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Master's Thesis of Science in Agriculture

Overexpression of *OsmiR171f*, a drought-inducible microRNA, enhances drought tolerance in rice

마이크로RNA 171f 과발현 벼의 내건성 향상에 대한 연구

February 2019

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Abstract

Overexpression of *OsmiR171f*, a drought-inducible microRNA, enhances drought tolerance in rice

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Abiotic stresses, mainly drought, salinity and cold, are the major constraints of agricultural productivity. Recent studies have shown that the stresses induce aberrant expression of many noncoding RNAs including microRNAs (miRNAs). Generally, plant miRNAs are involved in developmental processes and regulation of drought-responsive genes. It has been previously identified that *OsmiR171f* was induced upon exposure of rice plants to drought stress. This study was aimed to examine whether the *OsmiR171f* is responsible for drought tolerance. In doing so, two vectors were generated: one with *OsmiRNA171f* gene connected to the constitutive GOS2 promoter and the other with CRISPR/Cas9 system with a sgRNA targeting specifically to the stem-loop region of *OsmiR171f*. The *Agrobacterium*-mediated transformation of rice produced several lines of transgenic plants. A total of four overexpressing lines and one knockout plant were chosen for further analysis. Overexpression of *OsmiR171f* conferred drought resistance as demonstrated in soil by withholding of water and in polyethylene glycol (PEG) solution by modulating osmotic potential. Moreover, it is widely known that stress-induced miRNAs exert their functions by downregulating

their target genes, thereby emphasizing that prediction of miRNA targets is crucial. To determine the molecular mechanism by which *OsmiR171f* regulates its downstream targets, the putative targets were predicted and validated in different temporal-spatial manners using qRT-PCR. It was observed that the transcript level of *OsmiR171f* displayed an inverse relationship with that of its putative targets, suggesting *OsmiR171f* as a negative regulator of the targets. Based on the gene expression patterns and phenotypes, it can be speculated that the overexpressing *OsmiR171f* gene mediates drought tolerance in rice by regulating its targets and that this can aid crop improvement.

Keywords: microRNA, abiotic stress, drought tolerance, target genes, overexpression, *Oryza sativa*

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List of Abbreviations

ABA	Abscisic acid
bar	*Bialaphos-resistance gene* encodes a *phosphinothricin acetyl transferase*
CaMV 35S	Cauliflower mosaic virus 35S promoter
cDNA	Complementary DNA
Dip1	*Dehydration stress inducible protein*
GFP	Green fluorescent protein
GOS	Gene from *Oryza sativa* 2
LB	Left border
mRNA	messenger RNA
miRNA	microRNA
nos	nopaline synthase gene
NT	Non-transgenic
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
qRT-PCR	quantitative real-time PCR
RB	Right border
RbcS	*Rubisco small subunit*
RNA	Ribonucleic acid

Introduction

Due to their sessile nature, plants have developed defense mechanisms to cope with abiotic and biotic stresses. Drought, a major adverse environmental condition, extensively affects plant growth and development and even limits agricultural production. There are ongoing attempts to make drought-tolerant crops using biotechnology. One of them is microRNA (miRNA) which plays an essential role in modulating the expression of drought-responsive genes. For instance, *miR159a* and *miR169g* showed a strong drought-induction in *Arabidopsis thaliana* and *Oryza sativa*, respectively. Also, for most of miRNA target genes are transcription factors that miRNAs' downregulating their target genes possibly results in inhibition of drought-responsive protein synthesis (Reyes and Chua, 2007; Zhao et al., 2007).

miRNAs are a highly conserved class of small non-coding RNAs with a length of 20-24 nucleotides; their maturation necessitates complex processes in vivo. Transcribed from a MIR gene by an RNA polymerase II, the primary miRNA transcript (pri-miRNA) forms an imperfect stem-loop duplex. Then, a Dicer-like1 (DCL1), an RNase III enzyme, facilitate the pri-to-pre-miRNA conversion and mature miRNA processing through a two-step cleavage in the nucleus (Reinhart et al., 2002; Voinnet, 2009). The mature miRNA duplex is composed of an active miRNA strand and a complementary miRNA*, a passenger strand, which was thought to have no specific function and to be degraded. In certain cases, two mature miRNAs are produced instead of one passenger strand. The mature miRNA is exported via HASTY to the cytosol where it is preferentially incorporated into the RNA-induced silencing complex (RISC), and guides Argonaute (AGO) to cleave the target mRNA through base-paring. Since both of strands can be loaded into the (RISC), accumulate in specific tissues, and act as important regulatory factors, the released strands are termed with the '5p' and '3p' suffixes according to their position in the hairpin

precursor. Whereas the 5p strand is present in the forward (5'-3') position, the 3p-arm is located in the reverse position with a near-perfect complementarity to the 5' strand. (Liu et al., 2017).

The miRNA transcription is not followed by translation into proteins but regulates gene expression of their target genes at the post-transcriptional level. Their sequence-based interactions with target mRNAs result in either target cleavage or translational inhibition (Phelps-Durr, 2010; Khandal et al., 2017). A number of evidence indicate that plant miRNAs are involved in various physiological processes: development, nutrient homeostasis, signal transduction, and abiotic/biotic stress responses by changing the amount of themselves or that of their target mRNA transcripts (Ding et al., 2013; Ferdous et al., 2015). Originally identified from *Arabidopsis*, plant miRNAs are known to serve as imperative riboregulators during plant stress responses (Zhang, 2015). Unlike animal miRNAs, which have imperfect matches with their targets, most plant miRNAs showed near-perfect complementarity to their targets (Bartel, 2004) so the identification of target genes is crucial for functional analysis of microRNAs. The miRNA sequences can be analyzed for target prediction using various software platforms available, such as psRNATarget (Dai and Zhao, 2011). There are several examples of stress-driven miRNAs regulating their target gene mRNA (Shriram et al., 2016). Overexpression of *OsmiR319* downregultated its target genes and enhanced cold tolerance in rice (Yang et al., 2013) and constitutive expression of *miR172c* and *miR394a*, which target AP2-like transcription factor and F-box protein, respectively, showed enhanced drought tolerance in Glycine max (Li et al., 2016; Ni et al., 2012). Also, under salt stress, decrease in *AtmiR398* level allowed upregulation of a positive regulator, superoxide dismutase 1, enabling plant stress tolerance (Sunkar et al., 2007).

In the previous study, transcriptome profiling of drought-responsive non-coding RNAs and their targets was performed and revealed that 66 miRNAs changed their expression substantially under drought treatments (Chung et al., 2016). Among

those candidates, the *OsmiR171f* was selected for analyzing its biological functions in this current study. In doing so, *OsmiR171f* overexpressing transgenic rice plants using GOS2 constitutive promoter were prepared. Also, CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats /CRISPR-associated Cas9 endonuclease)-mediated genome editing was used to develop knockout mutants. In brief, the drought-tolerant visual tests on *OsmiR171f* overexpression, knockout transgenic and non-transgenic plants were performed using a PEG (polyethylene glycol)-mediated method and natural drought stress mimicry treatment. The expression patterns of the reference genes, *OsmiR171f*, and its target genes were analyzed using qRT-PCR. These results showed that overexpression of *OsmiR171f* improved drought tolerance of rice.

Material and methods

1. microRNA identification and Target prediction

The precursor and mature sequences of *OsmiR171* from rice were obtained from miRBase, (http://www.mirbase.org), and sequence alignment of precursor *OsmiRNA171* was carried out using web based ClustalW (Thompson et al., 1994). The identified mature sequences were used for the prediction of target genes with a plant microRNA Target Analysis Server, psRNATarget, (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011). The putative functions of the identified target genes were characterized after Basic Local Alignment Search Tool searches of mature *OsmiR171f* sequences against that of another database Rice Annotation Project Database RAP-DB (http://rapdb.dna.affrc.go.jp).

2. Vector construction and rice transformation

To generate *OsmiR171f* overexpressing transgenic plants, the *OsmiR171f* sequence was amplified form rice (*Oryza sativa* L. ssp. Japonica cv Dongjin and Hwayoung) cDNA using DNA polymerase PrimeSTAR (TaKaRa). The amplified *OsmiR171f* fragment was ligated into the rice transformation vector, p700-GOS2 for constitutive overexpression (Jeong et al., 2010). For the suppression of *OsmiR171f* expression in rice, a recombinant CRISPR/Cas9 construct was generated with a rice codon-optimized *Streptococcus pyogenes* Cas9 expression vector carrying two Cauliflower mosaic virus 35S (CaMV 35S) promoters whereas a single guide RNA (sgRNA) was derived by a U3 promoter. The transgenic rice was obtained by *Agrobacterium tumefaciens* (LBA4404)-mediated transformation (Hiei et al., 1994).

3. Drought-stress treatments

Transgenic and non-transgenic (NT) (*Oryza sativa* L. ssp. Japonica cv Dongjin and Hwayoung) seeds were uniformly germinated on MS (Murashige and Skoog) media and placed in dark (28°C) for 3 days. Seedlings were transferred into soil pots (4 × 4 × 6 cm; 3 plants per pot) and grown in a greenhouse (16 h-light / 8 h-dark cycle) at 30°C. At the age of 3 weeks after transplanting, the plants were treated with drought stress by withholding water for 3 days and recovered by re-watering for 7 days. The soil water content in each pot was adjusted to nearly 80%. Soil moisture was monitored during the drought treatment using a Soil Moisture Sensor SM150 (Delta-T Devices). Leaves were harvested at 0 and 3 days of treatment and 7 days after re-watering, kept in liquid nitrogen and stored at -80°C.

Rice was germinated on MS medium at 28°C, kept in dark for 3 days and transferred into light conditions for 4 days. Seedlings were transferred to Yoshida's culture solution and grown in a chamber with a 16-h light (30°C) / 8-h dark (26°C) photoperiod. After 14 days of incubation in culture solution, which was renewed every 4 to 5 days, the seedlings were used for PEG-induced drought experiments. The seedlings were transferred from Yoshida's culture solution into 22.5% PEG-8000 solution. The leaves and roots of the treated plants were pooled at 0, 4 and 8h and ground in liquid nitrogen. Drought-induced phenotypes were visualized by imaging of treated plants using a Canon EOS 600d camera.

4. Agronomic trait analysis of rice plants grown in a paddy field

Evaluation of the agronomic traits of the transgenic and nontransgenic (NT) plants under normal field conditions was performed in a rice paddy field at the Kyungpook National University, Gunwi (128:34E/36:15N), Korea. The yield parameters were scored from 30 plants collected from three different plots for normal conditions.

5. Quantitative reverse transcription PCR

The pooled and frozen leaf and root tissues were ground to a fine powder, and the total RNA was isolated from using Trizol reagent (Invitrogen). 2 μg of total RNA was used to synthesize the first strand cDNA using a RevertAid™ First Strand cDNA Synthesis Kit. To analyze gene expression levels, Real-time PCR analysis was carried out by thermocycling and fluorescence detection using an Mx3000P Real-Time PCR machine (Stratagene, CA, USA) with the Solg™ 2x real-time PCR smart mix and EvaGreen (SolGent, Deajeon, Korea). The reactions were performed at 95°C for 15 min followed by 40 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 20 s. For normalization, the *OsUbi1* (Os06g0681400) gene was co-amplified as an internal control. The gene-specific primers used for the qRT-PCR are listed in Table 2.

6. Reverse Transcription PCR assay

After withholding water for one, two and three days, total leaves of 10 whole plants were sampled and kept in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen) and purified with an RNeasy (Qiagen). DNase I (Invitrogen) was treated to remove contaminating genomic DNA from the sample, according to the manufacturer's instructions. The cDNAs were synthesized and used to determine expression levels of miRNAs and target genes. RT-PCR conditions are as follows: 94°C for 2 min, 94°C for 30 s, 57.5°C for 30 s, 72°C for 30 s, and 72°C for 30 s, 33 cycles. The primer pairs used for RT-PCR are listed in Table 2.

7. Quantification of mature microRNA

To detect mature miRNAs, stem-loop reverse transcription and RT-PCR were performed as described in protocol (Varkonyi-Gasic et al., 2007). Digested with

RNAase-free DNase I (Promega), 200 ng of RNA was transcribed into cDNA using gene-specific RT primers and a thermostable reverse transcriptase (Invitrogen). The miRNA was hybridized with the miRNA-specific stem-loop RT primer and reverse transcribed. A 20 μl mixture, containing 50 U Superscript III RT (Invitrogen), 4 U RNaseOUT (Invitrogen) and 1 μM stem-loop RT primer, was incubated at 16°C for 45 min, and followed by 60 cycles of 30°C for 45 s, 42°C for 45 s, and 50°C for 1 s. Then quantitative RT-PCR, using EvaGreen 2×qPCR MasterMix-ROX(Abm), with a miRNA-specific forward and universal reverse primer was used for quantifying the RT products. The reactions were performed in triplicate and the expression was normalized to *OsUbi1*. The primer sequences are presented in Table 2.

Result

1. Identification of *OsmiR171f*

Previously, upregulation of *OsmiR171f* under drought stress, and presence of the MYB binding site, a drought response cis-acting element in *OsmiR171f* were identified (Chung et al., 2016; Zhu et al., 2015). To characterize *OsmiR171f*, the stem-loop sequence was obtained from the miRBase (a miRNA database, http://www.mirbase.org/). It was predicted that the primary transcript of *OsmiR171f* is encoded from the rice chromosome 3, and resides in the same locus with a non-protein coding transcript (Os03g0733200) (Figure 1A). Processing of the pri-miRNA brings forth a 21 nt-long stem-loop structure as shown in Figure 1B, posessing two mature sequences presented in blue letters. Also, the precursor sequences of *OsmiR171* family members were aligned using clustalW (https://www.genome.jp/tools-bin/clustalw) (Figure 1C). From the multiple sequence cluster, high sequence conservation among nine family members was detected.

2. Expression profiling of *OsmiR171f*

In determination of abiotic stress responses, the *OsmiR171f* transcript level was examined using qRT-PCR analysis. Two-week-old rice seedlings (*Oryza sativa* L. ssp. Japonica cv Dongjin) were exposed to drought (air-drying), high salinity (400 mM NaCl), low temperature (4°C) and abscisic acid (100 uM ABA) for the indicated time courses (2, 4 and 6 hours). Among these four stresses, the *OsmiR171f* expression level elevated under ABA treatments in leaves and under drought and ABA stress in root tissues (Figure 2A). Since the regulatory role of *OsmiR171f* can be speculated from expression in developmental stages, the organ-specific expression profiles from coleoptile to flowering stages were analyzed. Transcript accumulation of *OsmiR171f*

was detected in coleoptile, root, and flower (Figure 2B). These expression changes upon different stress treatments and in developmental stages reflect the possible role of *OsmiR171f*.

3. Overexpression of *OsmiR171f* enhances drought resistance

To investigate physiological functions of *OsmiR171f*, transgenic rice plants overexpressing pri-*OsmiR171f* were generated using two cultivars, Dongjin and Hwayoung. Driven by the whole-body expression promoter, GOS2, the overexpression vector (Figure 3A) was used to transform rice by using the *Agrobacterium*-mediated methods (Hiel et al., 1994); the synthetic green fluorescent protein (sGFP) was translationally fused to *OsmiR171f* with a stress-inducible promoter, Wsi18, for detecting transgenic plant seeds using a luminescent-image analyzer (Figure 3B). Overexpression of *OsmiR171f* in the transgenic rice plants was determined by the qRT-PCR analysis using total RNA of two-week-old leaf tissues. The increased expression levels were found in all eight transgenic lines of Dongjin, and four lines (2, 3, 5 and 9) with similar expression levels were chosen to study further (Figure 3C).

To observe a drought tolerant phenotype, the non-transgenic (NT) and transgenic rice seedlings were germinated on a MS agar medium, transferred to Yoshida solution for three weeks of growth, and exposed to PEG-mediated dehydration stress. Three hours of immersion in the 22.5% PEG-8000(m/v) solution caused a drought-induced symptom such as leaf rolling, and the symptom was less severe in *GOS2:miR171f* lines than the NT plants indicating drought tolerance of the transgenic plants. Through the two days of rehydration, a faster recovery was observed in the *OsmiR171f* -overexpressing lines (Figure 4A). Along with the drought-tolerant phenotypes, expression level assessments of *Dip1* (Dehydration stress-inducible protein 1; Os02g0669100), a drought-inducible marker gene, and those of *RbcS1* (Small subunit

of Rubisco; Os12g0274700), a drought-repressed gene, were performed. As the *Dip1* expression increased, the *RbcS1* expression decreased under the three hours of PEG-solution treatment; this inverse relationship between two marker genes indicated that PEG-induced drought stress mimicry was properly applied to the treated plants so that the overexpression lines displayed dehydration tolerance over the NT control plants (Figure 4B).

To determine whether the genetic background influences the role of *OsmiR171f*, phenotypic evaluation was performed in a greenhouse on the *GOS2:miR171f* plants with two cultivars, Dongjin (upper panel) and Hwayoung (lower panel of Figure 5A). Four-week-old NT and transgenic plants were subjected to drought stress by withholding water for 2 days; both of the *OsmiR171f*-overexpressing lines displayed improved drought tolerance, compared to their NT controls. The drought-induced visual symptoms, including wilting and leaf rolling, were monitored to set a proper time to induce recovery of the plants by re-watering. Overexpression and drought responsiveness of *OsmiR171f* and NT were identified at the molecular level (Figure 5B). Consistent with the phenotypes, about a 5-fold differance of recovery rate was observed after 9 days of rehydration (Figure 5C). In general, manipulation of miRNA may produce side effects, including growth retardation, so the yield components of transgenic plants were evaluated under field conditions. In doing so, four independent T_2 *OsmiR171f* transgenic and NT plants were planted in a rice paddy field and grown to maturity. The plant height, filling rate, and 1000 GW of the *GOS2:miR171f* plants were similar to that of NTs, indicating no serious defects in growth and reproduction from overexpression of *OsmiR171f* (Figure 5D).

4. Development of CRISPR/Cas9-mediated *OsmiR171f* mutants

To examine a loss-of-function mutation of the *OsmiR171f* gene, knockout mutants were generated using the CRISPR/Cas9 approach. A rice codon-optimized *Streptococcus pyogenes* Cas9 expression vector was constructed carrying two Cauliflower mosaic virus 35S (CaMV 35S) promoters and an U3 promoter-driven single guide RNA (sgRNA) (Figure 6A). A 20-nt long guide RNA enables the nuclear-localized Cas9 protein to produce a double-strand break (DSB) at the target sequence allowing insertions and/or deletions (indels) during repair process. This repair mechanism can mutate one or both of alleles for each gene in a way that the four different mutant genotypes can be formed: wild-type (no mutation), monoallelic (mutation on one allele), biallelic homozygous (same mutations on both alleles) and biallelic heterozygous (different mutations on two alleles) (Figure 6B). Here, for the suppression of *OsmiR171f* expression, a gRNA was designed to target the mature *OsmiR171f* sequence and eventually mutated the gene. To verify *OsmiR171f* knockout mutation, the mutagenized sequences were PCR-amplified and analyzed by aligning with the wild-type sequence. About half of the fifty independent variants had mutation in the expected region. The biallelic homozygous knockout (KO) line 2, was selected to study further. A deletion of 16 bp (lowercase letters shaded in gray) found in the T_0 plant of the KO line 2 was maintained in all the progeny lines of the T_1 generation (Figure 6C). Also, mRNA transcript level profiling implied preservation of the primary *OsmiR171f*, and a considerable decrease in both of mature miRNAs, 5p and 3p, (Figure 6D). With a substantial deletion, a secondary structure of the mutated *OsmiR171f* was predicted that the remaining mature 5p sequence (shaded in light blue) presented in the loop, instead of in the stem where the enzymatic activity usually occurs (Figure 6E). This implied that the remaining 3p strand might not function as well. Taken together, the CRISPR/Cas9-mediated knockout mutants of the *OsmiR171f* were obtained and these mutations were stably inherited to the next generation.

5. Phenotypic difference between *OsmiR171f* overexpression and knockout plants under drought stress

To understand the mechanism by which the *OsmiR171f* conferred drought tolerance, the phenotypic difference of *OsmiR171f* overexpressing and knockout transgenic plants under drought stress conditions were investigated (Figure 7A). Four-week-old *GOS2:miR171f* (OX) and loss-of-function mutant (KO) plants were exposed to drought stress for two days by withholding water while soil moisture content decreased uniformly over time (Figure 7C). The KO2 displayed rapid visual symptoms caused from drought stress whereas the NT showed a relatively acceptable performance. Nonetheless, drought tolerance of the *OsmiR171f* overexpressing lines (2, 3, 5 and 9) was evident in both visualization and survival rate (Figures 7A and 7D). Moreover, infra-red (IR) thermography was applied to show a consistent result. Likewise, a thermograph in Figure 7B showed a consistent result. In general, plants with less water display higher temperature, and emit more heat and infra-red radiation that the red color in the plants indicates a higher temperature, a less water content and a less stomatal conductance (Kwon et al., 2015). Here, temperature variance was detected under drought conditions among the different lines; the knockout mutant (24.9°C) and non-transgenic (24.5°C) plants displayed a higher temperature than did the overexpression lines (20.3 to 22.9°C). Together with results of visual test, it was indicated that the *OsmiR171f* overexpression contributed in enhancing plant drought tolerance during the vegetative stage (Figure 7B).

6. Target gene prediction of *OsmiR171f*

Processing of the Dicer-like 1 endonuclease gives rise to a duplex that is composed of two mature RNAs: 5p and 3p (gray and light blue highlighted, respectively, in Figure 8A). From the expression analysis, the precursor and mature miRNAs were unevenly expressed (Figure 8B). Here, the *OsmiR171f*-3p showed a constitutive, high level of

expression, indicating its role as a guide strand under normal growth conditions. Also, the expression *OsmiR171f*-5p suggested it possible role. As previously mentioned, target prediction is critical in determining function of miRNAs. Having different inclination and complementarity to different target sites, two *OsmiR171f* mature sequences were utilized to predict their target genes and present the miRNA-target pair alignments using the psRNAtarget web server (http://plantgrn.noble.org/ psRNATarget/) (Table 1). To examine the expression profiles of *OsmiR171f* and its target genes, an RT-PCR analysis was performed on NT plants under a progressive drought treatment (Figure 9A); drought stress was initiated four weeks after germination and lasted for 3 days. Under water-deficit conditions, *OsmiR171f* exhibited an increased expression while the putative target genes showed a gradual repression in their transcript levels during the drought treatments. This antagonistic manner of expression was consistent with the previously reported RNA-seq data (right panel) in Chung et al. (2016) and together suggesting drought induction in the putative target gene expression. A constitutive gene, *OsUbi1* was used as an internal control to verify the quantitative amplification of each transcript. In addition, to validate the predicted target genes the expression levels of mature *OsmiR171f* and its putative target genes in various tissues at different developmental stages were measured using qRT-PCR. When the *OsmiR171f*-5p sequence targets CSTF77 and non-protein coding transcript, the other strand, *OsmiR171f*-3p matches the ARC3, DEGP10, SCL6-1 and SCL6-2 gene sequences. While the mature *OsmiR171f*-5p showing up-regulation in roots and flowers, its corresponding target mRNAs accumulated in coleoptiles, shoots, and leaves (Figure 9B). Also, the root and flower tissue expressions were increased in the mature *OsmiR171f*-3p, and its predicted targets displayed an inverse relationship in expression patterns, confirming the target predictions (Figure 9C).

7. *OsmiR171f* targets several genes and modulates their expressions

Generally, miRNA regulates target gene expression at the post-transcriptional level. Here, the transcript levels of six putative target genes in *OsmiR171f* -overexpressing plants were investigated using the qRT-PCR. Under normal growth conditions, the *GOS2:miR171f* line 2 showed a slight or no significant decrease in target gene expression whereas most of the transgenic line 3 displayed a major reduction in mRNA levels of target genes. Nonetheless, drought induction caused the most of target genes decreased their transcript levels in the leaves of *OsmiR171f*-overexpressing plants, compared to the NT controls (Figure 10). In sum, target gene expression was lower in *GOS2:miR171f* lines than in NT that *OsmiR171f* could cleave the predicted target mRNAs directly.

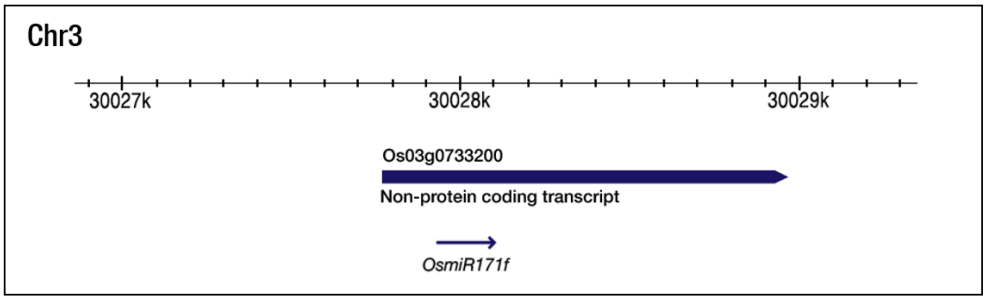
Figure 1. Characterization of *OsmiR171f*.

(A) The scheme of *OsmiR171f* gene locus. Chr, chromosome.

(B) The stem-loop structure of *OsmiR171f*. The mature RNA sequences are highlighted in blue. (upper: *OsmiR171f*-5p; lower: *OsmiR171f*-3p)

(C) The sequence cluster of *OsmiR171* family in rice. The mature sequences of miRNAs are underlined and the consensus sequences are shaded.

A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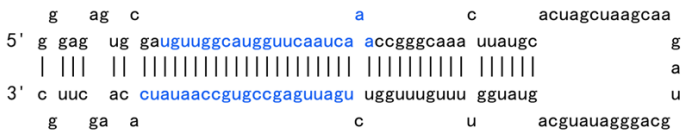
30028k
30029k
Os03g0733200
Non-protein coding transcript
OsmiR171f

B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5' g gag ug gauguuggcaugguucaauca accgggcaaa uuaugc g
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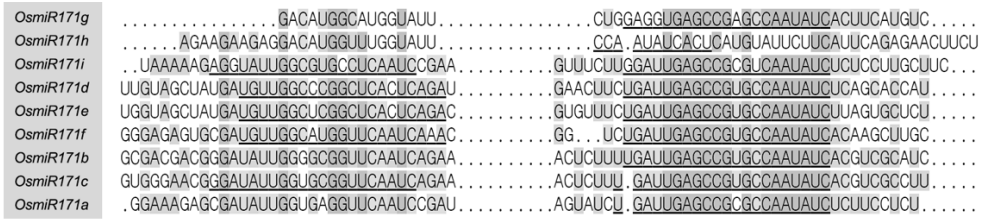
g ga a c u acguauagggacg

C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OsmiR171h .....AGAAGAAGAGGACAUGGUUUGGUAUU.....CCA.AUAUCACUCAUGUAUUCUUCAUUCAGAGAACUUCU
OsmiR171i ..UAAAAAGAGGUAUUGGCGUGCCUCAAUCCGAA.....GUUUCUUGGAUUGAGCCGCGUCAAUAUCUCUCCUUGCUUC...
OsmiR171d UUGUAGCUAUGAUGUUGGCCCGGCUCACUCAGAU.....GAACUUCUGAUUGAGCCGUGCCAAUAUCUCAGCACCAU.....
OsmiR171e UGGUAGCUAUGAUGUUGGCUCGGCUCACUCAGAC.....GUGUUUCUGAUUGAGCCGUGCCAAUAUCUUAGUGCUCU.....
OsmiR171f GGGAGAGUGCGAUGUUGGCAUGGUUCAAUCAAAC.....GG...UCUGAUUGAGCCGUGCCAAUAUCACAAGCUUGC.....
OsmiR171b GCGACGACGGGAUAUUGGGGCGGUUCAAUCAGAA.....ACUCUUUUGAUUGAGCCGUGCCAAUAUCACGUCGCAUC.....
OsmiR171c GUGGGAACGGGAUAUUGGUGCGGUUCAAUCAGAA.....ACUCUUU.GAUUGAGCCGUGCCAAUAUCACGUCGCCUU.....
OsmiR171a .GGAAAGAGCGAUAUUGGUGAGGUUCAAUCCGAU.....AGUAUCU.GAUUGAGCCGCGCCAAUAUCUCUUCCUCU.....

Figure 2. Expression analysis of pri-*OsmiR171f* by qRT-PCR.

(A) The relative expression patterns of *OsmiR171f* in response to four abiotic stresses. Two-week-old seedlings' leaves and roots of non-transgenic (NT) were exposed to air-drying (Drought), 400 mM NaCl (High salinity), 100 uM abscisic acid (ABA) and at 4°C (Low temperature). h, hour.

(B) The organ-specific *OsmiR171f* expression at the various developmental stages in the NT (*Oryza sativa* L. ssp. Japonica cv Dongjin). The rice *Ubi1* (Os06g0681400) was used as an internal control for normalization. The error bar represent the mean value and the ± standard deviation (SD) of three independent experiments (n=3). DAG, Days after germination.

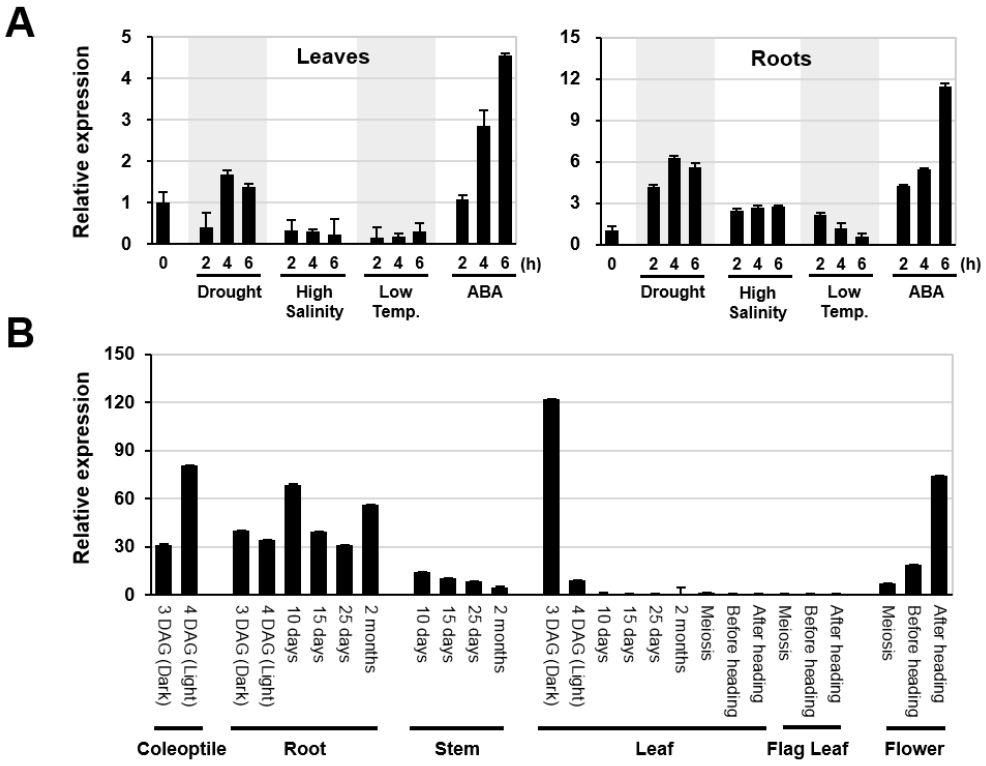

A
Relative expression
Leaves
Roots
0
1
2
3
4
5
6
9
12
15
0 2 4 6 (h)
Drought
High Salinity
Low Temp.
ABA
B
Relative expression
0
30
60
90
120
150
3 DAG (Dark)
4 DAG (Light)
10 days
15 days
25 days
2 months
Meiosis
Before heading
After heading
Coleoptile
Root
Stem
Leaf
Flag Leaf
Flower

Figure 3. Transgenic rice using a GOS2 constitutive overexpressing promoter.

(A) The schematic diagram of pri-*OsmiR171f* overexpression construct. RB, right border; GOS2, constitutive overexpressing promoter; NOS, the nopaline synthase polyadenylation region (terminator); Wsi18, a stress-inducible promoter; sGFP, synthetic green fluorescent protein; PinII, potato PinII 3' region (terminator); 35S, cauliflower mosaic virus promoter; *Bar*, the bacterial *phosphinothricin acetyl transferase* gene (a selectable marker for rice transformation); LB, left border.

(B) The GFP fluorescence profiles in seeds of the Wsi18:GFP reporter gene were analyzed in comparison with the transgenic plants. GFP fluorescence in transgenic and nontransgenic (NT) seeds was detected using fluorescence analyzer.

(C) Relative expression patterns of pri-*OsmiR171f* in NT and transgenic plants. NT, nontransgenic, *Oryza sativa* L. ssp. Japonica cv Dongjin;

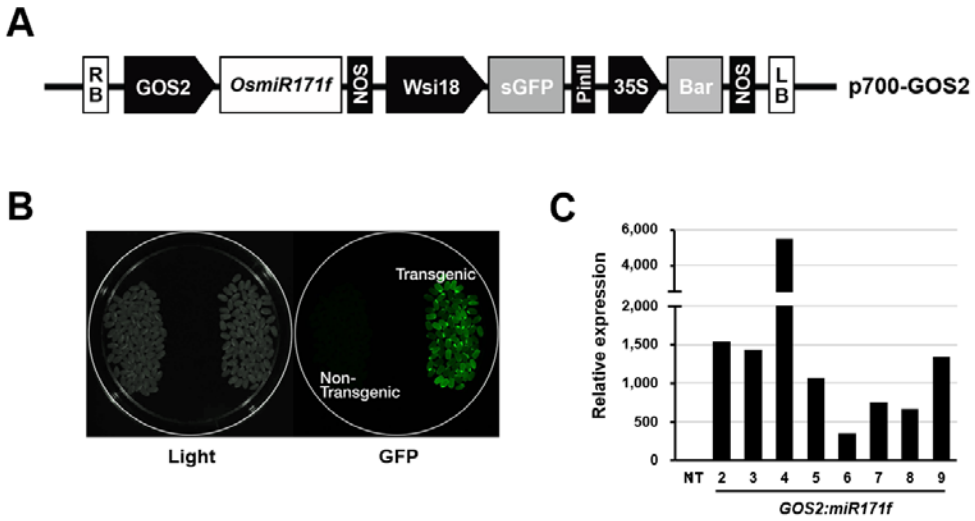
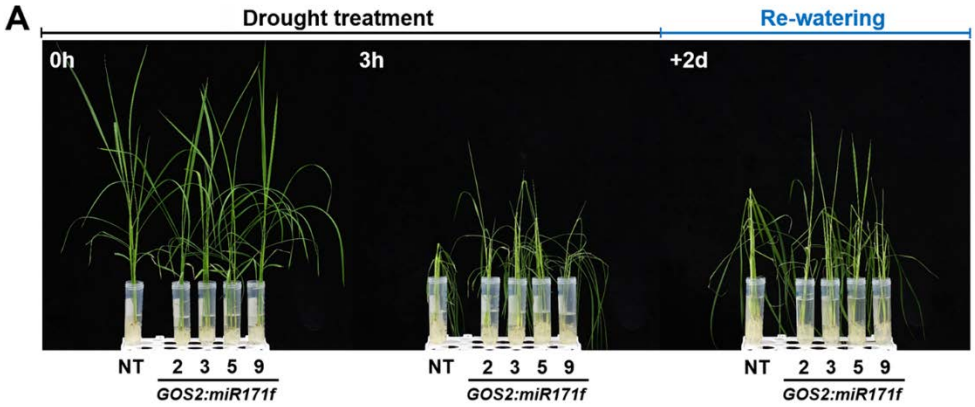

A
RB
GOS2
OsmiR171f
NOS
Wsi18
sGFP
PinII
35S
Bar
NOS
LB
p700-GOS2
B
Transgenic
Non-
Transgenic
Light
GFP
C
Relative expression
6,000
4,000
2,000
1,500
1,000
500
0
NT
2
3
4
5
6
7
8
9
GOS2:miR171f

Figure 4. PEG-induced drought treatment.

(A) Grown on MS agar medium for 7 days and transferred into Yoshida solution for 3 weeks of growth, the seedlings were treated with 22.5% PEG-8000 (m/v), and incubated at 28°C under long-day conditions (16 light/8 dark). NT (nontransgenic, *Oryza sativa* L. ssp. Japonica cv Dongjin)

(B) Relative expression of *OsmiR171f* transgenic lines in PEG-solution. *Dip1*, Dehydration stress-inducible protein 1, Os02g0669100; *RbcS1*, Small subunit of Rubisco, Os12g0274700. 4-week-old plants were exposed to 3 hours of PEG-mediated drought treatment (gray) and recovered for 2 days (blue).


A
Drought treatment
Re-watering
0h
3h
+2d
NT 2 3 5 9
GOS2:miR171f
NT 2 3 5 9
GOS2:miR171f
NT 2 3 5 9
GOS2:miR171f

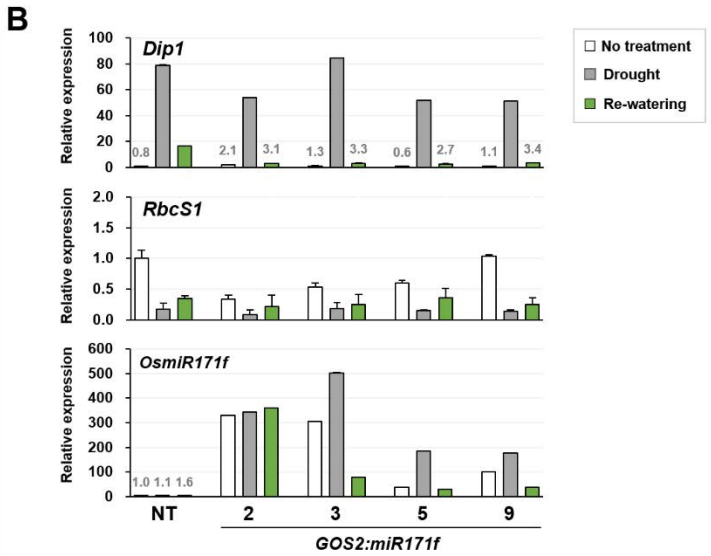

B
Dip1
Relative expression
100
80
60
40
20
0
0.8
2.1
3.1
1.3
3.3
0.6
2.7
1.1
3.4
No treatment
Drought
Re-watering
RbcS1
Relative expression
2.0
1.5
1.0
0.5
0.0
OsmiR171f
Relative expression
600
500
400
300
200
100
0
1.0 1.1 1.6
NT
2
3
5
9
GOS2:miR171f

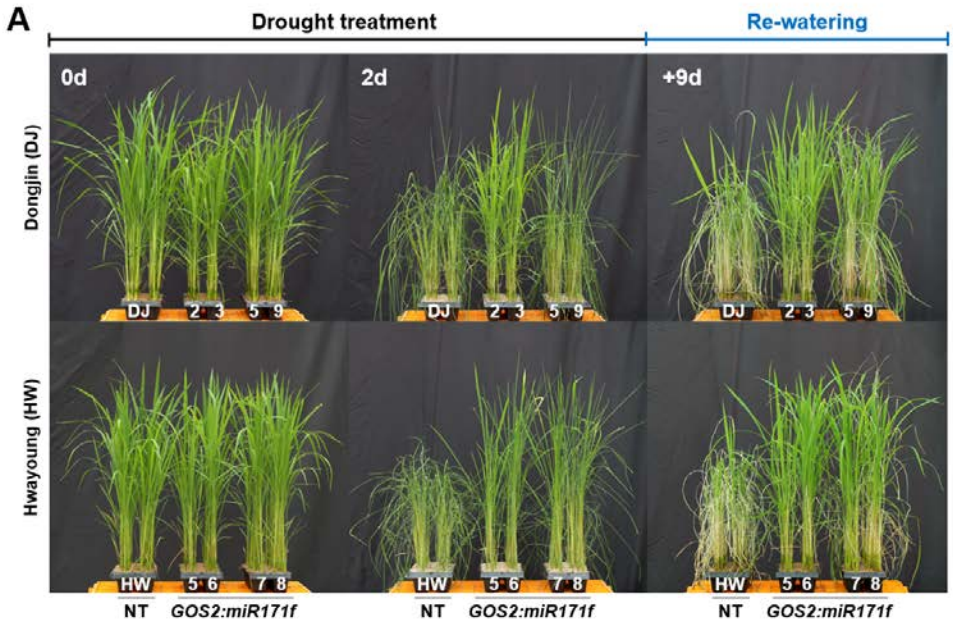
Figure 5. Phenotypic analysis of drought-resistance traits.

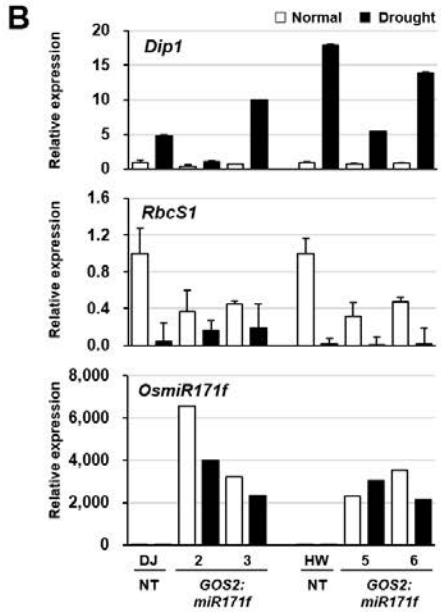
(A) Drought stress resistance of *GOS2:miR171f* transgenic plants. 4-week-old plants were exposed to no watering for 2 days and re-watering for 9 days. Each number indicates individual lines of *GOS2:miR171f* in two cultivars: Dongjin (DJ) and Hwayoung (HW).

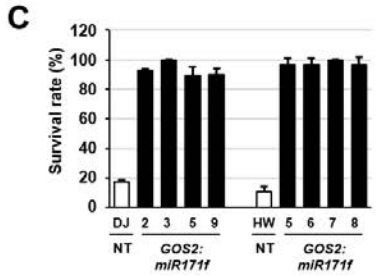
(B) Expression analysis of drought induction (upper panel), drought repression (middle panel) and overexpression of *OsmiR171f* (lower panel). *Dip1*, dehydration stress-inducible protein 1 (Os02g0669100); *RbcS1*, small subunit of Rubisco (Os12g0274700). The rice *Ubi1* (Os06g0681400) was used as an internal standard.

(C) The survival rate was scored from the transgenic and NT plants at the 9^{th} day after rehydration. Values are the means ± SD (n=30).

(D) Agronomic traits of *GOS2:miR171f* and NT plants under field conditions. The spider plot of yield parameters with four independent T_2 homozygous plants of *OsmiR171f* represented by the percentage of the mean value (n=30). Mean measurements from the NT control were assigned as a 100% reference value. CL, culm length; PL, panicle length; NP, number of panicles per hill; NTS, number of total spikelets; NSP, number of spikelets per panicle; NFG, number of filled grains; FR, filling rate; TGW; total grain weight; 1000 GW, 1000 grain weight.


A
Drought treatment
Re-watering
0d
2d
+9d
Dongjin (DJ)
Hwayoung (HW)
DJ
2 3
5 9
HW
5 6
7 8
NT
GOS2:miR171f


B
Normal
Drought
Dip1
RbcS1
OsmiR171f
Relative expression
DJ
2
3
HW
5
6
NT
GOS2:
miR171f


C
Survival rate (%)
DJ 2 3 5 9
HW 5 6 7 8
NT
GOS2:
miR171f

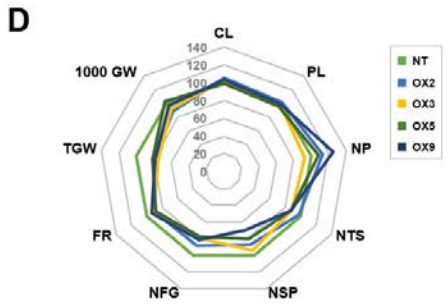

D
CL
PL
NP
NTS
NSP
NFG
FR
TGW
1000 GW
NT
OX2
OX3
OX5
OX9

Figure 6. *OsmiR171f* gene knockout mutant using rCRISPR/Cas9

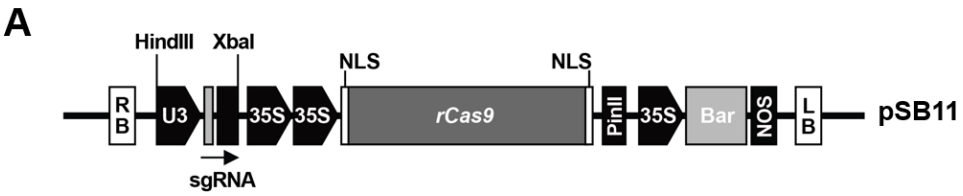
(A) The diagram of a recombinant CRISPR/Cas9 construct. A rice codon-optimized *Streptococcus pyogenes* Cas9 expression vector, including N-terminal and C-terminal nuclear localization signal (NLS), self-cleaving 2A peptide (P2A), and GFP, driven by two cauliflower mosaic virus 35S (CaMV 35S) promoters. A single guide RNA (sgRNA) is derived using a U3 promoter.

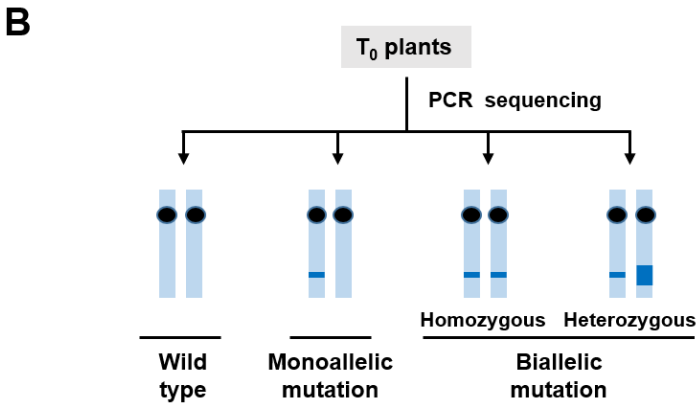
(B) The scheme of genotypic determination by CRISPR/Cas9 cleavage.

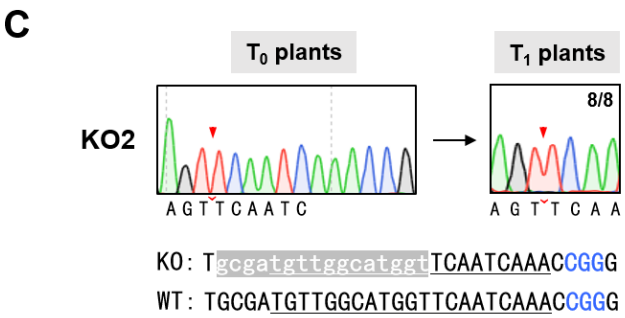
(C) Inheritance of mutated genes in the T_0 and T_1 generations. The 21 nucleotides target sequences for the rCas9/sgRNA complex are underlined, and the PAM site is depicted in blue characters; the rCas9/sgRNA-mutagenized DNA sequences are lowercase letters shaded in gray.

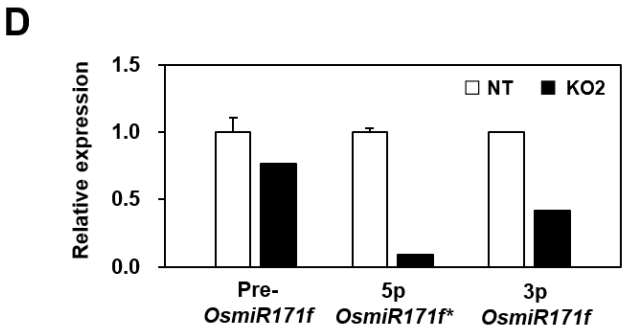
(D) Relative expression of the precursor and mature *OsmiR171f* in nontransgenic (NT: Dongjin) and CRISPR/Cas9 knockout line 2 (KO2).

(E) The stem-loop structures of CRISPR/Cas9-mutated *OsmiR171f* line 2 with the mature miRNAs (3p) sequences are highlighted in light blue.


A
HindIII
XbaI
NLS
NLS
RB
U3
35S
35S
rCas9
PinII
35S
Bar
NOS
LB
pSB11
sgRNA


B
T0 plants
PCR sequencing
Homozygous
Heterozygous
Wild type
Monoallelic mutation
Biallelic mutation


C
T0 plants
T1 plants
KO2
8/8
A G T T C A A T C
A G T T C A A
KO: TgcgatgttggcatggtTCAATCAAACCGGG
WT: TGCGATGTTGGCATGGTTCAATCAAACCGGG


D
Relative expression
1.5
1.0
0.5
0.0
NT
KO2
Pre-OsmiR171f
5p OsmiR171f*
3p OsmiR171f

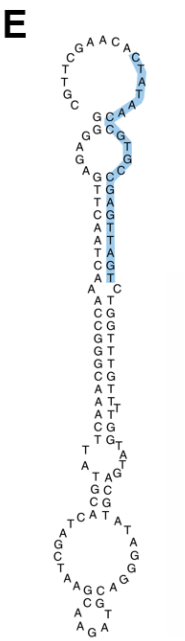

E

Figure 7. Drought response phenotypes of rice at the vegetative growth stage.

(A) Visualization of drought treatment on four-week-old *OsmiR171f*-overexpressing transgenic (OX2, 3, 5 and 9), CRISPR/Cas9-mutated (KO2) rice plants and their NT (DJ).

(B) Thermal imaging of transgenic lines and non-transgenic plants. Different leaf temperatures are expressed in colors: lower in blue and higher in red.

(C) Measurement of soil moisture content (%). Data represent mean value ± SD of 15 measurements performed at different soil locations.

(D) The survival rate (%) was measured after 7 days of re-watering. Data represent mean value ± SD of 15 measurements performed at different soil locations.

KO (Knockout): CRISPR/Cas9-mediated *OsmiR171f* mutant
NT (Nontransgenic): *Oryza sativa* L. ssp. Japonica cv Dongjin
OX (Overexpression): *GOS2:miR171f*

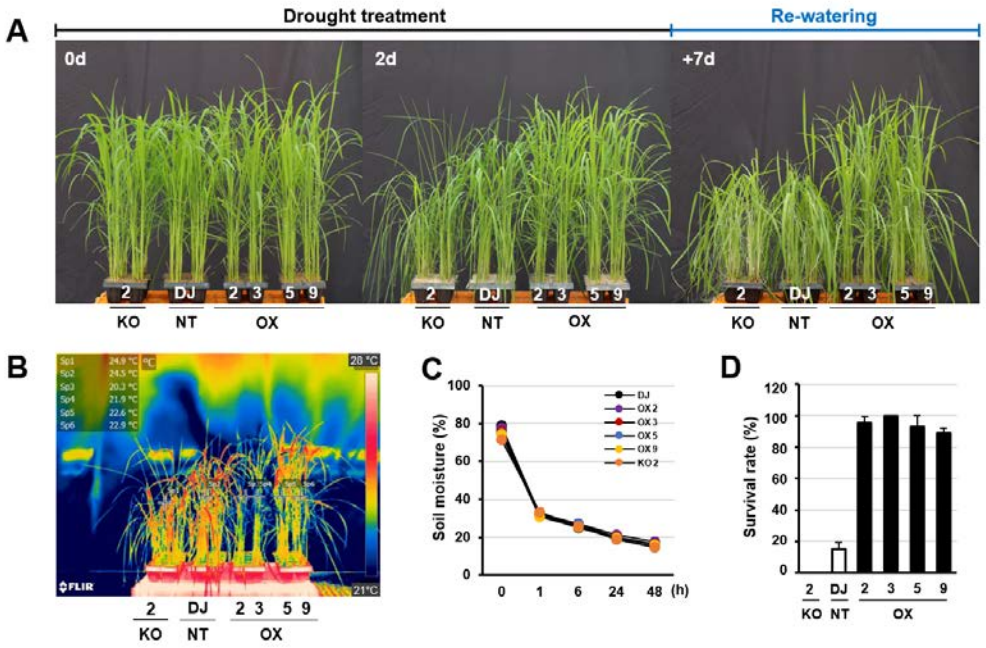


Figure 8. Primary and mature *OsmiR171f*.

(A) The stem-loop structure of *OsmiR171f*. The mature sequences are highlighted: 5p (gray) and 3p (light blue).

(B) Expression patterns of the precursor and mature *OsmiR171f*. Each number in the x-axis indicates individual lines of *GOS2:miR171f*. The rice *Ubiquitin1* was used as a reference gene, and data were shown as the mean ± SD of three biological and three technical replicates.

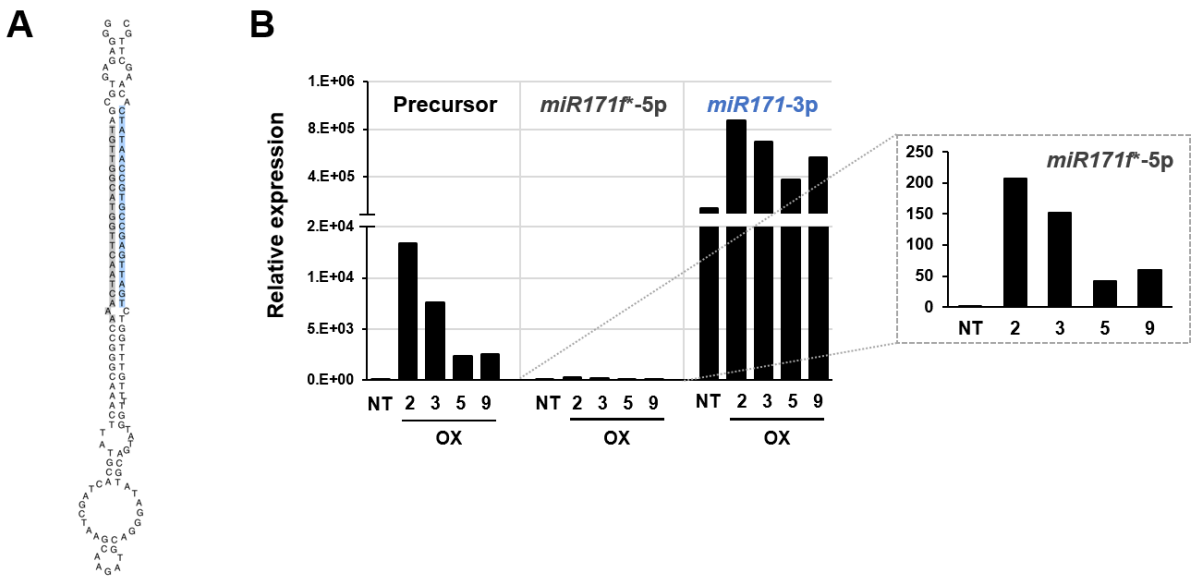

A
B
Relative expression
1.E+06
8.E+05
4.E+05
2.E+04
1.E+04
5.E+03
0.E+00
Precursor
miR171f*-5p
miR171-3p
NT 2 3 5 9
OX
250
200
150
100
50
0

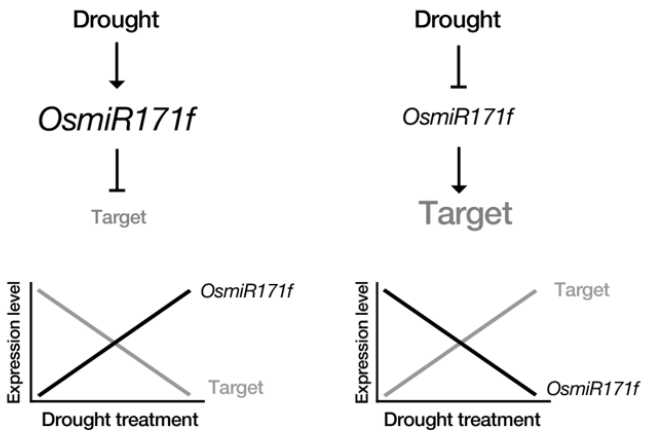
Figure 9. Target prediction of *OsmiR171f*.

(A) Possible regulatory mechanisms of *OsmiR171f* under drought stress.

(B) RT-PCR detection of *OsmiR171f* putative targets genes under a progressive drought stress treatment. The lane 1 to 4 indicate days of drought treatment. RNA seq. data are written in Log2 ratio. C: well-watered control, *OsUbi1*: internal standard.

(C) Expression analysis of mature *OsmiR171f*-5p and *OsmiR171f*-3p with its putative targets at various developmental stages.

A



B

		RT-PCR				RNA sequencing (Log2 Ratio)			
		C	1	2	3 (d)	C	1	2	3 (d/C)
	OsmiR171f					-	3.32	3.70	4.64
5p targets	CSTF77					-	0.10	-0.76	-2.51
5p targets	Non-coding transcript					-	0.52	-1.36	-3.19
5p targets	ARC3					-	0.08	-2.42	-5.07
3p targets	DEGP10					-	0.29	-2.71	-3.37
3p targets	SCL6-1					-	0.63	-3.28	-0.84
3p targets	SCL6-2					-	0.01	-0.92	-0.15
	OsUbi1					-	-	-	-

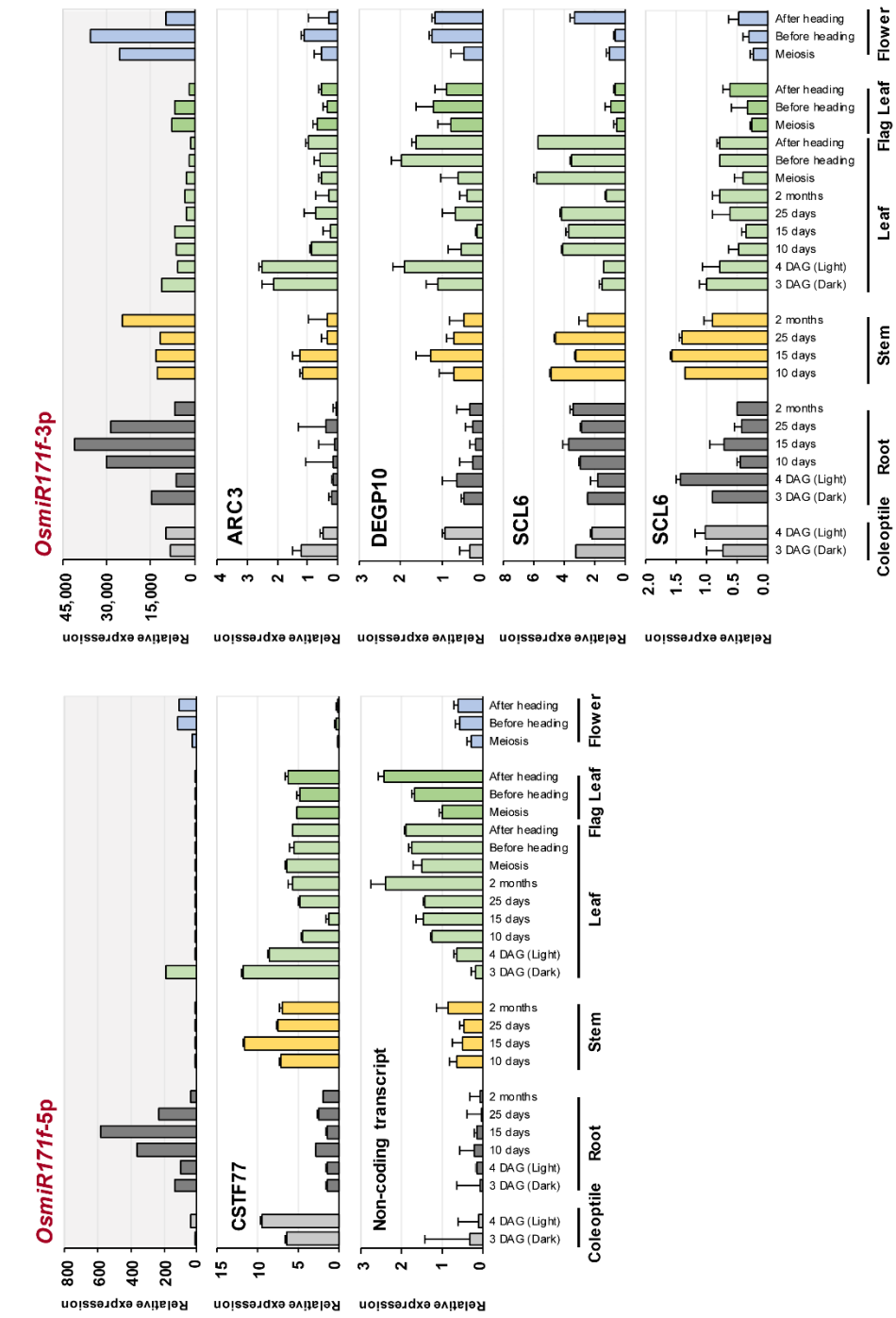

C
OsmiR171f-5p
OsmiR171f-3p
CSTF77
Non-coding transcript
ARC3
DEGP10
SCL6
SCL6
Relative expression
Coleoptile
Root
Stem
Leaf
Flag Leaf
Flower
4 DAG (Light)
3 DAG (Dark)
10 days
15 days
25 days
2 months
After heading
Before heading
Meiosis

Figure 10. Target validation of *OsmiR171f*.

Expression analysis of *OsmiR171f* putative target genes in *OsmiR171f*-overexpressing lines (OX 2,3) and non transgenic plants (NT) under normal and drought conditions. The expression level of NT was set as 1.0. The rice *Ubi1* gene was used as an internal control. The error bars indicate standard deviation of triplicate.

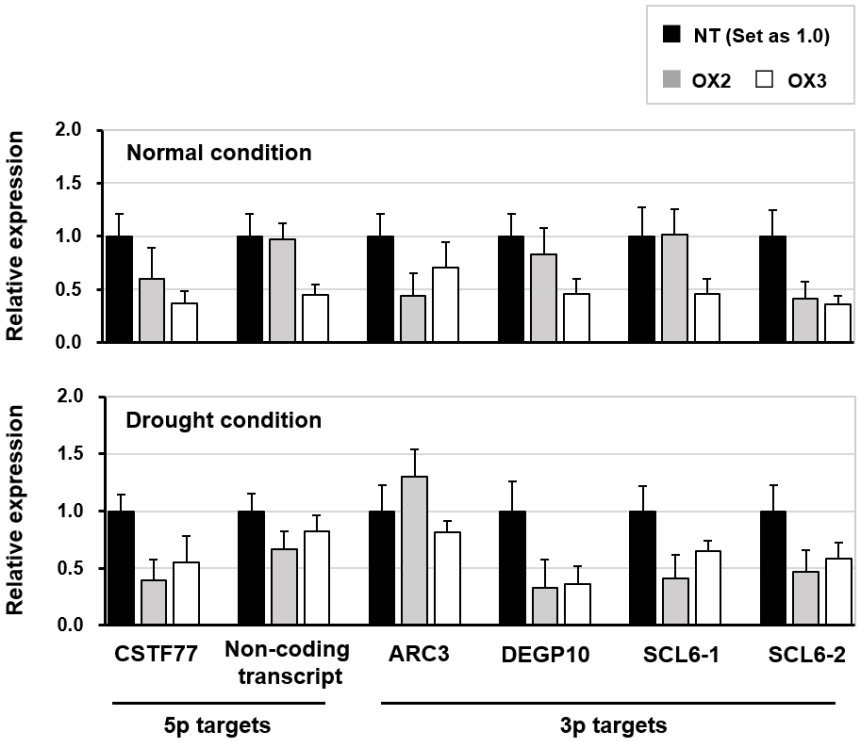

NT (Set as 1.0)
OX2
OX3
Normal condition
Drought condition
Relative expression
2.0
1.5
1.0
0.5
0.0
CSTF77
Non-coding transcript
ARC3
DEGP10
SCL6-1
SCL6-2
5p targets
3p targets

Table 1. Target prediction of *OsmiR171f*

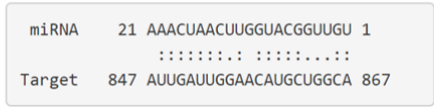
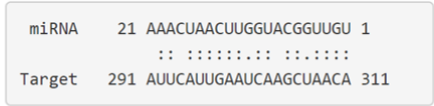
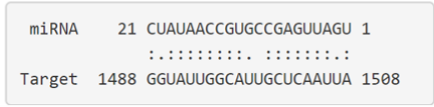
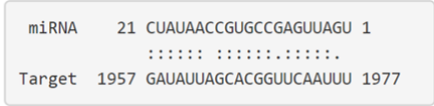
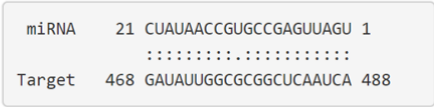
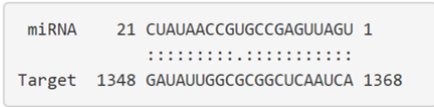
	Genes	Alignment	Annotation
5p	Os12g0571900		Cleavage stimulation factor subunit 77 (CSTF77)
	Os03g0828701		Non-protein coding transcript
3p	Os09g0555600		Accumulation and replication of Chloroplast 3 (ARC3)
	Os05g0417100		Protease Do-like 10 (DEGP10)
	Os06g0105350		Scarecrow-like protein 6 (SCL6-1)
	Os02g0662700		Scarecrow-like protein 6 (SCL6-2)

Table 2. Primer sequences used for this study

Primer name	Forward	Reverse	Experiments
Ubi1	ATG GAG CTG CTG CTG TTC TA	TTC TTC CAT GCT GCT CTA CC	RT-PCR
RbcS1	GGC AGG TAC TGG ACC ATG TG	TTG TCG AAG CCG ATG ATA CG	RT-PCR
Dip1	GAG CTT GTC ACC GGC ATG GA	AGC TGG AGC TGG AGC TGG AT	RT-PCR
pri-*OsmiR171f*	TGG GAG AGT GCG ATG TTG GC	CAG GCA AGC TTG TGA TAT TGG C	RT-PCR
Os12g0571900	AGA CCT CCA AGA GAC GGG CA	ACA GTG CCG CTG ATC CTG AC	RT-PCR
Os03g0828701	GCC CGC CCA ATG GTA TGA TGT G	GAG TCT GCA GGA ATG GGG ATC G	RT-PCR
Os09g0555600	CAG TTG CTA ATC GTG CTG CG	CAC CAT TAG CCC TCC CCT TC	RT-PCR
Os05g0417100	AGA TGC GGC TAT CAA CCC AG	AGG CAC ACC GTC AAA CTC AA	RT-PCR
Os06g0105350	CCA AAG CTG CTT GTG ATA TGT TAG C	TCA CAA GGT TGG GGG CTT GC	RT-PCR
Os02g0662700	TGG AGC TGC ACC TTA CCC AG	CCA ACC GAA GAA TCG CTG GC	RT-PCR
***OsmiR171f*-5p_RT**	GCG GCG GTG TTG GCA TGG TTC AAT C	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT TTG ATT G	Stem-loop RT-PCR
***OsmiR171f*-3p_RT**	GCG GCG GTG ATT GAG CCG TGC C	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG ATA TTG GC	Stem-loop RT-PCR
universial_R		GTG CAG GGT CCG AGG T	Stem-loop RT-PCR

Discussion

For understanding of miRNA functions, it is crucial to investigate the spatio-temporal expression of miRNAs during development and stress response. Previously, the RNA sequencing and the expression profiling demonstrated that *OsmiR171f* expresses in an organ-specific and stress-inducible manner (Chung et al., 2016). This study presented the temporal expression pattern of *OsmiR171f* differing in various tissues throughout the rice life cycle (Figure 2B), indicating its possible roles in specific developmental processes. Upon dehydration stress, abscisic acid (ABA) accumulates and regulates downstream genes to optimize growth (Singh et al., 2015). It was found that expression of *OsmiR171f* was up-regulated by treatments with drought and ABA (Figure 2A). Several studies showed that regulation of a single miRNA expression can improve or reduce plant resistance to abiotic stresses. For example, *OsmiR393*-overexpressing plants displayed sensitivity to salt and drought in rice (Xia et al., 2012), whereas transgenic *Arabidopsis* plants overexpressing *Glycine max miR394a* exhibited an enhanced drought tolerance (Ni et al., 2012). Likewise, overexpression of *miR169* increased plant resistance to drought stress by decreasing its transpiration rate (Zhang et al., 2011). To find out whether the *OsmiR171f* works in drought tolerance I set out to make transgenic rice plants overexpressing *OsmiR171f* under the constitutive *GOS* promoter. For a preliminary screening of drought tolerant phenotype of the plants, 22.5% polyethylene glycol (PEG)-8000 solution (Burnett et al., 2005) was applied to the plants at the seedling stage. The PEG caused drought-induced morphological changes as shown in Figure 4A and anatomical modifications as indicated by the marker gene expressions. Under drought stress conditions, the expression of *Dip1* was induced but the expression of *RbcS1* was repressed. The expression levels of *RbcS1* were less reduced in transgenic lines by 30 to 50% than were in the NT plants (Figure 4B). The *GOS2:OsmiR171f* plants also displayed

reduced and delayed drought-induced symptoms. It is possible that miRNAs respond to abiotic stress in a genotype-dependent manner. Under salt stress conditions, the *miR167a* was differently regulated in two different cotton cultivars, SN-011 and LM-6 (Yin et al., 2012). Here, to see if there is any genotype-dependency of our *OsmiR171f*, transgenic plants with two different cultivars *Oryza sativa* L. ssp. Japonica cv Dongjin and Hwayoung were generated (Figure 5). At the vegetative stages, both transgenic plants exhibited drought tolerance with survival rates far exceeding that of the NT plants. About 20% of NT plants recovered whereas the transgenic lines showed nearly full survival. Thus, overexpression of *OsmiR171f* conferred drought tolerance to rice plants, regardless of their genotypes (Figure 5). To investigate roles of *OsmiR171f* in drought tolerance further, the CRISPR/Cas9-mediated knockout vector was constructed and transformed. The KO mutant displayed a higher degree of drought sensitivity than did the NT and OX lines (Figure 7A). A thermograph also demonstrated different leaf temperature and water content in response to drought stress, advocating the functional role of *OsmiR171f* in drought tolerance (Figure 7B).

Since miRNAs exert their functions by regulating their downstream target mRNAs, it is central to identify their targets to elucidate their biological roles (Figure 9A) (Yang et al., 2013). In rice, *miR156* is known to target the squamosal promoter biding like (SPL) genes, which are known as important in leaf development. Plants overexpressing *miR156* displayed increased leaf and tiller numbers, and semi-dwarf traits (Xie et al., 2012). Expression of *miR156a* was induced in barley leaves under dehydration treatments (Kantar et al., 2010). Interestingly, the *miR156*-SPL pathway displayed an inverse correlation between development and abiotic stress response in *Arabidopsis* (Cui et al., 2014). Although seemed as quite distinct processes, plant development and stress acclimation are closely linked for they are halted under extreme environments but accomplished under normal conditions (Sunkar, 2010). In addition, a star (passenger) strand from a pre-miRNA duplex is released and degraded

typically; however, in some circumstances, it can be retained and perform its own function (Liu et al., 2017). For example, in *Arabidopsis*, the *AtmiR171a**-SUVH8 pair regulated plant growth and in broccoli, *miR171b*-5p was expressed differently from the guide strand, 3p and mediated sulfate assimilation by targeting adenylylsulfate reductase 3 (APR3) (Manavella et al., 2012; Li et al., 2018). Similarly, the mature sequences of *OsmiR171f* were expressed differentially under normal conditions; the *OsmiR171f*-3p showed constitutively high expression (Figure 8B). The *OsmiR171f*-5p was highly expressed in roots and flowers whereas its candidate targets were expressed in organs other than roots and flowers. In a similar manner, the *OsmiR171f*-3p and its targets displayed an inverse relationship except for SCL6-1 and SCL6-2, which were expressed in roots and flowers as well. These two Scarecrow-like 6 (SCL6) proteins are GRAS family transcription factors that are known to function in optimal growth and development (Llave et al., 2002). The role of *OsmiR171f* that targets SCL6 in stress tolerance is demonstrated in Figure 9B and also in previous studies (Kantar et al., 2010; Lima et al., 2012). Identified as ARC3 and DEGP10 respectively, Os09g0555600 and Os05g0417100 are predicted to mediate chloroplast accumulation, which can be related to development and stress response processes as well; however, the studies on the predicted targets of *OsmiR171f* other than SCL6 remain to be elucidated further. Here, our expression analysis showed that the six predicted targets were expressed significantly lower in *GOS2:miR171f* plants than in NT plants under both conditions (Figure10). Collectively, *OsmiR171f* is implicated as a co-regulator of both developmental process and stress response.

In conclusion, this study demonstrated that *OsmiR171f* could play a crucial role in drought response. The overexpression of *OsmiR171f* increased drought tolerance in transgenic plants without severe growth defects. Putative targets of *OsmiR171f* that include SCL6, ARC3 and CSTF77 were identified and shown to have an inverse correlation in expression profiles with their corresponding mature *OsmiR171f*. These findings provide new insights into the functional and regulatory

role of *OsmiR171f* and indicate that manipulating *OsmiR171f* can enhance drought tolerance in rice. As a potential application, CRISPIR/Cas9-mediated target knockdown plants can be developed to improve drought tolerance without insertion of foreign genes. By doing so, this strategy could be utilized as a promising crop improvement through manipulating miRNA and its targets.

References

Bartel, D. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116(2), pp.281-297.

Burnett, S., Pennisi, S., Thomas, P. and Iersel, M. (2005). Controlled Drought Affects Morphology and Anatomy of Salvia splendens. *Journal of the American Society for Horticultural Science*, 130(5), pp.775–781.

Chung, P., Jung, H., Jeong, D., Ha, S., Choi, Y. and Kim, J. (2016). Transcriptome profiling of drought responsive noncoding RNAs and their target genes in rice. *BMC Genomics*, 17(1).

Cui, L., Shan, J., Shi, M., Gao, J. and Lin, H. (2014). The miR156-SPL9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. *The Plant Journal*, 80(6), pp.1108-1117.

Dai, X. and Zhao, P. (2011). psRNATarget: a plant small RNA target analysis server. *Nucleic Acids Research*, 39(S2), pp.W155-W159.

Ding, Y., Tao, Y. and Zhu, C. (2013). Emerging roles of microRNAs in the mediation of drought stress response in plants. *Journal of Experimental Botany*, 64(11), pp.3077-3086.

Ferdous, J., Hussain, S. and Shi, B. (2015). Role of microRNAs in plant drought tolerance. *Plant Biotechnology Journal*, 13(3), pp.293-305.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994). Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal*, 6(2), pp.271-282.

Jeong, J., Kim, Y., Baek, K., Jung, H., Ha, S., Do Choi, Y., Kim, M., Reuzeau, C. and Kim, J. (2010). Root-Specific Expression of OsNAC10 Improves Drought Tolerance and Grain Yield in Rice under Field Drought Conditions. *Plant Physiology*, 153(1), pp.185-197.

Kantar, M., Lucas, S. and Budak, H. (2010). miRNA expression patterns of Triticum dicoccoides in response to shock drought stress. *Planta*, 233(3), pp.471-484.

Khandal, H., Parween, S., Roy, R., Meena, M. and Chattopadhyay, D. (2017). MicroRNA profiling provides insights into post-transcriptional regulation of gene expression in chickpea root apex under salinity and water deficiency. *Scientific Reports*, 7(1).

Kwon, T., Kim, K., Yoon, H., Lee, S., Kim, B. and Siddiqu, Z. (2015). Phenotyping of Plants for Drought and Salt Tolerance Using Infra-Red Thermography. *Plant Breeding and Biotechnology*, 3(4), pp.299-307.

Llave, C. (2002). Cleavage of Scarecrow-like mRNA Targets Directed by a Class of Arabidopsis miRNA. *Science*, 297(5589), pp.2053-2056.

Li, H., Zhang, Q., Li, L., Yuan, J., Wang, Y., Wu, M., Han, Z., Liu, M., Chen, C., Song, W. and Wang, C. (2018). Ectopic Overexpression of bol-miR171b Increases Chlorophyll Content and Results in Sterility in Broccoli (Brassica oleracea L var. italica). *Journal of Agricultural and Food Chemistry*, 66(37), pp.9588-9597.

Li, X., Xia, K., Liang, Z., Chen, K., Gao, C. and Zhang, M. (2016). MicroRNA393 is involved in nitrogen-promoted rice tillering through regulation of auxin signal transduction in axillary buds. *Scientific Reports*, 6(1).

Lima, J., Loss-Morais, G. and Margis, R. (2012). MicroRNAs play critical roles during plant development and in response to abiotic stresses. *Genetics and Molecular Biology*, 35(4-S1), pp.1069-1077.

Liu, W., Meng, J., Cui, J. and Luan, Y. (2017). Characterization and Function of MicroRNA∗s in Plants. *Frontiers in Plant Science*, 8.

Manavella, P., Koenig, D., Rubio-Somoza, I., Burbano, H., Becker, C. and Weigel, D. (2012). Tissue-Specific Silencing of Arabidopsis SU(VAR)3-9 HOMOLOG8 by miR171a. *Plant Physiology*, 161(2), pp.805-812.

Ni, Z., Hu, Z., Jiang, Q. and Zhang, H. (2012). Overexpression of gma-MIR394a confers tolerance to drought in transgenic Arabidopsis thaliana. *Biochemical and Biophysical Research Communications*, 427(2), pp.330-335.

Phelps-Durr, T. L. (2010) MicroRNAs in Arabidopsis. *Nature Education*, 3(9), pp.51

Reinhart, B. (2002). MicroRNAs in plants. *Genes & Development*, 16(13), pp.1616-1626.

Reyes, J. and Chua, N. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *The Plant Journal*, 49(4), pp.592-606.

Shriram, V., Kumar, V., Devarumath, R., Khare, T. and Wani, S. (2016). MicroRNAs As Potential Targets for Abiotic Stress Tolerance in Plants. *Frontiers in Plant Science*, 7.

Singh, D. and Laxmi, A. (2015). Transcriptional regulation of drought response: a tortuous network of transcriptional factors. *Frontiers in Plant Science*, 6.

Sunkar, R., Chinnusamy, V., Zhu, J. and Zhu, J. (2007). Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends in Plant Science*, 12(7), pp.301-309.

Sunkar, R. (2010). MicroRNAs with macro-effects on plant stress responses. *Seminars in Cell & Developmental Biology*, 21(8), pp.805-811.

Thompson, J., Higgins, D. and Gibson, T. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), pp.4673-4680.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. and Hellens, R. (2007). Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*, 3(1), pp.12.

Voinnet, O. (2009). Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell*, 136(4), pp.669-687.

Xia, K., Wang, R., Ou, X., Fang, Z., Tian, C., Duan, J., Wang, Y. and Zhang, M. (2012). OsTIR1 and OsAFB2 Downregulation via OsmiR393 Overexpression Leads to More Tillers, Early Flowering and Less Tolerance to Salt and Drought in Rice. *PLoS ONE*, 7(1), pp.30039.

Xie, K., Shen, J., Hou, X., Yao, J., Li, X., Xiao, J. and Xiong, L. (2012). Gradual Increase of miR156 Regulates Temporal Expression Changes of Numerous Genes during Leaf Development in Rice. *Plant Physiology*, 158(3), pp.1382-1394.

Yang, C., Li, D., Mao, D., Liu, X., Ji, C., Li, X., Zhao, X., Cheng, Z., Chen, C. and Zhu, L. (2013). Overexpression of microRNA319 impacts leaf morphogenesis and leads to enhanced cold tolerance in rice (Oryza sativa L.). *Plant, Cell & Environment*, 36(12), pp.2207-2218.

Yin, Z., Li, Y., Yu, J., Liu, Y., Li, C., Han, X. and Shen, F. (2011). Difference in miRNA expression profiles between two cotton cultivars with distinct salt sensitivity. *Molecular Biology Reports*, 39(4), pp.4961-4970.

Zhang, B. (2015). MicroRNA: a new target for improving plant tolerance to abiotic stress. *Journal of Experimental Botany*, 66(7), pp.1749-1761.

Zhang, X., Zou, Z., Gong, P., Zhang, J., Ziaf, K., Li, H., Xiao, F. and Ye, Z. (2010). Over-expression of microRNA169 confers enhanced drought tolerance to tomato. *Biotechnology Letters*, 33(2), pp.403-409.

Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K. and Jin, Y. (2007). Identification of drought-induced microRNAs in rice. *Biochemical and Biophysical Research Communications*, 354(2), pp.585-590.

Zhu, X., Leng, X., Sun, X., Mu, Q., Wang, B., Li, X., Wang, C. and Fang, J. (2015). Discovery of Conservation and Diversification of Genes by Phylogenetic Analysis based on Global Genomes. *The Plant Genome*, 8(2).

Abstract in Korean

마이크로RNA 171f 과발현 벼의 내건성 향상에 대한 연구

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가뭄을 포함하는 비생물적 스트레스는 식물 생장에 영향을 주어 결과적으로 작물 생산성 손실을 유도한다. 일반적으로 식물 마이크로 RNA는 발달과 스트레스에 반응하는 유전자 조절에 관여하는 것으로 알려져 있다. 최근 연구에서 스트레스가 마이크로RNA를 포함한 많은 논코딩 RNA의 특이적 발현 유도가 확인되었다. 선행연구에서 가뭄에 노출 되었을 때 *OsmiR171f* 발현이 확인되어 가뭄에 따른 영향을 알아보기 위한 연구를 진행하였다. 전신발현형 GOS2 프로모터를 사용한 *OsmiR171f* 과발현체와 CRISPR/Cas9 시스템을 이용하여 *OsmiR171f*의 스템 루프 영역을 표적하는 guide RNA를 포함하는 돌연변이체를 제작하였고 Agrobacterium으로 유도된 형질전환 벼를 생산하였다. 총 네 라인의 과발현체와 하나의 기능소실 돌연변이체를 선발하여 실험을 진행하였다. 토양과 폴리에틸렌 글리콜 (PEG) 용액을 사용하여 물 부족 환경을 유도한 결과 과발현체에서 가뭄저항성 향상을 확인했다. 스트레스에 의해 발현된 마이크로 RNA는 타겟 유전자를 하향 조절하여 기능을 하기 때문에 타겟 예측이 매우 중요시 된다. *OsmiR171f*에 의해 조절되는 유전자를 알아보기 위해 qRT-PCR을 사용하여 다

양한 시공간적인 발현을 확인했다. *OsmiR171f*의 발현과 타겟의 발현이 상반된 것으로 나타나 *OsmiR171f*에 의한 타겟의 감소로 예측 되었다. 유전자 발현 양상과 가뭄에 의한 표현형을 통해 벼에서 *OsmiR171* 유전자 과발현은 타겟 유전자 조절과 가뭄 저항성 매개 작용에 관여하는 것을 확인하였다.

키워드: 마이크로RNA, 비생물적 스트레스, 가뭄저항성, 타겟 유전자, 과발현, 벼