In-frame editing of transcription factor gene *RDD1* to suppress miR166 recognition influences nutrient uptake, photosynthesis, and grain quality in rice

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The transcription factor-encoding gene *RDD1* increases the uptake of nutrient ions, photosynthetic activity under ambient and high CO2 conditions, and grain productivity, and microRNA166 (miR166) regulates its transcript levels. This study found that CRISPR/Cas9 genome editing of rice plants to inhibit miR166–*RDD1* transcript pairing (*R1-Cas plants*) increased *RDD1* transcript levels, NH4+ and PO43− uptake, and photosynthetic activity under high CO2 conditions in rice. However, the panicle weight of the *R1-Cas plants* decreased compared with the wild-type (WT) plants. Adversely, changes in environmental conditions, such as high CO2 or high temperatures, showed insignificant differences in the panicle weight between the WT and *R1-Cas plants* despite a largely increased panicle weight observed in the transgenic *RDD1*-overexpressing plants. Moreover, both the *R1-Cas* and transgenic *RDD1*-overexpressing plants that were matured in a growth chamber demonstrated an improved grain appearance quality or a decrease in the number of chalky grains compared with the WT plants. These results suggest that the in-frame mutagenesis of *RDD1* to suppress miR166–*RDD1* transcript pairing contributes to the improved grain appearance of rice.

There are 58 families of transcription factors in higher plants, which regulate the transcript levels of target genes. DNA-binding with one finger (DoF) proteins are plant-specific transcription factors with a highly conserved 52-amino acid DoF domain at the N-terminal region. The DoF domain has a single C2–C2 zinc-finger structure that recognizes the (A/T)AAAG sequence in the promoter region of target genes. Furthermore, the C-terminal region of DoF proteins contains a transcriptional activation domain. In rice, 30 genes encoding DoF transcription factors have been identified through database searches. We previously reported the characterization of a DoF transcription factor-encoding gene in rice—rice DoF daily fluctuations 1 (*RDD1*). *RDD1* is a circadian clock-regulated gene, and daily oscillations in its expression were retained when rice plants were transferred to continuous dark or light conditions. Antisense *RDD1* transgenic plants, produced to examine the *RDD1* role, have observable reductions in grain size and weight, thus indicating that this gene is associated with rice productivity. Conversely, we found that microRNA166 (miR166) regulated the *RDD1* transcript levels, and inhibition of miR166–*RDD1* mRNA pairing was necessary for the constitutive expression of the *RDD1* transgene. We previously generated transgenic rice plants overexpressing an *RDD1* transgene with nucleotide residue substitutions in the miR166 recognition site under the control of a constitutive promoter (m*RDD1*-OX plants). These transgenic rice plants experienced increased uptake and accumulation of various nutrients, including essential plant nutrients, as well as an increase in photosynthetic CO2 assimilation and grain productivity. Phylogenetic analysis revealed three *RDD1*-like genes (*RDD2*, *RDD3*, and *RDD4*), which had circadian-regulated expression levels like *RDD1*. However, *RDD2* function and expression patterns differed from those of *RDD1*, despite *RDD2* exhibiting the highest sequence similarity to *RDD1* among the rice DoF transcription factor genes. However, *RDD4* contributed to the control of the content of various nutrient ions and the control of photosynthetic CO2 assimilation, as was the case with *RDD1*. Additionally, it has been reported that plants overexpressing *OsDof12*...
The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonuclease 9 (CRISPR/Cas9) system is a powerful tool for site-directed mutagenesis in various organisms, including plants. This system induces DNA double-strand breaks at given genome sites, repaired via nonhomologous end joining. However, nonhomologous end joining is error-prone. Therefore, it is commonly used to disrupt genes by generating random insertions or deletions at target sites. The CRISPR/Cas9 system requires two components, Cas9 nuclease and a guide RNA (gRNA) for directing Cas9 to the target site, and sequence specificity is achieved by changing a 20-nucleotide (nt) guide sequence into the gRNA. Additionally, a trinucleotide protospacer adjacent motif (PAM) is needed immediately after the target site for binding and cleavage by Cas9. In this study, we performed an in-frame mutagenesis of RDD1 to suppress miR166–RDD1 transcript pairing using the CRISPR/Cas9 system and examined whether endogenous RDD1 without miR166 recognition increased its transcript levels, nutrient ion uptake, photosynthetic activity, and productivity, as observed in similar experiments involving mRDD1–OX plants.

Methods

Plant materials and growth conditions. The rice (Oryza sativa L. cv. Nipponbare) plants used in this study were grown in soil or in a hydroponic nutrient solution, as described previously. Seedlings were maintained in a growth chamber at 28 °C under long-day conditions consisting of a 16 h light/8 h dark cycle, and white light (80 µmol·m⁻²·s⁻¹) was provided using cool, white fluorescent light fixtures. Plants were grown hydroponically in a growth chamber to examine transcript levels and nutrient ion uptake. A hydroponic nutrient solution composed of 0.5 mM NH₄H₂PO₄, 1 mM KNO₃, 0.5 mM MgSO₄, 0.5 mM CaCl₂, 12.5 µM Fe-EDTA, and additional micronutrients (23.1 µM boron, 3.2 µM manganese, 0.1 µM copper, 1.4 µM zinc, and 0.2 µM molybdenum) was diluted 2.5 times to prepare a low-concentration hydroponic nutrient solution. To measure photosynthetic parameters and productivity, plants were transferred to growth chambers at 28 °C (light period) and 23 °C (dark period) under a 14 h light/10 h dark cycle. The CO₂ concentration in the growth chambers was set at 400 µmol mol⁻¹ for plants under ambient CO₂ conditions and 1000 µmol mol⁻¹ for those under high CO₂ conditions. The light was provided using a metal halide lamp. Examination of productivity under high-temperature conditions was conducted using plants that were initially grown in growth chambers at 28 °C (light period) and 23 °C (dark period) under a 14 h light/10 h dark cycle and then at 30 °C (light period) and 26 °C (dark period) under a 14 h light/10 h dark cycle after flowering. The collection of plant materials and the experimental research on plants were performed in accordance with relevant institutional, national, and international guidelines and legislation, and permission was obtained to collect the rice seeds.

Construction of a gRNA/Cas9 vector and plant transformation. The construction of a gRNA/Cas9 all-in-one vector (pZH_OsU6-gRNA_MMCas9) was described. Oligonucleotide pairs for the target sequence (5′-GGGATCAAGCTTGGAGACCCC-3′) were annealed, after which the resulting fragment was cloned into a BbsI site of the gRNA cloning vector, pU6_cclB_gRNA. This construct was digested with I-SceI, and inserted into the binary vector pZH_gYSA_MMCas9. The plasmid construct was introduced into Rhizobium radiobacter (Agrobacterium tumefaciens) EHA105, following which the bacteria were utilized to transform the rice plants as previously described.

RNA extraction and reverse transcription polymerase chain reaction. Plants were harvested 2 h before, or 2, 4, 6, 10, or 14 h after the onset of illumination, and total RNA was extracted using an RNaseq Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was conducted using one-step reactions (Superscript One-Step RT-PCR system; Invitrogen, Carlsbad, CA, USA), as previously described. The primers are listed in Supplementary Table S1. The primers used for targeting RUBQ2 were previously reported. PCR was conducted in a DNA thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA, USA). The RT-PCR product levels were quantified using a Qubit dsDNA HS assay kit (Invitrogen) and measured using a Qubit 3.0 Fluorometer (Invitrogen).

Determination of nutrient ions. The low concentration hydroponic nutrient solution was replaced with a fresh nutrient solution, and samples were collected 48 h after replacement. They were filtered using a 0.2-µm 13A1 filter (GL Sciences, Tokyo, Japan). The nutrient ions in the hydroponic nutrient solution were determined by ion chromatography using the ICS-900 ion chromatography system and IonPac AS12A or CS12A column (Dionex, Sunnyvale, CA, USA).

Photosynthesis and chlorophyll contents. The photosynthetic parameters of the upper parts of the youngest fully expanded leaf blades (no flag leaves) on the main stems at the vegetative stage, including photosynthetic carbon assimilation (A), stomatal conductance to water vapor (g_s), and intercellular CO₂ concentration (C_i), were determined at approximately midday using a portable CO₂/H₂O gas exchange analyzer (LI-6400; LI-COR, Lincoln, NE, USA), as previously described. The incident photosynthetic photon flux density was 1200 µmol m⁻² s⁻¹ (light-saturated level of A for the rice plants), and the flow rate and leaf temperature were set to 500 µmol s⁻¹ and 28 °C, respectively. CO₂ concentrations within the leaf chambers were maintained at 400 and 1000 µmol mol⁻¹ for plants grown under ambient and high CO₂ conditions, respectively.
The chlorophyll content (soil and plant analyzer development [SPAD] values) of the leaves was examined using a chlorophyll meter (SPAD-502 Plus, Konica Minolta, Tokyo, Japan). The SPAD values were determined at the upper portion of the youngest fully expanded leaf blades on the main stems at approximately midday when the photosynthetic parameters were measured.

**Determination of glucose and starch contents.** The glucose and starch contents in the rice grains were quantified using an F-kit system (Starch, R-Biopharm AG, Darmstadt, Germany) following the manufacturer's instructions.

**Statistical analysis.** Data are presented as the average ± standard error of the mean. Significant differences based on the two-sided Student’s t-test were indicated by asterisks (*p < 0.05, **p < 0.01, and ***p < 0.001).

**Results**

**Mutagenesis of RDD1 mediated by the CRISPR/Cas9 system.** CRISPR/Cas9 genome editing was used to generate in-frame deletions around the miR166 recognition site in RDD1, and three independent lines of edited rice plants (R1-Cas plants) were isolated (Fig. 1a). The untranslated and coding regions are indicated by black and white bars, respectively. The nucleotide sequences of the miR166 recognition site are boxed in gray, and the 20-nt target sequence and protospacer adjacent motif (PAM) are indicated by single and double underlines, respectively. Black and white arrowheads show the boundary sites of the 32-bp and 23-bp regions, respectively. The primer used to detect the absence of the 32-bp region (−32-bp primer) is indicated by an interrupted arrow. The wild-type (WT) and three independent lines of R1-Cas plants (#1–3) are shown in black and white bars, respectively. The nucleotide sequences of the miR166 recognition site are boxed in gray, and the 20-nt target sequence and protospacer adjacent motif (PAM) are indicated by single and double underlines, respectively. Black and white arrowheads show the boundary sites of the 32-bp and 23-bp regions, respectively. The primer used to detect the absence of the 32-bp region (−32-bp primer) is indicated by an interrupted arrow. A substituted nucleotide residue (adenine) in the R1-Cas #3 plants is shown framed by a square. (b) The amino acid sequences of RDD1 in the WT and R1-Cas #1–3 plants. Two highly conserved motifs (motifs II and III) are boxed in gray, and a substituted amino acid (glutamic acid) in the R1-Cas #3 plants is framed by a square.

The chlorophyll content (soil and plant analyzer development [SPAD] values) of the leaves was examined using a chlorophyll meter (SPAD-502 Plus, Konica Minolta, Tokyo, Japan). The SPAD values were determined at the upper portion of the youngest fully expanded leaf blades on the main stems at approximately midday when the photosynthetic parameters were measured.

**Effects of RDD1 mutations on RDD1 expression and miR166 recognition.** The RDD1 transcript levels oscillate daily, peaking after dawn under long-day conditions. Examination of the RDD1 transcript levels in the R1-Cas plants indicated that the RDD1 transcript levels in the peak (L2) increased 1.2- to 1.3-fold in the R1-Cas plants compared with the wild-type (WT) plants (Fig. 2). A decline in the RDD1 transcript levels before and after the peak (D6 and L6, respectively) was also detected in the R1-Cas and WT plants, although the RDD1 transcript levels in the R1-Cas #1 and #2 plants increased compared with the levels in the WT plants at D6 and L6.
The full-length cDNA for RDD1 (DDBJ/EMBL/GenBank database accession number AK063318) lacks a 32-bp region in the coding region. Our previous study reported that the miR166 recognition site is located within the 32-bp region, which deletes artifically during a reverse transcription reaction in RT-PCR analysis, and that adding synthetic miR166 RNA strongly inhibited cDNA synthesis of the RDD1 transcript lacking the 32-bp region. The 32-bp region appears to form a stem-loop structure, an architecture that might cause the 32-bp region's deletion during reverse transcription. The inferred stem-loop structure is possible in the R1-Cas #1 and #2 plants but not in the R1-Cas #3 plants (Fig. 3a). RT-PCR analysis was conducted to ascertain miR166 RNA binding to the miR166 recognition site in the R1-Cas plants. We found that RT-PCR equally amplified the RT-PCR product lacking the nucleotide sequence corresponding to the 32-bp region with or without synthetic miR166 RNA in the R1-Cas #1 and #2 plants, although adding synthetic miR166 RNA inhibited the amplification of the RT-PCR product lacking the 32-bp region in the WT plants (Fig. 3b). These results showed that the binding of synthetic miR166 RNA to the miR166 recognition site was suppressed in the R1-Cas #1 and #2 plants. Conversely, there was no amplification of the RT-PCR product in the R1-Cas #3 plants because of a 2-bp deletion of the 32-bp region. However, adding synthetic miR166 RNA strongly inhibited cDNA synthesis of the transcript lacking the 32-bp region in the coding region. Our previous study reported that the miR166 recognition site is located at the 5′ boundary sequence of the 32-bp region, which deletes artificially during an reverse transcription reaction in RT-PCR analysis, forming a 23-bp stem-loop structure (Fig. 1a and Supplementary Fig. S2a,b). We examined whether the inferred 23-bp stem-loop structure was formed in the R1-Cas plants. The RT-PCR analysis indicated that the RT-PCR product lacking the 32-bp region was equally amplified, irrespective of the addition of synthetic miR166 RNA during reverse transcription (Supplementary Fig. S2c), suggesting that there was no synthetic miR166 RNA binding to the miR166 recognition site in the R1-Cas #3 plants.

**Nutrient ion uptake in R1-Cas plants.** Next, we examined the nutrient ion uptake in the R1-Cas plants under low-nutrient conditions as mRDD1-OX plants have been reported to increase the absorption of nutrient ions under low-nutrient conditions. The nutrient ion uptake was examined by comparing the nutrient ion content in the hydroponic nutrient solution at 48 h after replacement with the fresh hydroponic nutrient solution containing the initial nutrient ion content. The absorption of the nutrient ions, except for SO_4^{2-} and Cl^−, per shoot fresh weight (FW) was greater in two of the R1-Cas plant lines than in the WT plants (Table 1). However, the shoot FW of the R1-Cas plants was significantly lower than that of the WT plants grown in the hydroponic nutrient solution (Supplementary Table S2). Also, the values of NH_4^+ and NO_3^− uptake in the R1-Cas plants relative to the WT plants in Table 1 were identical to those of the shoot FW of the WT plants relative to the R1-Cas plants in Supplementary Table S2, indicating that most of the NH_4^+ and NO_3^− in the hydroponic nutrient solution were absorbed in both the WT and R1-Cas plants. Alternatively, when the remaining nutrient ion content in the hydroponic nutrient solution at 48 h after replacement was compared between the WT and R1-Cas plants, the NH_4^+ and PO_4^{3−} content in the hydroponic nutrient solution was significantly lower in the R1-Cas plants than in the WT plants, despite the lower shoot FW in the R1-Cas plants (Table 1). Our previous study showed that RDD1 overexpression induced the expression of genes for glutamine synthetase 1;1 (GS1;1) and phosphate transporters 1 and 8 (PT1 and PT8, respectively), which are associated with the transport of NH_4^+ and PO_4^{3−}, respectively. Therefore, transcript levels of GS1;1, PT1, and PT8 were examined in R1-Cas plants, and it was found that except for the transcript level of GS1;1 in the R1-Cas #1 plants, all transcript levels increased in the R1-Cas plants (Fig. 4).
The *R1*-Cas plants’ photosynthetic activity was examined to determine whether an in-frame deletion of *RDD1* to suppress the miR166 recognition promoted the photosynthetic activity. The photosynthetic CO₂ assimilation rates of the *R1*-Cas #1 and #3 plants exhibited no difference and a 1.3-fold increase, respectively, compared with those of the WT plants grown under ambient CO₂ conditions (400 µmol CO₂ mol⁻¹ air⁻¹) (Fig. 5a). Stomatal conductance was higher in the *R1*-Cas #3 plants than in the WT plants, and there were insignificant differences in intercellular CO₂ concentrations in either the *R1*-Cas #1 or #3 plants. Conversely, when plants were grown under high CO₂ conditions (1000 µmol CO₂ mol⁻¹ air⁻¹), we found that the photosynthetic CO₂ assimilation rates of the *R1*-Cas #1 and #3 plants were 1.3-fold higher than those of the WT plants (Fig. 5b). Additionally, we examined the chlorophyll content (SPAD value) of the plants and observed that there was no significant difference in the SPAD value between the WT and *R1*-Cas plants under both ambient and high-CO₂ conditions. However, the SPAD value of the *R1*-Cas #1 plants was statistically higher than that of the WT plants under high CO₂ conditions.

**Effects of *RDD1* mutation on productivity.** The increased photosynthetic CO₂ assimilation rates of the *R1*-Cas plants under high CO₂ conditions suggested that productivity may be higher in the *R1*-Cas plants than in the WT plants under high CO₂ conditions. The *R1*-Cas plants were matured in a growth chamber grown under both ambient and high CO₂ conditions to examine their productivity. No significant difference was found in the panicle dry weight (DW) between the WT and *R1*-Cas plants under high CO₂ conditions despite a decreased panicle DW in the *R1*-Cas plants under ambient CO₂ conditions (Table 2). Particularly, the panicle DW in the m*RDD1*-OX plants was 1.4-fold higher than that in the WT plants under high CO₂ conditions. Conversely, the shoot DW in the *R1*-Cas plants was lower than that in the WT plants under both ambient and high CO₂ conditions, as observed in the m*RDD1*-OX plants. Consequently, the panicle DW to shoot DW ratio was significantly higher in the *R1*-Cas plants than in the WT plants under high CO₂ conditions but not under ambient CO₂ conditions. Note that the m*RDD1*-OX plants showed the highest panicle DW to shoot DW ratio under both ambient and high CO₂ conditions.

High temperature is a major serious environmental problem caused by global climate change. High temperatures occurring during rice ripening periods decrease grain yield and quality. To examine whether the *R1*-Cas
plants exhibited improved productivity under high-temperature stress, the R1-Cas plants were initially grown in a growth chamber under normal temperature and transferred to high-temperature conditions after flowering. No statistically significant difference was found in the panicle DW between the WT and R1-Cas plants, although the panicle DW was 2.4-fold higher in the mRDD1-OX plants than in the WT plants (Table 3). Conversely, the shoot DW was significantly decreased in the R1-Cas plants compared with that in the WT plants under high-temperature conditions, as observed in the mRDD1-OX plants. Therefore, the panicle DW to shoot DW ratio in the mRDD1-OX plants was significantly higher than that in the WT plants. Because of high-temperature stress, the one-grain weight decreased in all plants examined. Nevertheless, the one-grain weight was 1.2-fold higher in the mRDD1-OX plants than in the WT plants under high-temperature conditions.

Grain appearance quality in R1-Cas plants. Chalky appearance is an important quality characteristic of rice grains. Chalkiness is the opaque part of the endosperm and is colored white compared to the relatively transparent rest part. When the WT and R1-Cas plants matured in a growth chamber under normal conditions, most of the WT plants' rice grains appeared chalky (Supplementary Table S3 and Fig. 6a). Contrarily, the number of chalky grains in both the R1-Cas and mRDD1-OX plants was smaller than that of chalky grains observed in the WT plants. Other studies have reported that the glucose content in the opaque parts of chalky grains is remarkably high compared with that in the translucent parts of perfect grains. Because of these findings, we measured the glucose content in the whole grains of the R1-Cas and mRDD1-OX plants and found that the glucose content was less in both the R1-Cas and mRDD1-OX plants than that in the WT plants (Fig. 6b). Regarding the starch content, no significant difference was found between the WT and R1-Cas plants, and it was slightly higher in the mRDD1-OX plants than in the WT plants (Fig. 6c).

Discussion

miRNAs are small, non-coding RNAs that regulate gene expression by binding to partially complementary sequences in target mRNAs. Research has shown that miRNAs affect the function of circadian clocks in flies and mammals. Our previous studies demonstrated that RDD1 overexpression driven by a constitutive promoter exhibited diurnal fluctuations through the miR166 recognition site. In this study, in-frame editing of RDD1 was conducted to increase the transcript levels of RDD1 by interfering with miR166’s binding to RDD1’s
recognition site. We found that the mutated RDD1 transcript levels driven by the endogenous RDD1 promoter were oscillated and increased 1.2- to 1.3-fold at the peak in the R1-Cas plants (Fig. 2). Conversely, the RDD1 transgene was constitutively expressed under the control of a constitutive promoter in the mRDD1-OX plants. Because oscillations in the transcript levels of mutated RDD1 were similar to those of the transcript levels of endogenous RDD1 in the WT plants, we suspect that the endogenous RDD1 promoter drives the oscillated expression in cooperation with miR166. Additionally, the RT-PCR analysis in the R1-Cas #3 plants suggested that synthetic miR166 did not bind to the RDD1 transcript's miR166 recognition site, which carries no deletion (Supplementary Fig. S2). These results suggested that the inferred 32-bp stem-loop structure is important for miR166's pairing to the RDD1 transcript.

Enhanced CO2 levels have been reported to stimulate photosynthesis by promoting the net rate of carboxylation catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)23. This study showed that the photosynthetic CO2 assimilation rates of the R1-Cas plants were higher than those of the WT plants under high CO2 conditions (Fig. 5). Our previous study reported that RDD1 functions to promote photosynthetic activity8, and we found that the RDD1 transcript levels in the wild-type plants grown under high CO2 conditions were significantly decreased compared with those under ambient CO2 conditions (Supplementary Fig. S3). These results suggested that the inferred 32-bp stem-loop structure is important for miR166's pairing to the RDD1 transcript.

Figure 4. Transcript levels of GS1;1 (a), PT1 (b), and PT8 (c) in the shoots of the wild-type (WT) and R1-Cas #1 and #3 plants at the 4-leaf stage. The plants were grown in a low concentration hydroponic nutrient solution under normal conditions and harvested 2 h (L2), 6 h (L6), 10 h (L10), and 14 h (L14) after the onset of illumination. The transcript levels are normalized to those of RUBQ2, and the highest transcript level is defined as 100. Data are presented as the average ± standard error of the mean (n = 3). Asterisks indicate significant differences relative to the WT plants based on the Student's t-test (*p < 0.05, **p < 0.01, and ***p < 0.001).
Figure 5. Photosynthesis-related characteristics in the R1-Cas plants. The photosynthetic carbon assimilation (A), stomatal conductance to water vapor (gsw), intercellular CO2 concentration (Ci), and SPAD value of the wild-type (WT) and R1-Cas #1 and #3 plants in a growth chamber under ambient CO2 (a) and high CO2 conditions (1000 µmol CO2 mol⁻¹) (b). Data are presented as the average ± standard error of the mean (n = 3). Asterisks indicate significant differences relative to the WT plants based on the Student’s t-test (*p < 0.05 and **p < 0.01). Values relative to those of WT as 100 are shown in parentheses.

### Table 2. Characterization of shoots and panicles of mature WT, R1-Cas #1 and #3, and mRDDI-OX plants (mR1-OX) at the mature stage under ambient and high CO2 conditions. Data are represented as the average ± standard error of the mean (n = 3–4). Asterisks indicate significant differences relative to the WT plants based on the Student’s t-test (*P < 0.05, **P < 0.01 and ***P < 0.001). Values relative to those of WT as 100 are shown in parentheses. DW, dry weight.

| CO2   | WT    | #1    | #3    | mR1-OX |
|-------|-------|-------|-------|--------|
| Shoot DW | 54.102 ± 2.179 (100) | 37.940 ± 3.626** (70) | 37.846 ± 3.770** (70) | 39.492 ± 2.934** (73) |
| Panicle DW | 10.271 ± 0.371 (100) | 7.983 ± 1.505 (76) | 7.625 ± 1.003* (74) | 11.905 ± 1.219 (116) |
| Panicle DW/Shoot DW ratio | 0.191 ± 0.009 (100) | 0.217 ± 0.040 (114) | 0.207 ± 0.030 (109) | 0.301 ± 0.016** (158) |

### Table 3. Characterization of shoots and grains of mature WT, R1-Cas #1 and #2, and mRDDI-OX plants (mR1-OX) at the mature stage under high-temperature conditions. Data are represented as the average ± standard error of the mean (n = 3). Asterisks indicate significant differences relative to the WT plants based on the Student’s t-test (*P < 0.05, **P < 0.01 and ***P < 0.001). Values relative to those of WT as 100 are shown in parentheses. DW, dry weight.

|         | WT         | #1          | #2          | mR1-OX       |
|---------|------------|-------------|-------------|--------------|
| Shoot DW (g) | 72.573 ± 1.038 (100) | 36.334 ± 2.359** (50) | 38.033 ± 0.654*** (52) | 41.889 ± 4.410* (58) |
| Panicle DW (g) | 3.770 ± 0.497 (100) | 1.992 ± 0.306 (53) | 4.100 ± 1.016 (109) | 9.151 ± 0.566** (243) |
| Panicle DW/Shoot DW ratio | 0.052 ± 0.007 (100) | 0.054 ± 0.005 (104) | 0.108 ± 0.027 (207) | 0.221 ± 0.017* (425) |
| One-grain DW (mg) | 14.214 ± 0.251 (100) | 14.050 ± 0.232 (99) | 15.179 ± 0.422 (107) | 17.052 ± 0.050** (120) |
#3 plants had a substitution of an Asp with a Glu residue. Therefore, the absence of Asp may have negatively influenced RDD1’s activity in the R1-Cas #3 plants, and the presence of Glu instead of Asp possibly caused higher NH$_4^+$ and PO$_4^{3-}$ uptake, GS1;1 and PT1 expression, and photosynthetic CO$_2$ assimilation rates under ambient CO$_2$ conditions in the R1-Cas #3 plants than was observed in the R1-Cas #1 plants.

An examination of the R1-Cas plants’ productivity indicated that the panicle weight decreased in the R1-Cas plants grown under ambient CO$_2$ conditions (Table 2). Therefore, the amino acid residues, which were deleted from RDD1 in the R1-Cas plants, may have been necessary to increase grain productivity. In-frame deletion lines of early heading date 1 (Ehd1), which acts as a key signal integrator in the networks that regulate floral transition, lacked one to three amino acid residues in the receiver domain using the CRISPR/Cas9 system, and their flowering time was delayed compared with that of the WT plants but earlier than that of the frame-shift ehd1 lines. These reports suggested that one to three amino acid deletions in RDD1 yield a moderate decrease in grain productivity. Because the suppression of miR168, which targets Argonaute1 (AGO1), by a target mimic improves grain yield in rice, transgenic plants expressing the RDD1 target mimic, including the 32-bp region, may show increased panicle weight via the enhanced transcript levels of endogenous RDD1.

High-temperature stress reduces growth, yield, and grain quality. The R1-Cas plants were grown under high-temperature conditions after flowering to ascertain the effect of high temperatures on productivity, and it revealed that there was no significant difference in the panicle and one-grain weights between the WT and R1-Cas plants (Table 3). This result indicated that in-frame editing of RDD1 had no significant effect on productivity under high-temperature stress. Interestingly, panicle and one-grain weights largely increased in the mRDD1-OX plants compared with the WT and R1-Cas plants under high-temperature conditions. These results suggested that constitutive RDD1 overexpression in the mRDD1-OX plants, but not fluctuations of its expression in the R1-Cas plants, can strengthen grain yield under high-temperature stress.

The number of chalky grains in rice can be increased by high-temperature stress during grain filling. The chalky appearance results from the air spaces between the loosely packed starch granules that randomly reflect light. We found that the number of chalky grains in both the R1-Cas and mRDD1-OX plants significantly decreased compared with that in the WT plants when the plants matured in a growth chamber under normal conditions. This indicated that in-frame mutations of RDD1 in the R1-Cas plants decreased the number of chalky grains, just like the constitutive RDD1 expression in the mRDD1-OX plants. Due to an increase in temperature (up to 35 °C) for a moment in a growth chamber after transferring from the dark period (23 °C) to the light period (28 °C), this may generate many chalky grains in the WT plants. Because it has been suggested that unusual starch degradation occurs in chalky grains of rice, RDD1 may negatively affect starch degradation of grains, thus decreasing the number of chalky grains. This is supported by the observation that constitutive RDD1 overexpression in the mRDD1-OX plants, but not fluctuations of its expression in the R1-Cas plants, can strengthen grain yield under high-temperature stress.

Figure 6. Differences in the rice grains’ chalky appearance and glucose as well as starch contents of plants grown in a growth chamber under normal conditions. (a) Rice grains of the wild-type (WT), R1-Cas #1 and #3, and mRDD1-OX plants (mR1-OX). (b,c) Glucose (b) and starch contents (c) of the rice grains of the WT, R1-Cas #1 and #3, and mRDD1-OX plants (mR1-OX). Data are presented as the average ± standard error of the mean (n = 5). Asterisks indicate significant differences relative to the WT plants based on the Student’s t-test (**p < 0.01 and ***p < 0.001). Values relative to those of WT as 100 are shown in parentheses.
by the results revealing that the glucose content in the grains decreased in the R1-Cas and mRDD1-OX plants compared with the WT plants (Fig. 6b). Since Asp and Glu can mimic the phosphorylation state of a protein, the presence of a Glu instead of an Asp residue in the R1-Cas #3 plants may decrease the glucose content in grains compared with the R1-Cas #1 plants, which had the RDD1 protein without an Asp residue.

In conclusion, this study demonstrated that in-frame editing of RDD1 to suppress miR166’s binding to the RDD1 transcript promoted the uptake of NH₄⁺ and PO₄³⁻, the expression of NH₄⁺ and PO₄³⁻-transport-associated genes, and grain appearance quality. Still, the panicle weight decreased in the R1-Cas plants. Therefore, we expect that editing RDD1 to substitute nucleotide residue(s) but not amino acid(s) within the 32-bp region, including the miR166 recognition site, largely improves rice grain yield.

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Author contributions
M. I. designed the research, carried out the experiments, analyzed data, and wrote the article.

Competing interests
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