Relationship between reduction in rice (Nipponbare) leaf blade size under elevated CO₂ and miR396–GRF module

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ABSTRACT
Elevated CO₂ (eCO₂; 1000 ppm) influences developing rice leaf formation, reducing leaf blade length and width as compared to rice grown under ambient CO₂ (aCO₂; 400 ppm). Since micro RNAs (miRNAs) are known to play multiple roles in plant development, we hypothesized that miRNAs might be involved in modulating leaf size under eCO₂ conditions. To identify miRNAs responding to eCO₂, we profiled miRNA levels in developing rice leaves (P4; plastochron number of the fourth-youngest leaf) under eCO₂ using small RNA-seq. We detected 18 mature miRNA sequences for which expression levels varied more than two-fold between the eCO₂ and aCO₂ conditions. Among them, only miR396e and miR396f significantly differed between the two conditions. Additionally, the expression of growth-regulating factors (GRFs), potential target mRNA of miR396e, were repressed under the eCO₂ condition. We used an antisense oligonucleotide approach to confirm that single-strand DNA corresponding to the miR396e sequence effectively downregulated GRF expression in developing leaves, reducing the leaf blade length, such as for rice grown under eCO₂. These results suggest that the miR396–GRF module is crucially relevant to controlling rice leaf blade length in eCO₂ environments.

Introduction
Carbon dioxide (CO₂) is an atmospheric gas essential for terrestri-al plant photosynthesis. Atmospheric CO₂ concentrations have gradually increased since the industrial revolution and are expected to reach 550–1000 ppm by the end of the century. In this context, many researchers have investigated the effects of elevated CO₂ (eCO₂) on plant growth and physiological processes, reporting that increases in crop plant biomass and yield are expected under eCO₂ conditions. However, plants will gradually acclimate to the eCO₂ environment; increased photosynthetic efficiency at eCO₂ onset cannot be maintained due to decreases in rubisco levels and activity and stomatal conductance. Consequently, the expected increases in biomass and yield in field cultivation have not been achieved under eCO₂ conditions. In the case of rice (Nipponbare) plants subjected to eCO₂ (eCO₂; 1000 ppm), the leaf blade (LB) length and width are reduced in newly developed leaves despite of sufficient nitrogen supply. Specifically, reduction in LB length and width are detectable in fully expanded second and third leaves later, representing leaf developmental stages P4 (plastochron number of the fourth-youngest leaf) and P3 (third-youngest leaf), respectively, at the onset of eCO₂ treatment. However, the primary internal regulator responsible for reducing leaf growth under eCO₂ has yet to be elucidated.

Plant micro RNAs (miRNAs) are small endogenous non-coding RNAs comprising 20–24 nucleotides (nt). miRNA-related gene silencing occurs through target mRNA cleavage, translational repression, and DNA methylation by binding to targets in a sequence-complementary manner. Numerous miRNAs have been identified in plants, several of which play essential roles in regulating gene expression during plant development processes, such as apical meristem, leaf, and root development, flowering transition, and floral patterning. Among plant miRNAs, miR396 is a well-established miRNA family. Many studies have examined the biological role of miR396 and its target transcription factor, the growth-regulating factor (GRF) family. The miR396–GRF module plays a central role in regulating leaf size, plant height, and seed size and yield. This module also functions in a regulatory pathway for plant hormones, including gibberellin and brassinosteroid, and in disease resistance against pathogen infection. Because the miR396–GRF module is essential for various developmental processes in plants, it may play a role in regulating phenotypic changes in response to environmental cues.

The antisense oligonucleotides approach is an effective tool for post-transcriptional gene regulation and has been extensively exploited to develop RNA-based therapeutic technologies. A typical antisense oligonucleotide drug is approximately 20 nt long with specific chemical modifications to enhance its biological stability in living organisms. Antisense oligonucleotides primarily bind to RNA by base pairing in a sequence-specific manner; the degradation of antisense oligonucleotide-RNA is dependent on RNase H-based degradation of the DNA–RNA
hybrid, thereby downregulating target gene expression.\textsuperscript{19,21,22} Although several studies have demonstrated the effectiveness of antisense oligonucleotides in mammalian cells and clinical trials,\textsuperscript{19,20,23–25} this approach is not widely utilized in the plant science field.\textsuperscript{26,27}

In this study, we hypothesized that eCO$_2$-induced miRNAs in developing rice leaves are relevant to reduced leaf size because miRNAs are crucially associated with plant development. To clarify this hypothesis, we investigated the role of miRNAs in leaf development under eCO$_2$ conditions using an antisense oligonucleotide approach. First, we performed miRNA profiling on developing leaves under eCO$_2$. We found that the miR396–GRF module may be a predominant regulator of reduced LB length under eCO$_2$ conditions using single-strand DNA corresponding to the miR396e sequence.

Materials and methods

Plant growth conditions and eCO$_2$ treatments

Rice seeds (Oryza sativa L. cv. Nipponbare) were germinated in tap water at 28°C for 2 days. The germinated seeds were sown in a soil mixture (1:1 non-fertilized granular soil [Kanuma Sangyou, Kanuma, Japan] and granular soil L [Sankensoiuru, Hachimatai, Japan]) and grown in a growth chamber at a relative humidity of 60% on a day/night cycle (28°C/23°C; 14 h light/10 h dark [6:00–20:00:00 daylight]). Light was provided using metal halide and fluorescent lamps at a photosynthetic photon flux density (PPFD) of 600–700 μmol m$^{-2}$s$^{-1}$ for 10 days. Seedlings were transplanted into 1/5000-are Wagner pots containing non-fertilized granular soil (Kanuma Sangyou) supplemented with N (0.5 g per plant to remove the effect of N deficiency; the standard N level is 0.3 g per plant), P$_2$O$_5$ (0.45 g per plant), and K$_2$O (0.35 g per plant).\textsuperscript{7} The rice plants were grown under waterlogged and ambient CO$_2$ (aCO$_2$; approximately 300–350 ppm CO$_2$ with ventilation) conditions in two identical growth chambers (60% relative humidity; 28°C/23°C; 14 h light/10 h dark cycle [6:00–20:00:00 h daylight] with 700–800 μmol m$^{-2}$s$^{-1}$ PPFD after transplanting and 800–900 μmol m$^{-2}$s$^{-1}$ PPFD from the tenth leaves stage, provided at the plant height). Pot positions were rotated three times per week after transplanting to avoid positional effects on rice growth. When the seventh leaves were fully expanded (plant age of leaf number = 7.0–7.2 stage), the CO$_2$ concentration inside one of the growth chambers was raised at 5:30 h to 1000 ppm using high-purity CO$_2$ gas (99.999%) to minimize possible ethylene contamination. As a result, the CO$_2$ concentration reached 1000 ppm at 6:00 h (beginning of the daytime period). Rice plants grown in aCO$_2$ were used as controls.

Leaf sampling

All rice leaves were sampled at designated time points after treatments for subsequent analysis. The part (10 cm) of seventh LBs, including one-third position from the tip, were cut and immediately frozen using liquid nitrogen and stored at −80°C for total RNA isolation. To sample the developing leaf (P4; plastochron number of the fourth-youngest leaf), the aboveground part of the plant was cut, and the stem and leaves older than the seventh leaf were removed. The lower part (2 cm), including P4, was dissected using a razor and immediately immersed into the fixative solution (5 mL, 3:1 ethanol/acetic acid) in a silicon tube. The silicon tube was placed in a vacuum desiccator for a few seconds and slowly released to facilitate infiltration of the fixative into the dissected tissue. The tissue in the silicon tube was then fixed under a vacuum for 30 min and stored at 4°C until P4 isolation. Isolation of the P4 leaf from the fixed tissue was conducted on a fixative using a stereomicroscope. The isolated P4 leaf was frozen using liquid nitrogen and stored at −80°C for total RNA isolation.

Total RNA isolation

Total RNA was extracted from rice leaves (seventh and P4) using the RNaseasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions with modifications for small RNA isolation.\textsuperscript{26} Briefly, frozen rice leaves were ground with liquid nitrogen using a mortar and pestle. Powdered rice leaf was lysed in 500 μL of RNaseasy RLT buffer containing 1% beta-mercaptoethanol (v/v). The lysate was homogenized in a QiAShredder spin column for 2 min at 15,000 g. The flow-through was collected and added to 750 μL of 99.5% ethanol to achieve final concentration of 60%. The solution was mixed and then transferred into the RNaseasy spin column and centrifuged for 20s at 10,000 g. The RNaseasy mini spin column was washed using RWT buffer (350 μL, Qiagen) for 20s at 10,000 g, and DNase (RNase-Free DNase Set, Qiagen) was added to the spin column and incubated for 15 min at room temperature. The spin column was washed using RWT buffer (350 μL), followed by RPE buffer (500 μL), each for 20s at 10,000 g. The spin column was washed with RPE buffer (500 μL) for an additional 2 min at 10,000 g. Finally, the spin column was transferred to a clean collection tube and centrifuged for 1 min at 15,000 g for drying. The spin column was transferred to a new collection tube to elute the pure total RNA fraction, and nuclease-free water (30 μL) was added directly into the column membrane and centrifuged for 1 min at 10,000 g. Total RNA, containing small RNA, samples were stored at −80°C. The final total RNA concentration and purity were determined spectrophotometrically at absorbances of 260 nm and 280 nm using a NanoDrop ND1000 (Thermo Fisher Scientific, Waltham, MA). The total RNA integrity was examined using an Agilent 2100 bioanalyzer with an RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). RNA integrity numbers greater than seven were used for the subsequent experiment.

Library construction and sequencing

Small RNA library construction and sequencing were performed using total RNA, containing small RNA, from the P4 leaf, with two biological replicates. The small RNA library was constructed from isolated total RNA using a TruSeq Small RNA Library Prep kit (Illumi, San Diego, CA) according to the manufacturer’s instructions. The libraries were sequenced.
with 50-bp single-end reads for small RNA-seq analysis using the Illumina HiSeq 2500 platform at Macrogen (Macrogen, Seoul, South Korea).

**Small RNA-seq data processing and analysis**

Low-quality reads were removed from the raw sequence reads, and adapter sequences were trimmed. A unique cluster was formed based on the sequence identity and read length. The unique clustered reads were aligned to the rice reference genome sequence (IRGSP-1.0, [https://rapdb.dna.affrc.go.jp](https://rapdb.dna.affrc.go.jp)), miRBase v21 ([http://www.mirbase.org](http://www.mirbase.org)), and non-coding RNA database (Rfam9.1, [http://rfam.xfam.org](http://rfam.xfam.org)) to classify known miRNAs and remove reads associated with other RNA types, including transfer, small nuclear, and small nucleolar RNAs. To quantitate the abundance of each miRNA, read counts were extracted from the mapped miRNAs. miRNA read counts were normalized by the total reads of each sample and standardized to reads per million (RPM). RPMs were transformed to log2 and quantile-normalized between samples. Two biological replicates were used for RNA-seq data analysis, and miRNAs with an absolute value of log2 fold change (eCO2/aCO2; \( n = 2 \) > 1 were included in the heatmap (Figure 1). miRNA target gene prediction was performed using psRNATarget v2017. Potential target genes were listed with the parameter of maximum expectation cutoff score set at 1.5. Small RNA-seq data were deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE186906 ([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)).

**Microarray data analysis**

Public microarray data deposited in the NCBI GEO database (accession numbers: GSE121054; [https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)) were used to analyze OsGRF gene expression patterns in developing rice leaves under aCO2 and eCO2 conditions. OsGRF expression between aCO2 and eCO2 conditions was compared using 75th percentile-normalized data with three biological replicates.

**Gene expression analysis**

Gene expression analysis (miRNA and mRNA) was performed via reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). First, template cDNA was obtained from miRbase ([http://www.mirbase.org/](http://www.mirbase.org/)).

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**Figure 1.** (a) P4 leaf of rice and the sampling time of the leaf after elevated CO2 (eCO2) treatment. (b) Heatmap showing variations in the level of miRNAs in developing leaves (P4) after eCO2 treatment. The colors indicate the mean value of log2 fold change (eCO2/ambient CO2 [aCO2]; \( n = 2 \)). miRNA sequences were obtained from miRbase ([http://www.mirbase.org/](http://www.mirbase.org/)).
synthesized from 500 ng of total RNA using a PrimeScript RT reagent kit with gDNA eraser (Takara bio, Kusatsu, Japan), miScript II RT kit (Qiagen), and miScript Plant RT kit (Qiagen) for mRNA, premature miRNA, and mature miRNA amplification, respectively, according to the manufacturer’s instructions. Target genes were amplified using SYBR Premix Ex Taq II (Takara Bio) for mRNA and miScript SYBR Green PCR kit (Qiagen) for miRNA. For mature miRNA amplification, we designed specific mature miRNA sequences for the forward primer and used the universal primer provided in the miScript SYBR Green PCR kit as the reverse primer. Primer sequences used in this study are listed in Table S1 (in supplementary data). RT-qPCR was performed using a LightCycler® 96 Instrument (Roche, Bazel, Switzerland). The relative gene expression level was calculated using the comparative Ct method.\textsuperscript{10} OsGAPDH and snoR31 were used as reference genes for mRNA and miRNAs, respectively. The relative expression level of premature miRNA calculated using snoR31 presented no significant difference compared with that calculated using U6, a previously known reference gene for miRNA analysis (Figure S1 in supplementary data).\textsuperscript{16}

**Treatmet with ssDNA, rice growth, and leaf size measurement**

To examine the effect of treatment with single-strand (ss) DNA, ssDNA was chemically synthesized by Eurofins Genomics (https://eurofinsgenomics.jp/). When the seventh leaves were fully expanded (plant age of leaf number = 7.1–7.3 stage), 1.5 mL of ssDNA solution (0.1 μM or 1 μM ssDNA, corresponding to the sequence of miR396e (ssd396e), 5 mM 2-(N-morpholino)ethanesulfonic acid [MES], pH 6.8) was sucked through the cutting edge of the tip of the seventh LB before beginning the daytime period. Nonspecific ssDNA (TCACGTATCCCGAAAAAAAG) was used as a ssDNA control. The solution was removed after two days, and rice plants were grown continuously under aCO\textsubscript{2} until the twelfth leaves fully expanded. Pot positions were rotated three times a week after transplanting to avoid positional effects on rice growth.

The lengths of the seventh LBs and each of the younger newly developed leaves were measured when the next LB was elongated to one-third of the full length. LB width was determined at the widest point of the blade (approximately one-third of the length from the tip).

**Dot blot analysis**

ssDNA treatment and total RNA isolation were performed as described above. The isolated total RNAs containing miRNA (8 μg) were applied on Amersham Hybond-N’ (GE Healthcare, Chicago, IL) and baked at 80°C for 2 h. Dot blot analysis was performed using a Digoxigenin (DIG) luminescent detection kit (Roche, Bazel, Switzerland) according to the manufacturer’s instructions.

**Statistical analysis**

All data are presented as mean ± standard deviation (SD). Student’s t-test was used to assess the statistical significance of differences between two groups of data. Graph Pad Prism 9 software was used for analyses.

**Results and discussion**

**miRNA level variation in P4 leaves between aCO\textsubscript{2} and eCO\textsubscript{2} conditions**

P4 leaves are the fourth smallest leaves in rice and surrounded by older leaves (Figure 1a). To investigate the changes in the miRNA level in P4 leaves under eCO\textsubscript{2} for 1 day, we harvested the leaves from both aCO\textsubscript{2} and eCO\textsubscript{2} treatments at 7 and 13.5 h after beginning the eCO\textsubscript{2} treatment at 6:00 am (Figure 1a). We performed miRNA profiling of the leaves using small RNA-seq analysis. A total of 328 miRNAs were detected in P4 leaves, among which the accumulation levels of 18 miRNA sequences changed more than two-fold (absolute values) between eCO\textsubscript{2} and aCO\textsubscript{2} treatments (Figure 1b). The levels of nine mature miRNA sequences (miR164a,b,f-5p; miR396d,g,h-3p; miR396e-5p; miR396f-5p; miR1423-5p; miR1863b-2–3p; miR1883,b-5p; miR5160-3p) were higher in the eCO\textsubscript{2} treatment at both sampling times, and levels of four mature miRNA sequences (miR159f-3p; miR319a-3p,2–3p; miR812o-5p; miR5072-5p) were lower at both sampling times. The remaining five miRNA sequences (miR168a-3p; miR171i-3p; miR390-3p; miR399a,b,c-3p; miR1878-3p) exhibited different accumulation patterns at 7 and 13.5 h after eCO\textsubscript{2} treatment.

In plants, several miRNAs are well-established as primary regulators of vegetative leaf development. Among the 18 miRNAs that were expressed more than two-fold between the aCO\textsubscript{2} and eCO\textsubscript{2} treatments, the functions of most have not been established; however, miR159, miR164, miR319, and miR396 are known to be involved in leaf development. miR159 regulates MYB33 and MYB65 transcription factors, reducing cell proliferation.\textsuperscript{31} Leaf serration and complexity are modulated by miR164-mediated repression of CUP-SHAPED COTYLEDON2 expression.\textsuperscript{32} miR319 promotes cell growth and proliferation and cellular differentiation during development by modulating TCP transcription factors.\textsuperscript{33,34} miR396 predominantly restricts cell proliferation and elongation by repressing the GPF transcription factor family.\textsuperscript{33,34} These findings prompt us to speculate that changes in the levels of miRNAs involved in leaf development may influence the reduction in leaf size of rice grown under eCO\textsubscript{2}.

To verify the significance of miRNA differences between aCO\textsubscript{2} and eCO\textsubscript{2}, we examined the relative levels of miRNA in P4 leaves using RT-qPCR analysis. Significant increases were only detected in the miR396 family; miR396e and miR396f levels were 6.0-fold and 4.2-fold higher, respectively, in the eCO\textsubscript{2} treatment compared with the aCO\textsubscript{2} treatment after 13.5 h (Figure 2). miR396d,g,h level increased in response to eCO\textsubscript{2} treatment, but not significantly, and showed a lower accumulation level compared with miR396e or f (data not shown).
The levels of other miRNAs were not significantly different between the aCO$_2$ and eCO$_2$ conditions both 7 and 13.5 h after treatment (Figure S2).

Next, we measured the levels of premature miR396d–f to determine whether mature miR396d–f are derived from their premature forms in P4 leaves. Compared with aCO$_2$ plants, premature miR396e and miR396f in eCO$_2$ treatment plants were significantly upregulated at 7 and 13.5 h, and premature miR396g levels were also significantly higher at 13.5 h (Figure 3). These results indicate that the P4 stage leaves contain more mature miR396e and miR396f under eCO$_2$, which is derived from their premature form in the same leaves.

To gain a more comprehensive understanding of the accumulation patterns of miR396e and miR396f in mature leaves (P6) under eCO$_2$, we examined mature and premature miR396e and miR396f levels in the seventh LB, leaf sheath (LS), and basal part (BP), including the stem and developing zone, after eCO$_2$ treatment. Mature miR396e and miR396f levels in the LB, LS, and BP were not significantly changed by eCO$_2$, and miR396e and miR396f levels were relatively low in the BP compared with the LB and LS (Figure S3). Because miR396 inhibits cell proliferation through repression of the GRF family in the developing zone, it is reasonable that the level of miR396 is relatively lower in BP compared with mature LB and LS. Otherwise, premature miR396e levels significantly increased in the LB 7 h after eCO$_2$ treatment, and the level increased up to 13.5 h, but not statistically significant (Figure S4). Premature miR396f was significantly upregulated 13.5 h after eCO$_2$ treatment in the LB and LS.

Several miRNAs are involved in systemic signaling, in which mature miRNAs are translocated between tissues through the vasculature system. For example, miR399 expression is upregulated under inorganic phosphate deficiency and translocated into the root to suppress PHOSPHATE 2 expression. miR395 is known as a mobile miRNA involved in systemic sulfate signaling, which regulates the expression of ATP sulfurylase. However, it remains unknown whether mature or premature miR396s are translocated between plant tissues to regulate target gene expression. In this study, mature miR396e and miR396f were upregulated...
in developing leaves but not mature leaves. However, premature miR396e and miR396f were accumulated in both developing and mature leaves under eCO₂, suggesting that premature miR396 accumulations in developing and mature leaves may influence mature miR396 accumulation in developing leaves. Further investigation of the translocation of premature miRNA could address this possibility.

**miR396 target gene analysis and OsGRF expression in developing leaves**

GRFs are plant-specific transcription factors involved in leaf growth and development and have been studied as miR396 family target genes.\(^1\) Twelve GRFs are found in rice, and most OsGRFs are more highly expressed in developing tissues, such as the elongation zone and shoot apical meristem, than in mature tissues.\(^2\) We analyzed the target genes of miR396e and miR396f using psRNATarget.\(^3\) Most OsGRFs were predicted as target genes (Table S2 and S3). To understand OsGRF expression patterns in response to eCO₂, we analyzed public microarray data of transcriptomic changes in developing rice leaves in response to eCO₂ (GSE121054; [https://www.ncbi.nlm.nih.gov/geo/]). The expression patterns of ten OsGRFs were available in the microarray data, indicating that OsGRF3, OsGRF6, OsGRF9, and OsGRF10 were significantly downregulated before the dark period under the eCO₂ condition (Figure 4a). We verified these transcriptional changes using RT-qPCR and found that the relative expressions of OsGRF3, OsGRF6, and OsGRF10 were significantly downregulated at 7 and 13.5 h after eCO₂ treatment (Figure 4b). These data demonstrate that OsGRF expression in developing rice leaves is influenced by eCO₂.

In Arabidopsis, AtGRFs are predominantly expressed in the shoot and root tips, including the apical meristem, with lower expression in young and mature leaves.\(^4\) Moreover, the leaf size of Arabidopsis triple mutants (AtGRF1, AtGRF2, and AtGRF3) was smaller than that of the wild-type, but the single mutants did not show any leaf size changes. Moreover, AtGRF1 and AtGRF2 overexpression lines both exhibit largely expanded leaf size compared with the wild-type, which was attributed to an increase in cell size, indicating that AtGRF1 and AtGRF2 have cell expansion functions in Arabidopsis.\(^5\) AtGRF5 overexpression also generates a larger leaf size, but the underlying mechanism is an increase in cell numbers rather than cell size.\(^6\) These reports show that GRFs play a predominant role in regulating cell size and cell numbers in developing zones, which dictate the mature leaf size.

Several studies have reported the relationship between OsGRFs and rice leaf growth. Liu et al.\(^7\) reported that OsGRF6 and OsGRF10 antisense transgenic lines produce plants with a reduced height compared to wild-type Dongjin. osgrf6 decreases cell length compared to the wild-type.\(^8\) In addition, rice overexpression line of MAKIBA3, classified with GRF interacting factor of Arabidopsis, exhibited increase in LB length and width significantly.\(^9\) In the P4 leaf stage, rice LBs are vigorously elongated and specific epidermal cells are

![Figure 4](image-url) Changes in OsGRF expression in developing leaves (P4) after eCO₂ treatment. (a) Daily Expression patterns of OsGRF family members in developing leaves (P4) under the eCO₂ condition in microarray (accession no. GSE121054). (b) RT-qPCR analysis of OsGRF3, OsGRF6, OsGRF9, and OsGRF10 in developing leaves after eCO₂ treatment. OsGAPDH was used as a reference gene. Asterisks indicate a significant difference (*p < 0.05, **p < 0.01) compared with aCO₂ (Student’s t-test). Values indicate means ± SD (n = 3).
differentiated. Decreases in rice LB length under eCO₂ were observed from the ninth leaves, which was the P4 leaf stage at the onset of eCO₂ treatment. It has been suggested that decreases in the epidermal cell number on the adaxial side and cell length on the abaxial side are associated with eCO₂-induced LB length reduction. In this context, the reduction in mature leaf size under eCO₂ may be attributed to the down-regulation of OsGRF3, OsGRF6, and OsGRF10 during the P4 leaf stage.

**Effects of exogenous ssd396e on leaf development and GRF expression**

Antisense oligonucleotides that bind to RNA by base pairing in a sequence-specific manner downregulate target gene expression depending on the RNase H-based degradation of the DNA–RNA hybrid. To investigate the relationship between eCO₂-induced miR396e and OsGRF3, OsGRF6, OsGRF9, and OsGRF10 downregulation, we examined the effects of exogenous treatment with ssDNA corresponding to the miR396e sequence (ssd396e) on GRF expression in P4 leaves. First, we verified whether the ssd396e treatment was applicable in our experiment. ssd396e was reverse-transcribed and amplified by RT-qPCR using a miR396e-specific primer based on the concentration of template ssd396e (Figure S5), indicating that our RT-qPCR system can estimate ssd396e levels in the target tissue after treatment, even though the value includes the original miR396e level in the tissue. Next, we checked whether ssd396e was accumulated in developing leaves (P4) using a DIG-labeled ssd396e treatment, in which the ssd396e solution was sucked from the tip of the uppermost fully expanded leaves (seventh leaf, Figure 5a). A large quantity of DIG-labeled ssd396e was detected in the LB of the seventh leaves, and the LS and P4 also exhibited chemiluminescent signals 12 h after treatment. We also tested ssd396e levels, including miR396e, in mature (LB) and developing (P4) leaves after ssd396e treatment using RT-qPCR. In both the LB and P4, ssd396e significantly increased 7 h after treatment with ssd396e solution compared with the control (Figure 5b). Although the mobility of DIG-labeled ssDNA in the biological tissue might differ from that of non-labeled ssDNA, these data indicate that both treated ssDNAs accumulate in the target P4 leaf.

After confirming that ssd396e was well-accumulated in the target P4 leaf, we examined OsGRF3, OsGRF6, OsGRF9, and OsGRF10 expression in P4 leaves under ssd396e treatment. Exogenous nonspecific ssDNA (21 nt) treatment was used as a ssDNA control, indicating no significant difference compared with controls. The OsGRF3, OsGRF6, and OsGRF10 expression in developing leaves was significantly downregulated 7 h after ssd396e treatment but showed similar transcript levels to the control after 13.5 h (Figure 6). Thus, ssd396e treatment is an effective tool for downregulating GRF3, GRF6, and GRF10 transcript levels in P4 leaves. These results suggest that the upregulation of miR396e is responsible for limiting GRF3, GRF6, and GRF10 gene expressions under eCO₂.

Finally, we investigated the effect of exogenous ssd396e on rice LB growth. The uppermost fully expanded seventh leaves were treated with 0.1 µM and 1 µM ssd396e solution for 2 days, and rice plants were cultivated until the twelfth leaves matured. The LB length and width were estimated after the leaf fully expanded. Treatment with nonspecific ssDNA did not change the LB length and width (Figure 7). Treatment with 0.1 µM and 1 µM ssd396e reduced the LB size of the fully expanded second leaf (ninth leaf) later, corresponding to P4 at the beginning of treatment. The 1 µM ssd396e treatment more significantly reduced LB length than the 0.1 µM treatment. Meanwhile, the LB width of the tenth leaves only decreased in the 0.1 µM treatment. These data indicate that ssd396e accumulation in target tissues leads to phenotypical changes by limiting GRF expression.

Several previous studies have investigated the efficacy of exogenous miRNA application. Zhang et al. examined the effects of exogenous miRNA on target gene and protein expression and reported that plant miR168a bound to mRNA coding the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1), inhibiting LDLRAP1 expression in mouse liver. Recently, exogenous miR156 and miR399 were shown to cause AGO1- and RDR6-mediated target transcript degradation in Arabidopsis. Thus, the exogenous miRNA approach seems to effectively regulate target gene expression in both mammals and plants. In our study, we used synthetic ssDNA instead of synthetic miRNA because ssDNA is...
Figure 6. RT-qPCR analysis of OsGRF3, OsGRF6, OsGRF9, and OsGRF10 in developing leaves (P4) after ssd396e treatment. ssd396e solutions were sucked from the cutting edge of the tip of the uppermost fully expanded seventh leaves (plant age of leaf number = 7.1–7.3 stage). Nonspecific ssDNA was used as a ssDNA control, and OsGAPDH was used as a reference gene. Asterisks indicate a significant difference (*p < 0.05, **p < 0.01) compared with control (5 mM MES) in the Student’s t-test. Values indicate means ± SD (n = 3).

Figure 7. Changes in the fully expanded leaf blade size after treatment with ssd396e. ssd396e solutions were sucked from the cutting edge of the tip of the uppermost fully expanded seventh leaf (plant age of leaf number = 7.1–7.3 stage) for 2 days, and fully expanded leaves were measured. Nonspecific ssDNA was used as a ssDNA control. Asterisks indicate a significant difference (*p < 0.05, **p < 0.01) compared with control (5 mM MES) in the Student’s t-test. Values indicate means ± SD (n = 6–8).
relatively stable in biological samples, and the time for ssd396e translocation from the treatment zone into target developing tissue was required for our experiment.

Antisense oligonucleotides have been extensively investigated for the development of RNA-based therapeutic technologies; however, little is known about the effect of this approach in the plant science field. In the present study, synthetic ssd396e was accumulated in the target developing tissue, downregulating target gene expression effectively; however, the detailed molecular mechanism of exogenous ssd396e remains to be elucidated. Although we tested only one miRNA sequence (miR396e, 21 nt) as a model ssDNA sequence, other miRNA sequences may also be useful model sequences to design antisense oligonucleotides, as the optimal nucleotide length and sequence are required for repression of target gene expression. Many miRNA sequences and their targets are disclosed, which will provide a valuable resource for facilitating antisense oligonucleotide applications in plant science field. To our knowledge, this is the first report to demonstrate that the application of ssDNA corresponding to the sequence of the miRNA effectively limits target gene expression in vivo, leading to phenotypic changes in plants.

The miR396–GRF module has been extensively investigated in rice plants because it directly regulates the rice leaf and inflorescence architecture and is closely associated with grain size and yield. Target GRFs preferentially regulated by miR396 isoforms with multiple functions have been described in previous studies. The repression of OsmiR396b causes significant development of auxiliary branches and spikelets with highly expressed OsGRF6, increasing the grain size and yield. OsGRF4 is regulated by the OsmiR396c via target cleavage, which influences the grain size and yield. OsmiR396d directly regulates OsGRF6 and OsGRF10 expression, which modulates spikelet development as accompanied by OsGRF–interacting factor 1. miR396e and miR396f cleave the target mRNA of OsGRF4, OsGRF6, and OsGRF8, resulting in miR396ef mutants with higher grain yield under nitrogen-deficient conditions. In addition, Mao et al. showed that OsGRF3, OsGRF4, OsGRF5, OsGRF8, and GRF10 are significantly upregulated in the leaves of miR396ef mutant rice which affects a grain size and architecture. In our experiment, GRF3, GRF6, and GRF10 were significantly downregulated in P4 leaves under eCO2 (Figure 4), concurrently with exhibiting significantly higher levels of miR396e and miR396f (Figure 2). Furthermore, treatment with exogenous ssd396e effectively repressed GRFs in P4 leaves (Figure 6). Therefore, it is possible that GRF3, GRF6, and GRF10 transcripts are degraded by miR396e via target cleavage in developing leaves under eCO2.

Conclusion

Overall, our findings provide crucial evidence that the miR396–GRF module is relevant to the regulation of LB length reduction under eCO2, and that the antisense oligonucleotide designed based on miRNA is a valuable tool for repressing target gene expression. Compared with plants grown under aCO2, mature miR396e and miR396f were both significantly upregulated in developing leaves of rice grown under eCO2, and their premature miRNAs simultaneously increased. In addition, GRF3, GRF6, and GRF10 in developing leaves were negatively regulated under eCO2 compared with aCO2. Utilizing the antisense oligonucleotide approach, we confirmed that ssd396e effectively repressed GRF3, GRF6, and GRF10 expression in target developing leaves. Furthermore, treatment with ssd396e significantly affected LB growth in developing leaves, resulting in LB length reduction at maturity like rice grown under eCO2. Therefore, our results strongly suggest that the miR396–GRF module is involved in eCO2-induced leaf phenotypic changes in rice plants and that the antisense oligonucleotide approach is a useful tool to clarify the role of GRFs in rice plants in eCO2 environments.

Developing new crops that are suitable for the changing atmospheric environment could help us secure food resources and sustain crop cultivation with climate change. eCO2-induced reductions in LB size appear to be an acclimation process to reduce the photosynthesis area in individual leaves. To engineer high-yield plants in response to eCO2, manipulating the miR396–GRF module may be crucial for retaining leaf size and expanding the photosynthesis area in eCO2 environments. These findings will facilitate the development of new crop plants suitable for the eCO2 atmospheric environment in the future.

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Author contributions

Y. K. and M. M. conceived and designed the research. Y. K. and S. T. performed the experiments. Y. K. analyzed the data and wrote the manuscript. All authors discussed the results.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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