention of leaves [32]. The main floral regulator miR156 and its SPL targets have been shown to be involved in salinity tolerance [33]. Moreover, miR390 overexpression increased salt tolerance in plants by regulating ARFs, which are key regulators of the auxin pathway [34]. Analyses of transgenic plants with modulated miR408 expression levels revealed that higher miR408 expression levels lead to improved tolerance to salinity stress [35].

In the present study, we preliminarily analyzed the role of miR1861c in regulating the rice response to salinity and the underlying molecular mechanisms behind this response using transgenic approach. First, we

generated transgenic rice plants by overexpressing the miR1861c gene of DXWR to evaluate the effect of miR1861c under salt stress. Our data showed that this transgenic rice had enhanced tolerance to salt stress. We then identified one miR1861c target gene, LOC_Os08g27860, using transcriptome sequencing and the psRNATarget database. Using qRT-PCR, we found that LOC_Os08g27860 expression was significantly down regulated in the transgenic plants. These results suggest that LOC_Os08g27860 could be a negative regulator of salt stress tolerance in rice.

Conclusion

In conclusion, we identified a miRNA involved in DXWR salt tolerance. We studied its overexpression phenotype and preliminarily explored its corresponding regulatory mechanism. Taken together, our results may provide novel molecular strategies that can be used to breed rice variants with improved salt stress tolerance.

Acknowledgement

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