gw2 mutation increases grain width and culm thickness in rice (Oryza sativa L.)

Kohei Yamaguchi¹, Tatsuya Yamamoto²,³, Shuhei Segami¹,³, Miho Horikawa¹,⁴, Genki Chaya¹, Hidemi Kitano⁵, Yukimoto Iwasaki¹ and Kotaro Miura*¹

¹ Faculty of Bioscience and Biotechnology, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka, Eiheiji-cho, Yoshida-gun, Fukui 910-1195, Japan
² Present address: Fukui Prefectural Livestock Experiment Station, 68-34 Hirayama, Mikuni-cho, Sakai, Fukui 913-0004, Japan
³ Present address: Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture, Shakudo, Habikino, Osaka 583-0862, Japan
⁴ Present address: Japan Agricultural Cooperatives Tsurugamikata, 2-11-11 Mishima-cho, Turuga, Fukui 914-0058, Japan
⁵ Bioscience and Biotechnology Center, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8601, Japan

Grain size is one of the most important agricultural traits in rice. To increase grain yield, we screened a large grain mutant from mutants with the ‘Koshihikari’ background. As a result, we obtained a mutant, KEMS39, that has a large grain size and increased yield. Cultivation tests revealed that this mutant had improved lodging resistance with thicker internodes. Next-generation sequencing analysis revealed the presence of a 67 bp deletion in the GW2 mRNA, owing to a mutation in the 3’ splice site of the sixth intron of the GW2 gene. To determine whether this mutation was responsible for the larger grain and thicker internodes, we performed gene editing and obtained a mutant with a 7 bp deletion, including this 3’ splice site. As this gw2 mutant had large grains and thicker internodes, the causal gene of KEMS39 was determined as GW2. Thicker internodes are attributed to the pleiotropic effect of gw2 mutation. On the basis of these results, we conclude that gw2 mutation has the potential to be an important genetic resource with the ability to achieve a well-balanced and high-yielding effect that simultaneously improves grain productivity and lodging resistance.

Key Words: rice, GW2, grain size, lodging resistance.

Introduction

Grain size is one of the most important agricultural traits in rice (Oryza sativa L.), and many genes regulating grain size have been identified (Li et al. 2018). Among these genes, GW2 encoding RING-type E3 ubiquitin ligase is thought to be one of the most useful genes that greatly affects grain size and improves grain yield potential (Song et al. 2007). Choi et al. (2018) demonstrated that GW2 functions as E3 ubiquitin ligase and ubiquitinates expansin-like1 (EXPLA1). Lee et al. (2018) demonstrated that chitinase14 (CHT14) and phosphoglycerate kinase (PGK) interact directly with the GW2 protein, and that protein disulfide isomerase-like 1-1 (PDIL1-1) accumulates in gw2 mutants. Xia et al. (2013) reported that the Arabidopsis GW2 homolog, DA2, directly interacts with the ubiquitin receptor, DA1, to control seed size. In maize, a genome-wide association study revealed that the GW2 orthologs, ZmGW2-CHR4 and ZmGW2-CHR5, were associated with kernel size and weight (Li et al. 2010). In wheat, mutation of TaGW2-A1 has been reported to enhance 1000-grain weight and increase grain width and length (Simmonds et al. 2016).

With the increase in grain productivity, panicles become heavier; therefore, it is necessary to improve lodging resistance. However, few studies have reported of a gene that controls grain yield along with internode thickness (Huang et al. 2009, Jiao et al. 2010, Ookawa et al. 2010). Although ‘Koshihikari’, the top variety in Japan, has a good taste and is highly popular, it easily elongates and lodges, and the yield tends to decrease. Therefore, lodging resistant genes are desired for rice breeding in Japan.

In this study, we selected a large grain mutant from ‘Koshihikari’ mutants, and found that this mutant has pleiotropic effect with thicker culm and improved lodging resistance. We report that GW2 is the causal gene of this mutant, and that gene editing revealed that gw2 mutation can increase internode thickness in rice.

Materials and Methods

Plant materials and growth conditions

KEMS39 was identified from M₂ individuals of ethyl
methanesulfonate (EMS)-treated ‘Koshihikari’ and maintained at Togo Field, Nagoya University. The M1 plants obtained from 3,000 seeds of ‘Koshihikari’ were treated with EMS for 6 h, with 6 mM solution prior to sowing. KEMS39 was screened from the M2 individuals in 2010.

Seedlings of ‘Koshihikari’ and KEMS39 were transplanted to a paddy field at the Research Center for Bio-resources Development in Fukui, Japan (36.2° N, 136.2° E) at the beginning of May in 2017. The planting density was one seedling per hill and 20 hills per m² with a spacing of 20 × 25 cm. Nitrogen fertilizer was applied at customary rates (basal dressing 0.8 kg/a; top dressing 0.3 kg/a). The experiments were designed with six randomly arranged replicates (2.5 m² for each plot).

For the yield test, 20 plants of each plot were harvested in bulk and threshed. After de-husking, immature grains were removed by a mill-grade sieve with a mesh size of 1.9-mm.

The Nipponbare cultivar was used for targeted mutagenesis. Mutated transgenic plants were grown using 10 × 10 cm pots in a closed growth chamber, TGH (ESPEC MIC, Osaka, Japan) at 28°C from 07:00 to 21:00 and 25°C from 21:00 to 07:00 for 3 months, and 28°C from 09:00 to 19:00 and 25°C from 19:00 to 09:00 for the next 3 months, in 2018. All data were statistically analyzed by Student’s t-test.

**Next generation sequence analysis**

Genomic DNA of ‘Koshihikari’ and KEMS39 were isolated from fresh leaf blades using NucleoBond HMW DNA kit (MACHEREY-NAGEL, Düren, Germany). DNAs were commissioned to TAKARA Bio to analyze as 100 bp pair ends using Hiseq 2000 (Illumina, California, USA). We obtained 5,886,657,800 and 5,865,389,600 bases from ‘Koshihikari’ and KEMS39 DNAs, respectively. The sequence data were analyzed using CLC Genomics Workbench 11 (QIAGEN, Hilden, Germany), and IRGSP 1.0 (https://rapdb.dna.affrc.go.jp/download/irgsp1.html) as the reference sequence.

**RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from the fully expanded leaf blade and total root of the vegetative plant at 4 weeks after transplanting, stem at the elongating stage before heading, young panicle at the 3–5 mm stage, and lemma at the 3–5 mm stage, using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized from total RNA using the PrimeScript RT Reagent Kit (TAKARA Bio, Shiga, Japan). For quantification of mRNA, qRT-PCR was performed using TB Green Premix Ex Taq (TAKARA Bio). The GW2 gene was amplified using the primers, GW2-RT-U: CATACGGATGTGCTGAGCAA and GW2-RT-L: GATGGCTCAGTTACAGGCA. OsUbi1 was used as an internal control, and amplified using the primers Ubi-U: CTTGGTCGTGTCCCGTTC and Ubi-L: TTCTTCCATGCTGCTACAC. The Thermal Cycler Dice Real Time System (TAKARA Bio) was used for qRT-PCR. The amplification conditions were as follows: 95°C 30 s—40 cycles of 95°C (5 s), 55°C (30 s), 72°C (30 s)—dissociation.

**Production of gw2 mutant**

The gw2 mutant was produced using the CRISPR/Cas9 system developed by Mikami et al. (2015). GW2-CRISPR-oligo-U: TCAATGTTATCATCAGTGA and GW2-CRISPR-oligo-L: TACAGTGATGATAACATTGA were annealed and cloned into pU6gRNA as the target sequence. The target sequence with the OsU6 promoter was replaced into the pZH_gYSA_MMCas9 vector. The construct was transformed into Nipponbare calli, as described in Mikami et al. (2015). Thereafter, regenerated M0 plants were transplanted and sequenced by 3130xl Genetic Analyzer (Thermo Fisher Scientific, MA, USA), using primers, GW2-RT-U and GW2-RT-L. M1 seeds were genotyped by sequencing and divided into homozygous and heterozygous plants.

**Lodging resistance test**

To compare lodging resistance, we measured lodging rates. Individuals with 50% over broken tillers were determined to be lodged plants, and their appearance rates were calculated (lodged plant number/total plant number).

**Breaking strength test**

The breaking strength of internodes was measured using a digital force gauge, FGP-0.5 (NIDEC-SHIMPO, Kyoto, Japan), using 5 cm of the central part of fresh internodes at 40 days after heading.

**Results**

**Large grain mutant KEMS39 improves grain yield**

To improve rice grain productivity, we screened a large grain mutant from the mutant collection of the Nagoya University. As a result, we obtained a large grain mutant, KEMS39 from the EMS-treated ‘Koshihikari’ mutant stock. Grain length, grain width, grain thickness, 1000-grain weight, brown rice length, brown rice width, brown rice thickness, and 1000 brown rice weight of KEMS39 were significantly higher than those of ‘Koshihikari’ (Fig. 1A, 1B, Table 1). There were no significant differences in plant height and grain number (Fig. 1C, Table 1). KEMS39 showed an increase of 4.59% in yield/plant; however, there was a slight decrease in panicle number/plant (Table 1).

**KEMS39 has a splicing abnormality in the GW2 gene**

To clarify the causal gene of large grain and higher grain weight in KEMS39, we performed next-generation sequence analysis and obtained the complete genomic sequences of ‘Koshihikari’ and KEMS39. Comparing the nucleotide sequences of previously reported grain size related 43 genes listed in Li et al. (2018), only a G→A mutation in the 3′
splicing site of the sixth intron of the GW2 gene was found (Fig. 2A, GenBank accession: XM_015772191). Therefore, primers were designed in the sixth and eighth exons, namely GW2-RT-U and GW2-RT-L, respectively, and qRT-PCR was performed using the cDNA as a template. It was revealed that the size of the amplicon was shorter in KEMS39 than in ‘Koshihikari’, and DNA sequencing revealed that the 67 bp of exon 7 was deleted in KEMS39 (Fig. 2B, 2C). From these data, KEMS39 was inferred to be a gw2 mutant.

**KEMS39 has thicker internodes and improved lodging resistance**

During the cultivation test, we found that KEMS39 had a lower lodging rate than ‘Koshihikari’ (Fig. 3A, 3B). No outward changes in internode length or pattern of internodes were observed; however, culm thickness did increase. To confirm this, the internode diameter of major axes was measured. The first to fifth internodes of KEMS39 were thicker than those of ‘Koshihikari’ (Fig. 3C, 3D). As the internodes were thicker in KEMS39, the breaking strength of each internode was measured. The breaking strength of each internode of KEMS39 was higher than that of ‘Koshihikari’ (Fig. 3E). From the qRT-PCR, GW2 expression was also detected in the stem and young panicle, suggesting that the thicker internodes of KEMS39 can be attributed to the pleiotropic effect of gw2 mutation (Fig. 3F).

**gw2 mutation improves lodging resistance owing to thicker internodes**

To confirm whether the large grain of KEMS39 is a result of gw2 mutation, and whether the thicker internodes are due to the pleiotropic effect of gw2 mutation, we attempted to induce mutations into the 3’ splicing site of the sixth intron by gene editing in Nipponbare (Fig. 4A). As a result, a heterozygous M0 gw2 mutant possessing a 7 bp deletion at the 3’ splicing site of the sixth intron and wild-type sequence was obtained (Fig. 4A). From the qRT-PCR

| Trait                  | ‘Koshihikari’ | KEMS39     | t-test | n       |
|------------------------|--------------|------------|--------|---------|
| Plant height (cm)      | 111.38 ± 3.25| 112.3 ± 4.47| n.s.   | 5 plants|
| Panicle number/plant   | 13.60 ± 0.89 | 11.80 ± 1.48| *      | 5 plants|
| Grain number/panicle   | 146.75 ± 30.71|159.20 ± 13.70| n.s.   | main panicles of 5 plants |
| Grain number/plant     | 1463.40 ± 465.15|1637.80 ± 419.84| n.s. | 5 plans |
| 1,000 grain weight (g) | 26.29 ± 1.02 | 30.48 ± 0.61| **     | 5 plants |
| Grain length (mm)      | 7.53 ± 0.18  | 8.03 ± 0.19  | **     | 50 grains from 10 plants |
| Grain width (mm)       | 3.52 ± 0.08  | 3.80 ± 0.12  | **     | 50 grains from 10 plants |
| Grain thickness (mm)   | 2.35 ± 0.04  | 2.43 ± 0.06  | **     | 50 grains from 10 plants |
| 1,000 brown rice weight (g) | 22.37 ± 0.07 | 26.02 ± 0.31 | **   | 10 plants |
| Brown rice length (mm) | 5.10 ± 0.21  | 5.24 ± 0.21  | **     | 50 grains from 10 plants |
| Brown rice width (mm)  | 3.10 ± 0.28  | 3.34 ± 0.15  | **     | 50 grains from 10 plants |
| Brown rice thickness (mm) | 2.13 ± 0.05 | 2.29 ± 0.06  | **     | 50 grains from 10 plants |
| Yield/plot (g/m²)      | 595.25 ± 19.57|622.57 ± 16.22| *    | 6 plots (20 plants/plot) |

Averages with standard deviation are shown. ** p < 0.01, * 0.01 ≤ p < 0.05, n.s.: not significant.
**Fig. 2.** Mutation of KEMS39 in the GW2 gene. (A) Gene structure of the GW2 gene. White and grey boxes indicate the coding and untranslated regions of the exon, respectively. The underlined nucleotide sequences indicate splice sites. Arrows above the 6th and 8th exon indicate primers. (B) qRT-PCR of GW2 gene using primers, GW2-RT-U and GW2-RT-L. (C) Comparison of cDNA sequences of the GW2 gene between ‘Koshihikari’ and KEMS39.

**Fig. 3.** Comparison of lodging resistance between ‘Koshihikari’ and KEMS39. (A) ‘Koshihikari’ and KEMS39 at the mature stage in the field. (B) Lodged plant rate of ‘Koshihikari’ and KEMS39. (C) Internode diameter of ‘Koshihikari’ and KEMS39. (D) Cross section of internodes. Bar indicates 1 mm. (E) Breaking strength of internodes of ‘Koshihikari’ and KEMS39. The 5th internodes were too short for this test. “n.t.” indicates not tested. (F) Organ expression of the GW2 gene in ‘Koshihikari’. Organ expression of GW2 was calculated using OsUbi1 as an internal control. Student’s t-tests performed between ‘Koshihikari’ and KEMS39 are indicated in (B), (C), and (E). ** p < 0.01. * p < 0.05. n = 5. Error bars indicate standard deviations.
using the selected homozygous 7 bp deletion M₁ plants, a 67 bp deletion of the seventh exon in the GW2 transcript was detected, as seen in KEMS39 (Fig. 4B, 4C).

The gw2 mutant showed thicker internodes and wider grain than the empty vector control, but there were no significant differences in heterozygous plants (Fig. 4D–4G). Consistent with KEMS39, there were no significant changes in plant height, panicle number, and grain number (Table 2).

These results prove that KEMS39 is the gw2 mutant and
its thicker internodes are due to the pleiotropic effect of gw2 mutation, indicating the thicker internodes and wider grain phenotypes are recessive traits.

Discussion

New gw2 mutant

In this study, we identified a large grain mutant, KEMS39, and found that the large grain size was due to gw2 mutation. The GW2 gene has a coding DNA sequence of 1272 bases, encodes 424 amino acid proteins, and it has a RING domain at 62–105th amino acid, which is important for E3 ubiquitin ligase activity. The mutation reported in Song et al. (2007) had a premature stop by inserting a single base at 316 base of exon 4, leading to the deletion of 310 amino acids, but its RING domain was intact.

The mutation of KEMS39 was a mutation in the splicing site of the 6th intron, resulting in the deletion of 67 bases in the mRNA of the 7th exon. This mutation causes a premature stop at 219th amino acid. Zhang et al. (2018) reported that a larger grain phenotype was observed when CRISPR mutations were introduced in 268th amino acid of the 425 amino acid TaGW2 protein in wheat. Both KEMS39 and TaGW2 mutations occurred in the C-terminal region and the RING domain was intact, indicating that GW2 may possess not only the RING domain but also the C-terminal region with an important function.

Does gw2 mutation improve lodging resistance?

In this study, we confirmed that KEMS39 has improved lodging resistance, and that gw2 mutation increased culm thickness and improved breaking strength. However, whether KEMS39 lodging resistance is directly conferred by gw2 mutation is yet to be genetically proven. In addition, it has been shown that RNA of GW2 is also highly accumulated in roots, which is consistent the results of Lee et al. (2018), and it possibly affects root growth and lodging resistance of KEMS39. Nonetheless, as reported by Ookawa et al. (2010) and Hirano et al. (2014) that the resistance to lodging was improved when the stem became thicker, we conclude that the improvement in gw2 mutant culm thickness is largely responsible for the improvement in lodging resistance of KEMS39.

gw2 mutation increases grain yield of rice

Previously, the possibility of utilizing gw2 mutation for high yields has been reported in the indica variety (Song et al. 2007), whereas the present study revealed that it also increased yield in the most popular Japanese variety, ‘Koshihikari’. The gw2 mutation was reconfirmed to be useful for increased yield of the japonica rice variety. In addition, gw2 mutants were shown to have thicker internodes. For gw2, increasing the grain yield makes the panicle heavier; therefore, it is important to take lodging resistance into consideration for breeding. Genes that improve lodging resistance by pleiotropic expression enable efficient breeding. From these observations, we conclude that gw2 mutation has the potential to be an important genetic resource that can contribute to future high-yield breeding in Japan.

Author Contribution Statement

KY, TY, MH, and GC performed the cultivation test. SS conducted the NGS analysis. HK identified KEMS39. YI and KM conceived and participated in the design and coordination of the study and helped draft the manuscript.

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