





# Rice *microRNA171f/SCL6* module enhances drought tolerance by regulation of flavonoid biosynthesis genes

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## Abstract

Plants have evolved sophisticated defense systems to enhance drought tolerance. These include the microRNA (miRNA) group of small noncoding RNAs that act as post-transcriptional regulators; however, details of the mechanisms by which they confer drought tolerance are not well understood. Here, we show that *osa-MIR171f*, a member of *osa-MIR171* gene family, is mainly expressed in response to drought stress and regulates the transcript levels of *SCARECROW-LIKE6-I (SCL6-I)* and *SCL6-II* in rice (*Oryza sativa*). The *SCL6* genes are known to be involved in shoot branching and flag leaf morphology. *Osa-MIR171f*-overexpressing (*osa-MIR171f*-OE) transgenic plants showed reduced drought symptoms compared with non-transgenic (NT) control plants under both field drought and polyethylene glycol (PEG)-mediated dehydration stress conditions. Transcriptome analysis of *osa-MIR171f*-OE plants and *osa-mir171f*-knockout (K/O) lines generated by clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) revealed that *osa-mature-miR171a-f (osa-miR171)* regulates the expression of flavonoid biosynthesis genes, consequently leading to drought tolerance. This upregulation in the *osa-MIR171f*-OE plants, which did not occur in NT control plants, was observed under both normal and drought conditions. Our findings indicate that *osa-miR171* plays a role in drought tolerance by regulating *SCL6-I* and *SCL6-II* transcript levels.

## KEYWORDS

drought, flavonoids, miR171, Osa-miR171f, Rice, SCL6

## 1 | INTRODUCTION

Drought is a common environmental condition and can profoundly affect plant growth and development (Mittler, 2006; Peet & Kramer, 1980). Accordingly, plants have developed mechanisms to cope with drought stress (Devi et al., 2017; Fu & Dong, 2013; Heil & Bostock, 2002) that have been extensively studied due to

the economic implications of reduced growth and yield on different crop species (Chung et al., 2016; Joshi et al., 2016; Vakilian, 2020). Yield reduction by drought is due to various factors, including reduction of photosynthesis efficiency, disturbance of assimilates allocation, and poor leaf development. Specially, drought stress at the pre-anthesis stage is critical in grain filling and yield (Fahad et al., 2017).

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One mechanism that is known to be involved in drought stress responses is the regulation of gene expression by microRNAs (miRNAs). A variety of miRNAs induced or repressed in response to drought stress and regulated its target genes to cope with drought stress (Sunkar et al., 2012; Wang et al., 2010; Yoshida et al., 2014). These are small (20–24 nucleotides) noncoding RNAs that act as negative post-transcriptional regulators to modulate gene expression (Lee et al., 1993; Mallory & Vaucheret, 2004; Ramachandran & Chen, 2008; Reinhart et al., 2002). Mature-miRNAs (miRNA) are processed from stem-loop precursor miRNAs (pre-miRNA), derived from primary miRNAs (pri-miRNA) by RNase III-type endonucleases, such as Dicer (Reinhart et al., 2002; Voinnet, 2009). This involves mRNA degradation where the mature-miRNA is incorporated into the RNA-induced silencing complex (RISC), which guides Argonaute (AGO) to cleave the target mRNA through base-pairing (Bartel, 2009; Filipowicz et al., 2008; Jones-Rhoades et al., 2006). The miRNA target sites are highly conserved (Axtell, 2008; Axtell & Bowman, 2008; Chorostecki et al., 2012) and so can be predicted, as has been reported in *Arabidopsis thaliana* and rice (*O. sativa*) (Jones-Rhoades & Bartel, 2004; Rhoades et al., 2002; Wang et al., 2004).

In *A. thaliana*, *ath-miR171c* regulates the GAI, RGA, and SCR (GRAS) transcription factor family members SCARECROW-LIKE6-II (SCL6-II), SCL6-III, and SCL6-IV (also known as HAIRY MERISTEM [HAM] or LOST MERISTEMS [LOM]). *Ath-MIR171c* overexpression leads to reduced shoot branching and increased plant height (Wang et al., 2010), consistent with the known roles of GRAS members in shoot, root, and chlorophyll development via regulation of gibberellic acid (GA)-auxin signaling (Bolle, 2004; Huang et al., 2017). In rice, *osa-miR171c*-mediated regulation of *OsHAM1* (SCL6-II), *OsHAM2*, *OsHAM3*, and *OsHAM4* (SCL6-I) affects the heading date and leaf morphology (Fan et al., 2015), and in tomato (*Solanum lycopersicum*), *Sly-miR171* overexpressing plants were found to be taller and to show earlier phase transition and flowering time than wild-type plants. Many studies have shown miRNA involvement in drought responses by transcriptional profiling (Chung et al., 2016; Ferdous et al., 2015; Zhou et al., 2010), and in rice, miR159, miR169, miR395, and miR474 transcripts are generally up-regulated by drought stress, whereas miR156, miR168, miR170, miR172, miR396, miR397, and miR408 transcripts are generally down-regulated (Khraiwesh et al., 2012; Zhou et al., 2010). However, within families of miRNAs, there can be different responses by individual members to drought stress. For example, in *A. thaliana* pre-miR171b transcript levels were observed to increase in response to drought stress, while pre-miR171a transcript levels decreased (Liu et al., 2008; Sunkar & Zhu, 2004). Similarly, in rice, pre-miR171f transcript levels were reported to increase following drought stress, whereas the *osa-pre-miR171a* transcript abundance decreased (Chung et al., 2016). Thus, the exact miRNA-based mechanisms controlling the drought response have yet to be clearly elucidated.

A diverse range of factors are involved in, and induced, by drought stress, including the content of flavonoid pigments. Flavonoids are secondary metabolites (Harborne & Williams, 2000) that mitigate against oxidative damage that can occur through accumulation of reactive oxygen species (ROS) caused by abiotic stress,

such as drought (Catalá et al., 2011; Koops et al., 2011; Kusano et al., 2011; Saito et al., 2013; Stracke et al., 2010). The metabolic pathway of flavonoid has been well defined, and core biosynthetic enzymes are identified (Galland et al., 2014; Shih et al., 2008). The expression of the corresponding genes is induced by drought stress in plants (Gai et al., 2020; Sharma et al., 2019; Wang et al., 2020).

In this current study, we investigated the role of *osa-miR171* in *SCL6-I* (*OsHAM4*) and *SCL6-II* (*OsHAM1*) regulation of the drought response in rice, using phenotypic and transcriptome analysis of *osa-MIR171f*-overexpressing and knockout plants generated by *Clustered Regularly Interspaced Short Palindromic Repeats* (CRISPR)/Cas9. We describe an association of this regulatory process with the expression of flavonoid biosynthesis genes and propose a model in which *osa-mature-microRNA171a-f* (*osa-miR171*) derived from *osa-pre-miR171f* regulates flavonoid biosynthesis as a response to drought using its *SCL6-I* and *SCL6-II* targets.

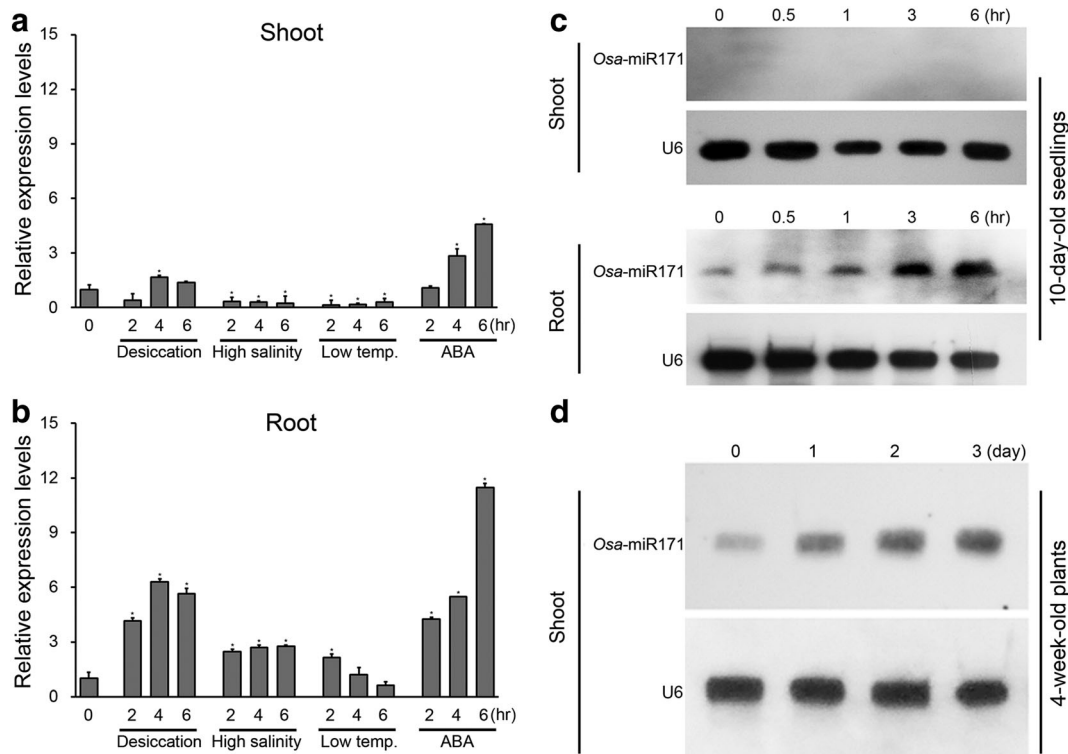
## 2 | RESULTS

### 2.1 | Drought stress induces expression of *osa-pre-miR171f* and *osa-miR171*

Previous studies showed that the precursor miR171f (pre-miR171f) accumulates under drought stress in rice (Chung et al., 2016). To investigate its function more broadly, we analyzed its expression pattern in response to a range of abiotic stresses (Figure 1a,b). We found that *osa-pre-miR171f* transcript levels increased in shoots following desiccation stress and abscisic acid (ABA) treatment (Figure 1a) and by high salt treatment, cold stress, and ABA treatment in roots (Figure 1b). We also analyzed *osa-mature-microRNA171a-f* (*osa-miR171*) expression in seedling roots and shoots and in the shoots of 4-week-old plants (Figure 1c,d). In the seedlings, *osa-miR171* transcript levels increased in roots during desiccation stress but were not detected in shoots under the same experimental conditions. However, *osa-miR171* transcript levels did increase in shoots in response to the drought treatment in the 4-week-old plants (Figure 1d). Previous our RNA seq data showed that *osa-MIR171e* and *f* were expressed dominantly in rice and other *osa-MIR171* members were hard to detect. Besides, *osa-MIR171e* expression was down-regulated, and miR171f expression was up-regulated response to drought treatment (Chung et al., 2016). Thus, the expression patterns of *osa-pre-miR171f* dominantly affected *osa-miR171* level under drought stress, suggesting that among members of the *osa-MIR171* gene family *osa-MIR171f* shows primarily association with drought responses.

### 2.2 | Overexpression of *osa-MIR171f* promotes drought tolerance

To understand the function of *osa-MIR171f* in drought stress, we generated transgenic rice lines overexpressing *osa-MIR171f* under the



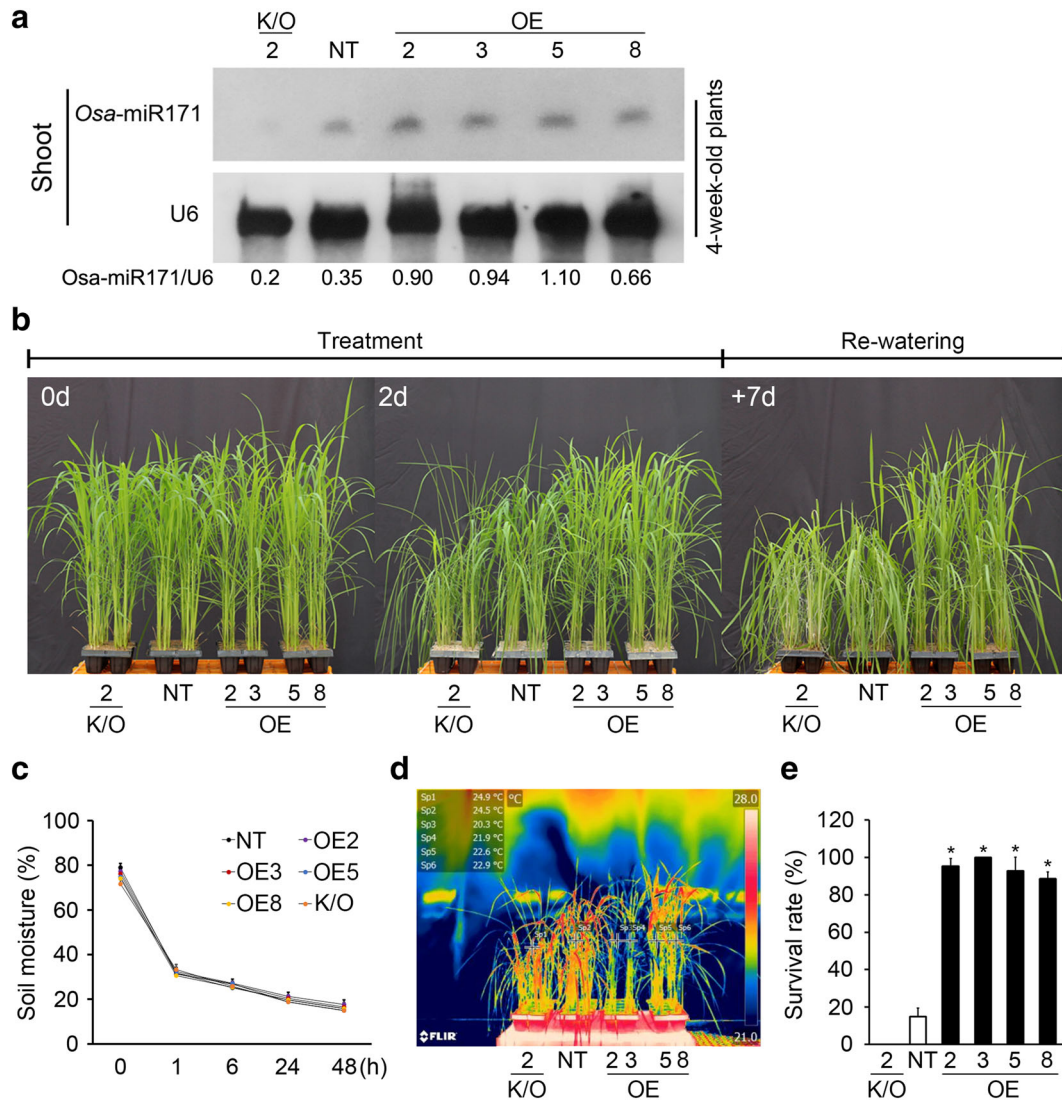
**FIGURE 1** Analysis of *osa-pre-miR171f* and *osa-mature-microRNA171a-f* (*osa-miR171*) response to abiotic stress in rice. (a and b) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis showing changes in *osa-pre-miR171f* expression levels in response to different stresses in shoots (a) and roots (b). Control plants were not treated with drought stress (0 h time). Error bars indicate SD of triplicate measurements. *OsTubA2* and *OsUbi1* were used as internal controls, and relative expression levels are shown as fold values. Asterisks indicate statistically significant differences between the corresponding samples and their control ( $p$  value < .01, Student's  $t$  test). ABA, abscisic acid. (c and d) *osa-miR171* expression levels in seedlings (c) and young plants (d) under drought stress. The expression level was assessed by Northern blot analysis. U6 transcript levels show equal loading of RNA. Total RNA was prepared from the 10-day-old and 4-week-old plants exposed to stresses for the indicated time periods

control of the constitutive *GOS2* promoter (Figure S1a) (Pater et al., 1992). We identified four independent transgenic lines (lines 2, 3, 5, and 8) with *osa-pre-miR171f* transcript levels using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays (Figure S1b) and analyzed the expression levels of *osa-mature-microRNA171a-f* (*osa-miR171*) in these transgenic lines using Northern blot analysis (Figure 2a). To investigate the drought resistance of these *osa-MIR171f* overexpressing (OE) plants, we compared their phenotypes to non-transgenic plants (NT) in response to polyethylene glycol (PEG)-mediated dehydration stress. The leaf-rolling of 10-day-old *osa-MIR171f* -OE plants clearly decreased compared to that of NT plants in the 22.5% PEG-8000 (m/v) treatment (Figure S1c). This result indicated that *osa-MIR171f* -OE plants are more tolerant of dehydration stress than NT plants. To investigate the effect of *osa-MIR171f* loss of function during drought stress, we then measured *osa-miR171* expression levels in a *osa-mir171f* -knockout mutant (line 2) generated using a CRISPR/Cas9 system (Chung et al., 2020) (Figures 2a and S2).

We next characterized 4-week-old *osa-MIR171f*-OE plants and knockout (K/O) mutants grown under drought conditions in a

greenhouse (Figure 2b-d). During the drought treatments, soil moisture content decreased uniformly over time (Figure 2c). *osa-MIR171f*-OE, K/O, and non-transgenic (NT) plants all showed induction of stress damage symptoms, such as wilting and leaf-rolling (Figure 2b); however, the *osa-MIR171f*-OE plants recovered better during re-watering for up to 7 days than did the K/O and NT plants. Notably, the survival rates of the *osa-MIR171f*-OE plants ranged from 85% to 98%, whereas the *osa-mir171f*-K/O and NT plants showed no signs of recovery (Figure 2b,e).

In general, plants with less water content have higher temperatures and emit more heat and infra-red radiation, and this can be measured using infra-red (IR) thermography, where a red color indicates a higher temperature, lower water content, and less stomatal conductance (Kim et al., 2020). Using this technique, leaves of *osa-MIR171f*-OE plants were observed to have lower temperatures (20.3–22.9°C) than those of NT and K/O plants, which had average temperature of 24.5°C and 24.9°C, respectively (Figure 2d). These results indicated that overexpressing *osa-MIR171f* in rice enhances drought resistance during the vegetative stage compared to wild-type plants.



**FIGURE 2** Analysis of *osa-MIR171f*-overexpressing transgenic plants and knockout mutants in response to drought stress. (a) Expression levels of *osa*-mature-microRNA171a-f (*osa*-miR171) in *osa-MIR171f*-overexpressing (OE) transgenic plants and knockout (K/O) mutants. The expression level was assessed by Northern blot analysis. U6 transcript levels show equal loading of RNA. Total RNA was prepared from the 4-week-old plants. *Osa*-miR171/U6 value was calculated by band image analysis. (b) The phenotype of *osa-MIR171f*-OE plants and K/O mutants during drought stress. Four-week-old plants from four independent lines of *osa-MIR171f*-OE plants (2, 3, 5, 8), the K/O mutant (2), and non-transgenic (NT) controls were exposed to drought stress for 2 days, followed by re-watering for 7 days. (c) Measurement of soil moisture contents (mV). Data represent mean value  $\pm$  SD of 10 measurements performed in different regions of the soil. (d), Thermal imaging of *osa-MIR171f*-OE plants (2, 3, 5, 8), K/O mutants (2), and NT plants. Different leaf temperatures are expressed as different colors: lower in blue and higher in red. (e) The survival rate of *osa-MIR171f*-OE plants (2, 3, 5, 8), K/O mutants (2), and NT plants 7 days after re-watering. Data represent mean value  $\pm$  SD ( $n = 30$ )

### 2.3 | Identification of *osa*-miR171 target genes during drought stress

To further determine the basis of the enhanced drought tolerance in *osa-MIR171f* overexpressing (OE) plants, we analyzed *osa*-mature-miR171a-f (*osa*-miR171) target mRNA in response to drought stress. To predict *osa*-pre-miR171f target genes and the associated mature miRNA-target pair alignments, we used psRNATarget software ([https://plantgrn.noble.org/psRNATarget/analysis](https://plantgrn.noble.org/psRNATarget/)) (Figure S3a). The *SCL6-I*, *SCL6-II*, *ARC3*, and *DEPG10*

transcripts were found to contain complementary sequences to *osa*-miR171, and the sequences of *SCL6-I* and *SCL6-II* showed 100% base pairing. These results suggested that *SCL6-I* and *SCL6-II* are regulated by *osa*-mature-microRNA171a-f (*osa*-miR171) in rice, as was shown recently for *SCL6-II*, *SCL6-III*, and *SCL6-IV* and pre-miR171a and pre-miR171c during shoot branching and elongation in *A. thaliana* (Llave et al., 2002; Wang et al., 2010).

We analyzed *SCL6-I*, *SCL6-II*, *ARC3*, and *DEPG10* transcript levels in response to desiccation and drought stress by RT-qPCR (Figures 3 and S2b-e). In 10-day-old plants, *SCL6-I* expression



decreased in roots in response to the desiccation stress but did not change in shoots (Figure S3b), while *SCL6-II* expression decreased in both shoots and roots (Figure S3c). *ARC3* and *DEPG10* expression did not change significantly in response to desiccation stress (Figures 3e and S3d). In 4-week-old plants, *ARC3* expression slightly decreased, while *DEPG10* expression increased during the drought stress (Figure 3a,b). Unlike *ARC3* and *DEPG10* expression, *SCL6-I* and *SCL6-II* expression strongly decreased due to the drought stress (Figure 3c,d). In contrast, *osa-pre-miR171f* and *osa-miR171* expression increased (Figure 1). These results suggest that in rice, the *SCL6-I* and *SCL6-II* are putative *osa-miR171* target genes under drought stress conditions.

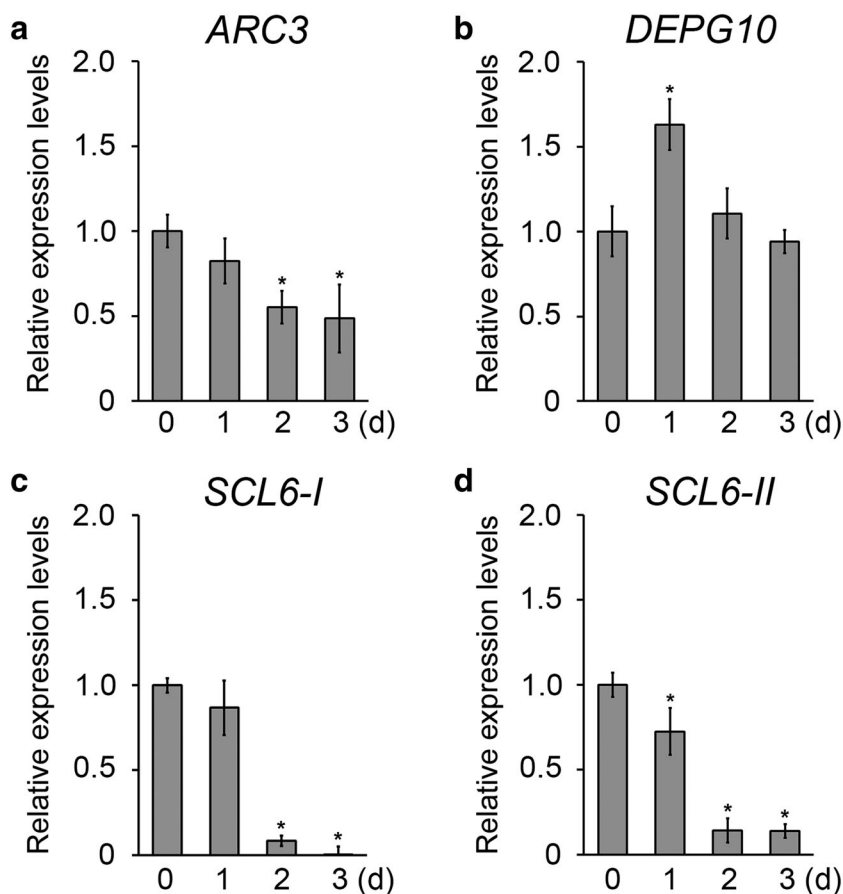
## 2.4 | *SCL6-I* and *SCL6-II* transcripts are regulated by *osa-MIR171f* expression

If *osa-mature-miR171f* regulates both *SCL6-I* and *SCL6-II* transcript levels, it would be expected that they would have an opposite expression pattern to that of *osa-mature-microRNA171a-f* (*osa-miR171*) during rice development. To determine the spatial and temporal expression patterns of *osa-miR171*, *SCL6-I*, and *SCL6-II*, we extracted total RNA from the coleoptile, roots, shoots, leaves, and flowers at different developmental stages and used it for RT-qPCR

analysis (Figure 4a). *Osa-miR171* expression was down-regulated in coleoptiles, whereas *SCL6-II* expression was up-regulated in 4-day-old plants. In the roots, *osa-miR171* expression was up-regulated, whereas *SCL6-II* expression was down-regulated in 10-, 15-, and 25-day-old plants. In leaves, *osa-miR171* expression was down-regulated, whereas *SCL6-I* expression was up-regulated during development, and in flowers, *osa-miR171* expression increased, while *SCL6-I* and *SCL6-II* expression decreased at meiosis and before heading stages. These results showed that tissue-specific expression of *SCL6-I* or *SCL6-II* correlates with *osa-miR171* transcript levels.

To validate the effect of *osa-MIR171f* expression on *SCL6-I* and *SCL6-II* expression, we performed RT-qPCR on the OE, K/O, and NT plants (Figure 4b,c). We checked the *SCL6-I* and *SCL6-II* expression in the leaf of *miR171f* OE and KO plants (4-week-old) because expression level of *miR171f* is significantly co-related with *SCL6-I* and *SCL6-II* in the leaf tissue at this developmental stage compared to other tissues, and it was expected that the *miR171f* overexpression effect on *SCL6-I* and *SCL6-II* expression could be maximized. In *osa-MIR171f*-OE plants, *SCL6-I* and *SCL6-II* transcript levels were down-regulated compared to NT plants, while the opposite was true for the *osa-miR171f* K/O mutants. These results indicated that *SCL6-I* and *SCL6-II* transcript levels were regulated by the expression of *osa-MIR171f*.

**FIGURE 3** Expression of putative *osa-mature-microRNA171a-f* (*osa-miR171*) target genes in response to drought stress. (a–d) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis shows changes in *ARC3* (a), *DEPG10* (b), *SCL6-I* (c), and *SCL6-II* (d) expression levels in response to drought stress. Total RNA was prepared from the 4-week-old plants exposed to drought stresses for the indicated time periods. Control plants were not treated with drought stress (0 days). X axis indicates days after drought treatment (d). Error bars indicate SD of triplicate measurements. *OsTubA2* and *OsUbi1* were used as internal controls, and relative expression levels are shown as fold values. Asterisks indicate statistically significant differences between the corresponding samples and the controls ( $p$  value < .01, Student's  $t$  test)

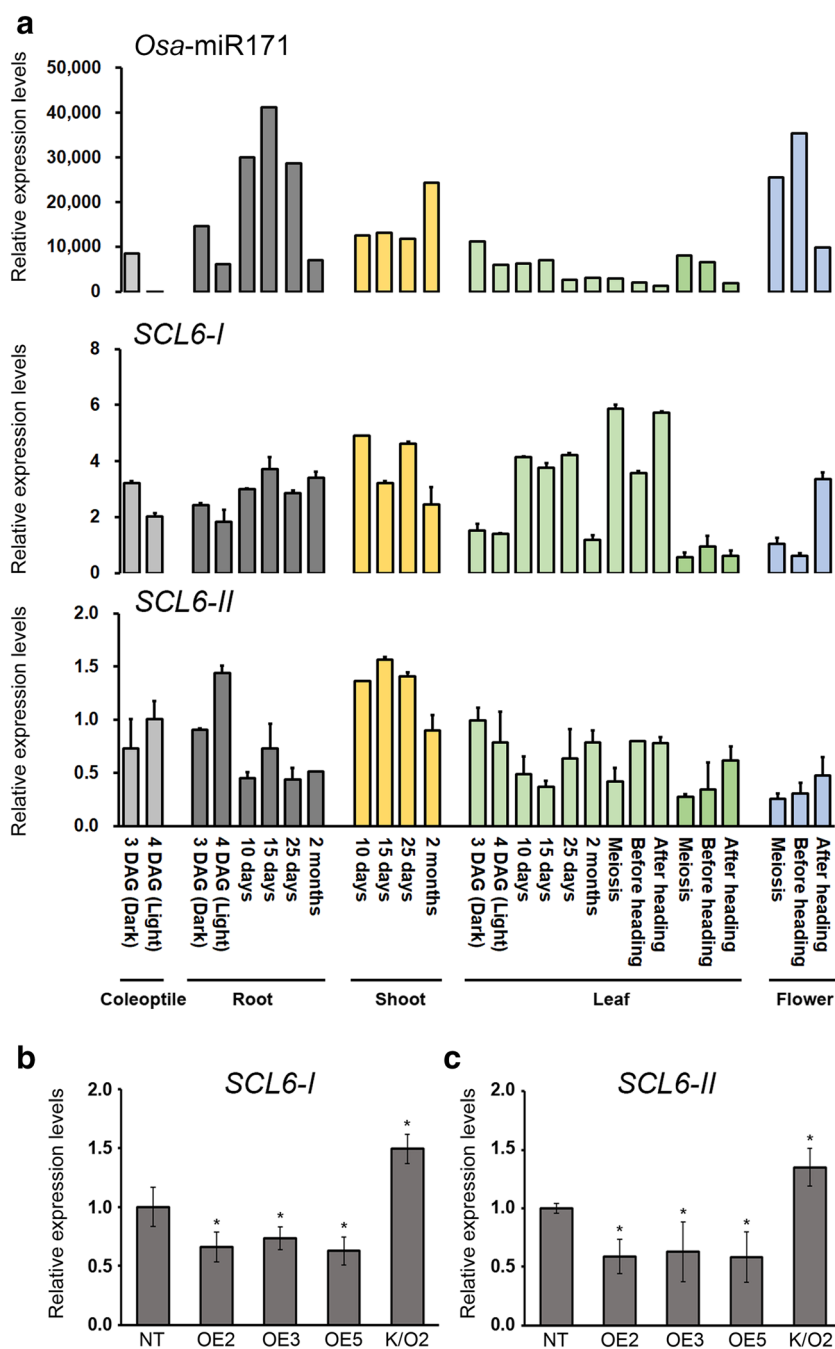


## 2.5 | Expression of *osa-MIR171f* enhances drought tolerance

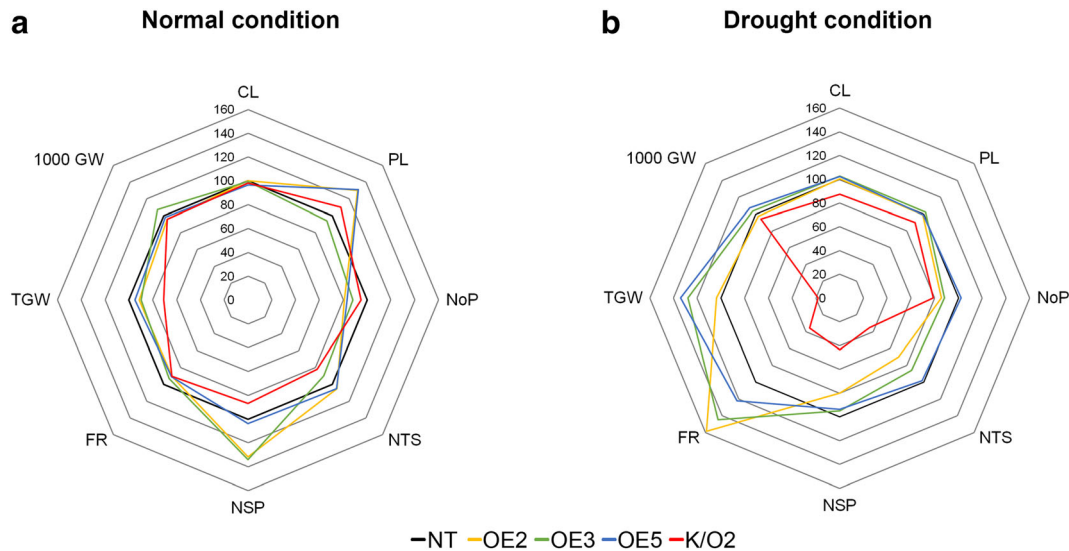
*SCL6-II*, *SCL6-III*, and *SCL6-IV* are downregulated by *ath-miR171c* and *osa-miR171a*, and this affects shoot branching and plant height in *A. thaliana* and rice, respectively. Overexpression of either *ath-MIR171c* and *osa-MIR171a* results in reduced shoot branching and increased plant height compared with wild-type (Wang et al., 2010). We, therefore, wanted to determine the phenotypic effect of *osa-MIR171f* expression (Figure S4). The OE plants exhibited a reduced tiller number compared to NT plants. However, we observed no significant difference in the number of tillers between K/O mutants and NT

plants (Figures 4b and S4a), and this was also true for plant height and culm length (Figure S4c). The flag leaf length of *osa-MIR171f* -OE plants was greater than in NT plants (Figures 4e and S4d), while the flag leaf width was similar (Figure S4f), suggesting that *osa-MIR171f* expression affects the number of tillers and flag leaf length but not plant height and flag leaf width.

The number of panicles with grains in *osa-MIR171f* -OE plants was 11–19% less than in NT plants (Figure 5a and Table S1), and the number of spikelets per panicle was 3–34% greater in the transgenic plants. However, values for total grain weight, filling rate, and 1,000-grain weight were not different between the transgenic and NT plants. In contrast, total grain weight and filling rate in the *osa-*



**FIGURE 4** Expression patterns of *SCL6* genes at various developmental stages and in *osa-MIR171f*-overexpressing (OE) plants and knockout (K/O) mutants. (a) Expression of *osa*-mature-microRNA171a-f (*osa-miR171*) (upper panel), *SCL6-I* (middle panel), and *SCL6-II* (lower panel) in various developmental stages and tissues. Total RNA was extracted, and the expression level was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (b) Expression level of *SCL6-I* and *SCL6-II* in *osa-MIR171f*-overexpressing plants and K/O mutants. The expression level was analyzed by RT-qPCR. *OsTubA2* and *OsUbi1* were used as internal controls, and relative expression levels are shown as fold values. Total RNA was prepared from 4-week-old plants. Data represent the mean value of three biological replicates, and error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control ( $p$  value < .01,  $t$  test)



**FIGURE 5** Agronomic traits of *osa-MIR171f*-overexpressing (OE) and -knockout (K/O) plants under normal growth conditions and drought stress conditions in the field. (a and b) Spider plots showing the agronomic traits of the *osa-MIR171f*-OE and -K/O plants grown under normal field conditions (a) and under drought stress conditions (b). *Osa-MIR171f*-OE and -K/O plants were analyzed together with non-transgenic rice (NT) counterparts grown under the same conditions. The values represent the percentage of the mean values ( $n = 30$ ). Mean values of controls were set at 100% as a reference. CL, culm length; PL, panicle length; NoP, number of panicles; NTS, total number of spikelets; NSP, the number of spikelets per panicle; FR, filling rate; TGW, total grain weight; 1,000 GW, 1,000 grain weight

*mir171f*-K/O mutants were reduced by 29% and 9%, respectively, compared with the NT plants. These results suggest that the expression of *osa-miR171* strongly affects the growth and productivity of rice under normal field conditions.

We next investigated whether *osa-MIR171f* expression correlates with grain yield under drought conditions in 14-week-old plants exposed to drought stress. After drought stress treatment, the growth and productivity of *osa-MIR171f*-OE, -K/O mutants, and NT plants were quantified at the reproductive stage (25-week-old) (Figure 5b and Table S2). The grain filling rate and total grain weight were higher in *osa-MIR171f*-OE plants than in NT plants, consistent with enhanced drought resistance. In contrast, *osa-mir171f*-K/O mutants showed reduced values for all parameters compared to NT plants, indicating that *osa-mir171f*-K/O mutants are more susceptible to drought stress than NT plants. Collectively, these findings suggest that *osa-MIR171f* expression promotes drought resistance in rice.

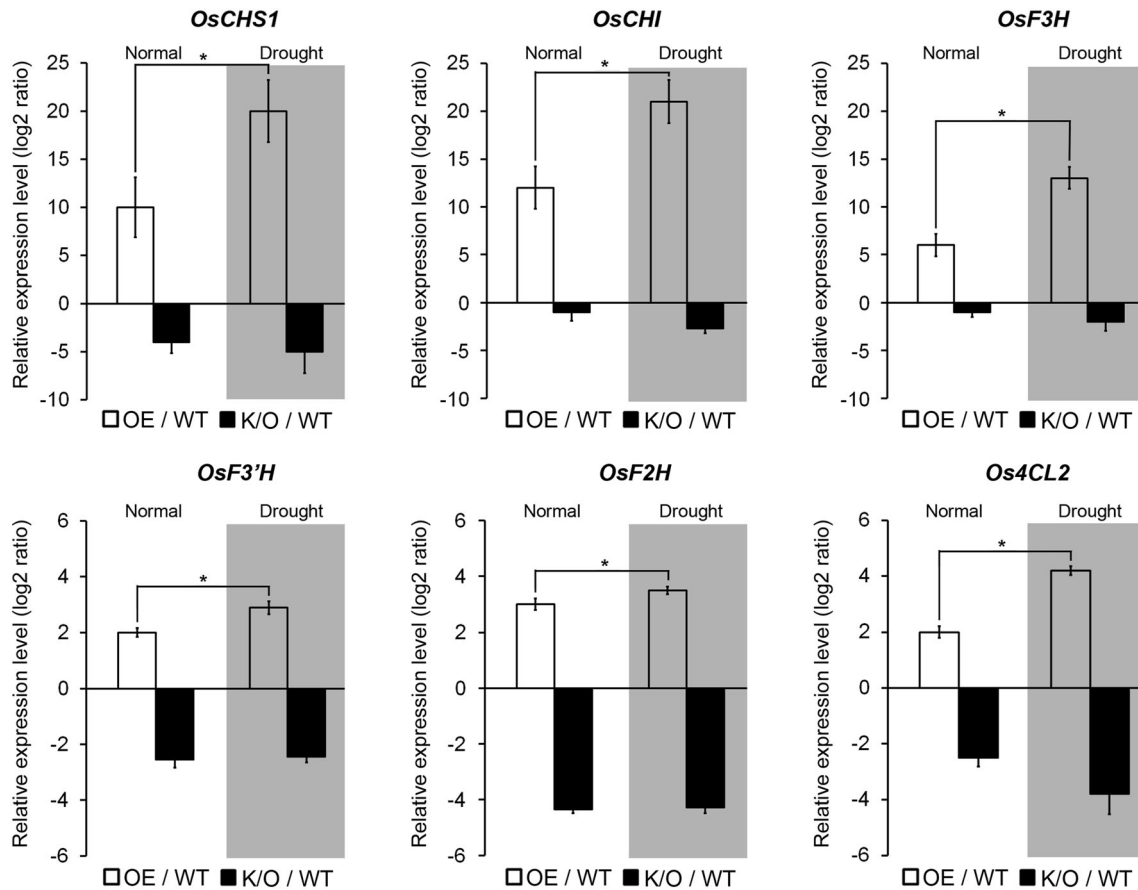
## 2.6 | The expression of flavonoid biosynthesis genes is regulated by *osa-MIR171f*

To better understand the mechanistic relationship between the expression of *osa-MIR171f* and drought tolerance, we analyzed the transcriptome in *osa-MIR171f*-OE plants, *osa-mir171f*-K/O mutants, and NT plants using RNA-sequencing analysis (Figure S5). We found that 45 of the differentially expressed genes (DEGs) overlapped between up-regulated genes in *osa-MIR171f*-OE and the down-regulated genes in *osa-mir171f*-K/O, compared to NT plants. In addition, 81 genes overlapped between down-regulated genes in *osa-*

*MIR171f*-OE and up-regulated genes in *osa-mir171f*-K/O, compared to NT plants (Figure S5a). Analysis of the DEG data using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) indicated that flavonoid synthesis genes were regulated by *osa-MIR171f* expression: CHALCONE SYNTHASE (*OsCHS*), CHALCONE ISOMERASE (*OsCHI*), FLAVANONE 3-HYDROXYLASE (*OsF3H*), FLAVONOID 3'-HYDROXYLASE (*OsF3'H*), FLAVONE SYNTHASE II (*OsFNSII*), 4-COUMARATE-COA LIGASE 2 (*Os4CL2*), and FLAVANONE 2-HYDROXYLASE (*OsF2H*) were all up-regulated in *osa-MIR171f*-OE and down-regulated in *osa-mir171f*-K/O plants (Figure S5b). We validated the expression patterns of the flavonoid synthesis genes by RT-qPCR (Figure 6). We also investigated whether *osa-MIR171f* expression correlated with the expression of flavonoid synthesis genes under drought stress conditions (Figure 6). We observed that the transcript levels of all the investigated genes (*OsCHS*, *OsCHI*, *OsF3H*, *OsF3'H*, *Os4CL2*, and *OsF2H*) were up-regulated by drought compared to normal conditions in *osa-MIR171f*-OE plants but were not significantly different in *osa-mir171f*-K/O mutants. These results indicated that *osa-MIR171f* regulates the expression of flavonoid biosynthesis genes in the drought response, suggesting that *osa-MIR171f*-mediated drought tolerance involves flavonoid biosynthesis under the control of *SCL6-I* and *SCL6-II* expression.

## 3 | DISCUSSION

In both *A. thaliana* and rice, there are three miR171 precursors (pre-miR171a to pre-miR171c) and nine miR171 precursors



**FIGURE 6** Expression patterns of flavonoid biosynthesis genes in *osa-MIR171f*-overexpressing (OE) and -knockout (K/O) plants. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis shows the expression levels of *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsF2H*, and *Os4CL2* in non-transgenic (NT), *osa-MIR171f*-OE, and K/O plants under normal and drought stress conditions (gray boxes). RT-qPCR analysis shows changes in *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsF2H*, and *Os4CL2* expression in response to drought stress. Total RNA was prepared from 4-week-old plants exposed to drought stress. Normal conditions mean plants were not treated with drought stress (0 h time). Error bars indicate SD of triplicate measurements. *OsTubA2* and *OsUbi1* were used as internal controls, and relative expression levels are shown as fold values. Asterisks indicate statistically significant differences between the corresponding samples and their control ( $p$  value < .01, Student's  $t$  test)

(pre-miR171a to pre-miR171i), respectively. Despite a high degree of conservation of the mature miR171 sequences, the expression of *MIR171* genes can be either down- or up-regulated, depending on specific drought stress conditions and species (Chung et al., 2016; Ferdous et al., 2015; Jian et al., 2016). *Osa-MIR171f* was previously identified as a drought stress-responsive gene (Chung et al., 2016). In this study, we showed that *osa-pre-miR171f* and *osa-miR171* expression is increased by drought stress and ABA treatment (Figure 1). Previous studies reported that the *osa-MIR171f* promoter contains both methyl-jasmonate (MeJA) responsive elements and ABA responsive elements, as well as a MYB binding site (Zhu et al., 2015). At the vegetative, the *osa-MIR171f*-OE plants showed drought resistance phenotypes, while the *osa-mir171f*-K/O plants showed drought-sensitive phenotypes (Figure 2a). Drought stress at the pre-anthesis stage is critical in grain filling and yield (Fahad et al., 2017). Therefore, we applied the drought stress at the panicle heading stage and observed the significant yield difference between miR171f transgenic plants and NT plant (Figure 5). These findings

suggest that rice *osa-MIR171f* is involved in regulating the drought stress response across the development stage.

The miR171/GRAS modules are known to mediate meristem maintenance via regulation of the gibberellin and auxin signaling pathways (Bolle, 2004; Fan et al., 2015; Huang et al., 2017; Wang et al., 2010). In *A. thaliana*, plants overexpressing *ath-MIR171c*, as well as triple *scl6* mutant plants, are less branched and have narrower leaves than wild-type plants. In addition, the triple *scl6* mutants have increased chlorophyll accumulation compared to the wild-type (Wang et al., 2010). In tomato, overexpression of *sly-miR171* increased plant height, whereas overexpression of the *sly-miR171* target gene, *SIGRAS24*, and *sly-miR171* knock-down (short tandem target mimic construct, STTM171) plants showed increased branching and a dwarf phenotype (Huang et al., 2017; Kravchik et al., 2019). Here, we observed that *osa-MIR171f* -OE plants had reduced panicle number and increased numbers of spikelets per panicle compared to NT plants at the reproductive stage (Figure S4). These results suggest that *osa-miR171* and its target genes *SCL6-I* and *SCL6-II* are important for





tiller formation in rice, and that tiller formation enhances drought tolerance.

Drought stress induces reactive oxygen species (ROS), such as hydroxyl radicals ( $\cdot\text{OH}$ ), superoxide radicals ( $\text{O}_2^{\cdot-}$ ), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and is critical for initiating stress signaling. However, excess ROS accumulation causes cell death that can be attributed to a disturbance in cellular homeostasis (Agati et al., 2012; Bolouri-Moghaddam et al., 2010; Nakabayashi et al., 2014; Vickers et al., 2009). Flavonoids can effectively scavenge ROS (Agati et al., 2020): for example, flavonoid over-accumulating plants showed resistance to oxidative stress and drought stress. Moreover, cyanidin-type anthocyanins and quercetin-type flavonols exhibited strong radical-scavenging activity in an 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay for analysis of flavonoids activity (Seyoum et al., 2006). Many studies show that the accumulation of flavonoids and expression of flavonoid biosynthesis genes increase under drought stress (Gai et al., 2020; Sharma et al., 2019; Wang et al., 2020). In a previous study in tobacco (*Nicotiana tabacum*), *NtCHS*-overexpressing plants showed higher oxidative stress tolerance and drought tolerance (Hu et al., 2019), and in soybean (*Glycine max*), *gma-miR171o*- and *gma-miR171q*-mediated expression of *GmSCL6* and *GmNSP2* were shown to regulate spatial and temporal aspects of nodulation (Hossain et al., 2019). Finally, in *A. thaliana*, *miR156e-3p*-overexpressing transgenic plants showed increased accumulation of anthocyanins in the lateral branches (Zhao et al., 2017). Here, we observed that key genes involved in flavonoid biosynthesis, such as *OsCHS*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsFNSII*, *Os4CL2*, and *OsF2H*, were up-regulated by *osa-MIR171f* under drought stress (Figures 6 and S5b).

However, it is still unclear how *osa-MIR171f* positively regulates the expression of the flavonoid biosynthesis genes via its repression of *SCL6* gene expression. DELLA (GAI, RGA, and RGLs) proteins belonging to the GRAS family function as master negative regulators of GA signaling and are convergent integrators of multiple signaling pathways via direct interaction with various transcription and co-transcription factors (Schwechheimer, 2011). It has been reported that DELLA proteins directly interact with MYB12/111 and positively regulate the expression of flavonoid biosynthesis genes, *CHS* and *F3H* (Tan et al., 2019), and other reports showed that *SCL3* directly interacts with DELLA and plays an antagonistic role in GA signaling (Heo et al., 2011; Zhang et al., 2011). Based on these results, it can be speculated that DELLA and SCL have opposite regulatory effects on the transcription of flavonoid biosynthesis genes and hypothesized that an *osa-miR171/SCL6* module regulates the transcription of flavonoid biosynthesis genes via interaction with DELLA proteins.

Although many transcriptome analyses have suggested that *osa-MIR171* genes are involved in drought stress responses, the underlying mechanisms have not been identified. Based on the results presented here, we propose that rice *osa-MIR171f* regulates the expression of flavonoid biosynthesis genes by regulating *SCL6-I* and *SCL6-II* expression under drought stress conditions, thereby conferring drought tolerance.

## 4 | MATERIALS AND METHODS

### 4.1 | Plant materials and growth conditions

*O. sativa* L. cv. Dongjin was used as non-transgenic control plants. Rice seeds were germinated in Murashige and Skoog (MS) agar medium in a growth chamber in the dark at 28°C for 3 days. After 3 days of germination, plants were grown under long-day conditions with 16-h light/8-h dark cycles at 28°C for 7 days.

### 4.2 | Vector construction

For the construction of the *GOS2::osa-MIR171f* plasmid, full-length *osa-MIR171f* cDNA was derived from total RNA extracted from Dongjin rice by reverse transcriptase polymerase chain reaction (RT-PCR). The *osa-MIR171f* cDNA was inserted into the p700 vector containing a promoter constitutively expressed in the whole body of the plant, *GOS2* (Pater et al., 1992). The recombinant plasmid was verified by sequencing and then introduced into *Agrobacterium tumefaciens* LBA4404 for *Agrobacterium*-mediated rice transformation. Primer sequences are listed in Table S3.

### 4.3 | Northern blot analysis

To analyze *osa*-mature-microRNA171a-f (*osa-miR171*) transcripts, total RNA was extracted from the indicated samples and 15 µg was separated on a 12.5% urea-polyacrylamide gel and electro-transferred to a Hybond N+ membrane (GE Healthcare). The miR171 probe was generated by labeling oligonucleotides complementary to the miR171 sequence (5'-GATATTGGCGCGCTCAATCA-3') using a DIG oligonucleotide tailing kit (Roche). U6 snRNA (5'-TTGCGTGCATCCTTGCGCAGG-3') was used as equal loading indicator of RNA samples. An AP-conjugated DIG antibody (Roche) was used for probe detection. Signals were visualized by treatment with an CSPD solution (Roche) and exposure to X-ray film.

### 4.4 | RT-qPCR

RT-qPCR analyses were performed using total RNA extracted from the indicated plants using TRIzol (Invitrogen). For cDNA synthesis, 20 µl reactions were performed using 2 µg of total RNA digested with RNAase-free DNase I (Promega) and the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific™). To detect *osa*-mature-microRNA171a-f (*osa-miR171*), 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 total 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  $\mu$ M 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. For quantitative PCR, a Mx3000P Real-Time PCR machine (Stratagene, CA, USA) with Solg™ 2 $\times$  real-time PCR smart mix and EvaGreen (SolGent, Deajeon, Korea) was used. PCR cycles included an initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. *OsUbi1* (Os06g0681400) and *OsTubA2* (Os11g0247300) were used as internal controls. Relative expression level was represented by the mean value of the two independent experiments with *OsUubA2* or *OsUBi1*. Primer sequences are listed in Table S3 and Figure S6.

#### 4.5 | Desiccation and drought stress

To analyze the effect of desiccation and drought stress on expressions of genes, 10-day-old plants grown on MS media or 4-week-old grown on soil were air-dried on 3 M papers or drought for the indicated times. For the drought tolerance test at the vegetative stage, seeds were uniformly germinated on MS (Murashige and Skoog) media and placed in the dark (28°C) for 3 days. Seedlings were transferred into soil pots (4  $\times$  4  $\times$  6 cm; 3 plants per pot) and grown in a greenhouse (16 h-light/8 h-dark cycle) at 30°C. Four weeks after transplanting, drought stress was induced by withholding water for 3 days and then re-watered for 7 days. The soil water content in each pot was adjusted to 80%. Soil moisture was monitored during the drought treatment using a Soil Moisture Sensor SM150 (Delta-T Devices). For analysis of agronomic traits under drought stress, *osa-MIR171f*-overexpressing or -knockout plants planted in pots (15 cm diameter  $\times$  14 cm tall) were grown in a rain-off shelter. At the panicle heading stage, the first round of drought stress treatments was applied by withholding water. After 7 days, plants were re-watered and allowed to recover. Ten days after the first treatment, a second round of drought stress was applied in the same manner as before. For analysis of agronomic traits under normal conditions, three independent lines from the T<sub>4</sub> (2020) generation of *osa-MIR171f*-overexpressing or *osa-mir171f*-knockout plants were grown in a paddy field located at Kyungpook National University, Korea (128:34E/36:15 N) together with corresponding NT plants.

#### 4.6 | Transcriptome analysis

Four-week-old NT, *osa-MIR171f*-OE and *osa-mir171f*-K/O plants were grown under normal conditions in a glasshouse. For the Illumina library preparation, total RNA was first extracted from NT, *osa-MIR171f*-OE and -K/O leaves using the Plant RNeasy mini kit (Qiagen, Valencia, USA). RNA from three independent lines of *osa-MIR171f*-OE, K/O, and NT plants was pooled for RNAseq according to the manufacturer's protocol and sequenced (Macrogen, Seoul, Korea) using the Illumina HiSeq2000 platform (Illumina, San Diego, USA). The

transcripts were assembled and normalized by Fragment Per Kilobase of transcript per Killion mapped reads (FPKM), and the DEGs in each *osa-MIR171f*-OE versus NT and *osa-mir171f* -K/O vs NT were selected by  $|\log_2(\text{fold change})| \geq 1$  and  $|\log_2(\text{fold change})| \leq -1$  using a *P* value <.05. The data set can be found at from GEO database with series accession number GSE180271 for RNA-sequencing data.

#### 4.7 | Accession numbers

Sequence data from this article can be found in the in the Rice Annotation Project (RAP) database (<https://rapdb.dna.affrc.go.jp/>) or the microRNA database (miRBase) (<http://www.mirbase.org/>) under the following accession numbers: *Osa-MIR171f* (MI0001137), *ARC3* (Os09g0555600), *DEPG10* (Os05g0417100), *SCL6-I* (Os06g0105350), and *SCL6-II* (Os02g0662700).

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#### AUTHOR CONTRIBUTIONS

T. U. and J-K K. designed this study; T. U., J. C., P. J. C., and J. S. S. (Jun Sung Seo) wrote the manuscript and prepared the figures; T. P., S. E. J., J. S. S. (Jae Sung Shim), Y. S. K., I-Y. C., S. C. P., S-J. O., and J-K. K. performed phenotyping and contributed to data analysis. All authors have read and agreed to the published version of the manuscript.

#### CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

#### DATA AVAILABILITY STATEMENT

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Ju-Kon Kim (jukon@snu.ac.kr).

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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