










The *gs3* allele from a large-grain rice cultivar, Akita 63, increases yield and improves nitrogen-use efficiency

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Abstract

The Green Revolution allowed a large amount of nitrogen (N) fertilization to increase crop yield but has led to severe environmental pollution. Therefore, increasing the crop grain yield must be achieved without such considerable input of N fertilization. A large-grain *japonica* rice cultivar, Akita 63, significantly increased grain yield and improved N-use efficiency (NUE) for yield per amount of N absorbed by plants. This study found that the nonsense mutated *GS3* gene, the *gs3* allele of Akita 63, has a superior yield production with enlarged grain size. The *gs3* allele increased the yield with improvements in harvest index and NUE for yields per plant N content by analyzing the near-isogenic line of rice plants with a large grain (LG-Notohikari), which was developed by introducing the *gs3* allele of Akita 63 into normal-grain *japonica* cultivar, Notohikari. Thus, the *gs3* allele would be promising for further yield increase without additional large input of N fertilization in non-*gs3*-allele rice varieties.

Highlight

The *gs3* allele transfer from large-grain *japonica* rice cultivar, Akita 63, into normal-grain *japonica* rice cultivar, Notohikari, increases yield and improves nitrogen-use efficiency.

KEYWORDS

gs3 allele, harvest index, large-grain, nitrogen-use efficiency, *Oryza sativa* L, yield

Dong-Kyung Yoon, Mao Suganami, and Keiki Ishiyama contributed equally to this article.

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1 | INTRODUCTION

By the middle of this century, the world's population is expected to reach 10 billion, and the Food and Agriculture Organization of the United Nations predicts that at that point, the world would need to produce 70% more food than today to feed all those people (da Silva, 2017). Modern cereal crops, including rice, which have improved the harvest index (HI) due to shorter and stiffer stems since the Green Revolution in the 1960s, have allowed a large input of nitrogen (N) fertilizer, resulting in an increased yield. Such large amounts of N fertilization increased photosynthesis and enhanced the source function and the increase in grain number with the enlargement of sink capacity in the crop (Evans, 1998). However, it is pointed out that excessive N fertilization on farmland is the cause of environmental pollution (Canfield et al., 2010; Good & Beatty, 2011). Therefore, increasing cereal crop yield by improving N-use efficiency (NUE) per amount of N fertilization is critical for a sustainable agricultural system in cereal crop production.

The enlargement of single grain size has a great impact on the increase in the yield of rice plants because single grain weight evaluated by grain size as well as the number of spikelets and ratio of filled spikelets is a critical component for yield potential. Unlike other cereal crops, rice grain size remained constant independent of the number of spikelets (Makino, 2011; Yoshida, 1981), and not influenced by environmental conditions, such as the water environment (Yoshida, 1981) and the amount of N fertilization (Makino et al., 2020). Thus far, dozens of different grain length and width genes that determine grain sizes have been found in rice plants (Li et al., 2018).

In our previous study, the yields of large-grain *japonica* cultivar, Akita 63, showed 20% higher than those of paternal cultivars; Akita 39 with normal-grain and Oochikara with ultra large-grain (Makino et al., 2020) and 20%–60% higher than those of the reference normal-grain *japonica* cultivars for the same amount of N fertilization (Mae et al., 2006; Makino et al., 2020). In Akita 63, the heavier single grain weight by the large grain, compared with normal-grain cultivars, led to high yields with higher HI and N-use efficiency (NUE) for the yield (Mae et al., 2006; Makino et al., 2020). The large-grain size of Akita 63 was mainly caused by nonfunctional *GS3* (*gs3*) and *qSW5* (*qsw5*) genes (Makino et al., 2020). The *GS3* gene is the first molecularly characterized QTL for the grain size of rice plants (Fan et al., 2006). The *gs3* allele is caused by one common single nucleotide mutation; a substitution of cytosine by adenine residue results in a premature stop codon in the second exon within the *GS3* gene. Elongating grain length by the *gs3* allele results in enlarged grain size (Fan et al., 2006; Makino et al., 2020; Mao et al., 2010; Takano-Kai et al., 2009). The *qsw5* allele, which contains the 1212-bp deletion in the *qSW5* gene, was isolated as a QTL for grain width in an *indica* cultivar, Kasalath, leading to an enlarged grain width (Shomura

et al., 2008). Also, combining of large grain alleles of *gs3* and *qsw5* effectively enlarged the grain size with increased single grain weight (Lu et al., 2013; Yan et al., 2011). However, the *qsw5* allele is widely observed in *japonica* rice cultivars (Shomura et al., 2008). Therefore, it is crucial to focus on the *GS3* gene to enlarge the grain size of *japonica* rice cultivars. In our previous study, near-isogenic lines (NILs) were developed to substitute the *GS3* segment of Koshihikari, which possessed a functional *GS3* allele (normal-sized grain allele), in the genetic background of Akita 63, after which Akita 63 and the NIL (referred to as Akita63NILGS3-Koshihikari) were compared (Obara et al., 2022). The grain (rough rice) length of the NIL was significantly reduced by approximately 8% compared with Akita 63, although grain width was the same level (Obara et al., 2022). The shorter grain length caused an approximately 13% decrease in the single grain weight of the NIL (Obara et al., 2022). Additionally, the NIL showed a decrease in grain yields due to lower single grain weight and the ratio of filled spikelets, although the total spikelet number increased (Obara et al., 2022).

These facts strongly suggested the possibility that the *gs3* allele not only enlarges grain size but also confers superior traits, high yield with improvements of HI and NUE for grain production per plant N content observed in Akita 63, compared with other rice cultivars. Here, to examine the positive effect of the *gs3* allele on rice yields, NIL in the genetic background of a *japonica* cultivar, Notohikari, with large-grain (LG-Notohikari) was developed by replacing the functional *GS3* gene with the *gs3* allele originated from Akita 63 according to repeated backcrossing and marker-assisted-selection techniques. The LG-Notohikari was cultivated with different levels of N fertilization in experimental paddy fields of three locations in 2019 and 2020. These field experiments demonstrated that the *gs3* allele partially confers superior traits observed in Akita 63 to LG-Notohikari, increasing grain yield with improvements in HI and NUE for yield.

2 | MATERIALS AND METHODS**2.1 | Development of large-grain Notohikari**

The NILs of rice plants with large-grain (LG-Notohikari) in the genetic background of Notohikari (*Oryza sativa* L.) was developed by replacing the *GS3* allele (identifier Os03g0407400 on the Rice Annotation Project database; <https://rapdb.dna.affrc.go.jp/>) (Sakai et al., 2013) with the *gs3* allele that originated from the large-grain *japonica* cultivar; Akita 63 (*O. sativa* L.), using repeated backcrossing and marker-assisted-selection techniques. We followed genotypic analyses for simple sequence repeats (SSR) and single nucleotide polymorphism previously described in Obara et al., 2022. Briefly, *GS3*, 864 SSRs, and 1468 Kompetitive Allele-Specific PCR (LGC Co.) markers were employed to detect the polymorphism between Akita 63 and the



normal-grain japonica cultivar, Notohikari. Akita 63 and Notohikari were crossbred to obtain the F_1 generation. The F_1 plant was backcrossed three times with Notohikari as a recurrent parent, and the resulting BC_3F_1 plants were self-pollinated to obtain BC_3F_3 . During the backcross procedure, progeny with the *gs3* allele as homozygous and minimize the chromosome segments originated from Akita 63 using 148 DNA markers, representing polymorphism between the parental lines (Table S1) was selected. The BC_3F_3 and its self-pollinated progeny were used in the following experiments as LG-Notohikari.

2.2 | Whole-genome resequencing of Notohikari and large-grain-Notohikari

Genomic DNA was extracted from the leaf blades of Notohikari and LG-Notohikari using DNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan) following the manufacturer's instructions. The quality and quantity of DNA were checked by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific KK., Tokyo, Japan). Sequencing DNA libraries were prepared using a TrueSeq DNA PCR-free kit (Illumina, Inc. San Diego, CA, USA). Whole-genome resequencing of the genomic DNA was performed using Illumina platform (Illumina, Inc.). Using Os-Nipponbare-Reference-IRGSP-1.0 reference genome, evaluation of genetic diversity of genomic DNA between Notohikari and LG-Notohikari was conducted.

2.3 | Field growth conditions

LG-Notohikari and Notohikari were cultivated in experimental paddy fields at the Kawatabi field Center (38°44' N, 140°45' E, at 140 m altitude) in 2019, at the Aobayama field (38°25' N, 140°83' E, at 160 m altitude) of Tohoku University in 2020, and at the Tsukuba field of the Japan International Research Center for Agricultural Sciences (36°05' N, 140°08' E, at 24 m altitude) in 2019. The soils in Kawatabi, Aobayama, and Tsukuba were sandy (Furukawa sandy alluvial soil), loam, and gray lowland soils, respectively. The paddy field of Kawatabi field had pH (H_2O) 5.8, 12.0 g total C kg^{-1} , 1.0 g total N kg^{-1} and 10.0 cmol (+) kg^{-1} cation exchange capacity. The Aobayama field had pH (H_2O) 6.6, 27.1 g total C kg^{-1} , 1.6 g total N kg^{-1} and 20.4 cmol (+) kg^{-1} cation exchange capacity. The Tsukuba field had pH (H_2O) 5.5, 18.4 g total C kg^{-1} , 1.6 g total N kg^{-1} and 22.1 cmol (+) kg^{-1} cation exchange capacity. For all fields, experimental plots were designed using different amounts of N fertilization without replication (Table S2).

Seedlings were grown in a greenhouse from April 16, 2019, and April 18, 2020, and transplanted to paddy fields on May 21, 2019 in Kawatabi field and May 31, 2020, in Aobayama field, respectively. In Tsukuba field, seedlings were grown in a greenhouse on April 23, 2019, and then transplanted into the paddy fields on May 20, 2019. The seedlings were transplanted in the paddy field at a hill spacing of 0.3×0.163 m (20.5 hills m^{-2}) with three to four seedlings per hill in the Kawatabi and Aobayama fields. A seedling was

transplanted in the Tsukuba field at a hill spacing of 0.3×0.18 m (18.5 hills m^{-2}). The sizes of the plots for Notohikari and LG-Notohikari were 6.0 m^2 (1.5 m wide and 4.0 m long) in 2019 at the Kawatabi field, 10.5 m^2 (4.5 m wide and 2.34 m long) in 2019 at the Tsukuba field, and 10.4 m^2 (2.3 m wide and 4.5 m long) in 2020 at the Aobayama paddy field. Plants were grown with two or three distinct amounts of N fertilization, along with ammonium sulfate or controlled-release fertilizers (LP70-type polyolefin-coated urea, JCAM AGRI Co.) for basal application and ammonium sulfate as a top-dressing fertilizer at the experimental paddy fields of Kawatabi and Aobayama (Table S2). LP70-type fertilizers linearly release 80% of their total N contents for 70 days at 20°C–30°C. Calcium superphosphate (4.37 g P m^{-2}) and potassium chloride (6.63 g K m^{-2}) was applied to all plots third to fifth day before transplantation at paddy fields of Kawatabi and Aobayama. At Tsukuba in 2019, organic fertilizer (guaranteed ingredient: 6% N-8% P-4% K, Katakura and Co-op Agri Co.) was applied to the paddy field (Table S2). Weeds, insects, and diseases were controlled as required to avoid yield loss. The full-heading time was defined as the time when 40% of the panicles had emerged. Full-heading dates were August 4, 2019, in Kawatabi field, August 8, 2020, in Aobayama field and July 27, 2019, in Tsukuba field. Two cultivars of rice plants were considered to have reached the ripening time when 95% of the grains had turned yellow. The plants were harvested on September 24, 2019, in Kawatabi field, September 30, 2020, in Aobayama and August 29, 2019, in Tsukuba field.

Table S3 presents the climatic data from May to October, covering the rice cultivation period at the Kawatabi, Tsukuba, and Aobayama fields in 2019 and 2020. In 2019, temperatures were relatively low during the tillering and panicle initiation stages and relatively high after the full-heading stage in Kawatabi and Tsukuba fields. In 2020, the sunlight duration observed was less than the average year from the end of June to July in the Aobayama field. In the panicle formation stage of meiotic phase of rice in the middle of July 2020, the lower temperature than average continued for about 1 week.

2.4 | Total dry matter and rice yield measurement

The total dry matter production at the full-heading stage was determined for five rice plant hills. Each sample with an average number of stems was collected as the above-ground part of plants from each plot at 1 day after heading (DAH) in 2019 at the Kawatabi field and 3 DAH in 2020 at the Aobayama field. To determine dry matter production at the harvesting stage, 15 rice plant hills with an average number of panicles were collected from each plot at 51 DAH in 2019 at the Kawatabi field and 53 DAH in 2020 at Aobayama field. The average number of panicles was calculated from 50 hills designated in advance. All samples collected at the Kawatabi and Aobayama fields were oven-dried (DRM620TM, Advantech Co.) at 80°C for several days.

The number of filled and unfilled spikelets was measured with a counting machine for plant seeds (WAVER IC-VA, Vai, AIDEX Co.). The filled spikelets were separated by submerging the hand-threshed



grains in an NaCl solution with a specific gravity of 1.06 g mL^{-1} . The ratio of filled spikelets was calculated by comparing the number of filled spikelets with the total spikelets. The filled spikelets were then hulled and oven-dried at 80°C for more than 1 week. The weight of the brown rice was adjusted to a fresh weight with a moisture content of $.15 \text{ gH}_2\text{O g}^{-1}$. The brown rice yield was calculated by multiplying the grain number per unit land area, the ratio of filled spikelets and the brown rice weight per grain, which constitutes the “yield component.” The sink capacity was calculated by multiplying the total spikelet number by the single filled grain weight (Mae et al., 2006; Yoon et al., 2020). At the Tsukuba field, 12 rice plants with an average number of panicles were collected from each plot at 33 DAH. The average number of panicles was calculated from 75 hills designated in advance. The samples collected at Tsukuba field were oven-dried (RY-120HL, ALP Co.) at 70°C for at least 5 days. Rough grain yield was determined by measuring the well-filled rough grain of 10 samples of a bulk sample consisting of six hills. Well-filled rough grains were selected by wind selection established exhaust air adjustment A for level 7 and B for level 4.5 (FV-459, Fujiwara Scientific Company Co).

2.5 | N and carbohydrate determinations

Dried plant materials were milled to fine powdered, and then the plant N content was determined using the Nessler's reagent after Kjeldahl digestion (Makino et al., 1984). Starch, sucrose, and glucose contents in these dried materials were determined by measurements using an F-kit (JK International, Tokyo, Japan) according to our previous experiment (Sudo et al., 2014).

2.6 | Statistical analysis

The data are expressed as the mean \pm standard error. After the Thompson tau test had been used for outliers, Fisher's exact test was conducted using Excel Tokei (BellCurve, Social Survey Research Information Co., SAS Institute Inc.). Scatter diagrams, regression lines, and Pearson's product-moment correlation coefficients (*R*-value) were created and calculated using Excel (Microsoft). Correlations were tested by Spearman's rank-order correlation coefficient (*P*-value) using Excel Tokei (BellCurve). Covariance analysis was conducted using Excel Tokei (BellCurve).

3 | RESULTS

3.1 | The *gs3* allele in the genomic gene in large-grain Notohikari and grain morphology

To investigate the effects on the *gs3* allele of Akita 63 on rice plants grain size and productivity, NILs of rice plants were developed by crossing the normal-grain *japonica* cultivar, Notohikari with Akita 63. The progenies of the NILs were continuously screened by backcrossing

with Notohikari three times using 148 DNA markers (Table S1). To confirm that the *gs3* allele derived from Akita 63 had been incorporated into the region of chromosome of LG-Notohikari, the entire genome was resequenced. Results indicated that 65.8 and 62.5 Gbp of the total read bases for Notohikari and LG-Notohikari were obtained from a 151-bp paired-end library with 142 and 149 coverage. Furthermore, 413561794 reads (44.14 GC%) for Notohikari and 435827151 (44.08 GC%) for LG-Notohikari were produced and calculated. A comparison of Notohikari and LG-Notohikari genomic sequences revealed that variants were concentrated at a segment of a chromosome for approximately 6-Mb length (approximately 15–21 Mb) on chromosome 3 compared with other chromosomal regions (Figure S1). This finding indicates that the 6-Mb-long segment of chromosome in LG-Notohikari originated from Akita 63, including the *gs3* allele.

The genomic genes' variant numbers and homology (based on the number of variants/rice genome size) between Notohikari and LG-Notohikari were 49,293 and more than 99.9%, respectively. Li et al. (2018) reported a summary of 43 previously reported genes that influenced rice grain size. It was examined whether mutations existed between the 43 genes of the two genotypes. Variant sequences were found in three of the 43 Notohikari and LG-Notohikari genomic sequences against a reference variety, Nipponbare (Table S4). One was found on chromosome 3 that was substituted by the Akita 63 segment in LG-Notohikari. Likewise, although the functional *GS3* was found in the Notohikari genomic gene, *gs3* was homozygous, inserted at the stop codon of the *GS3* exon in LG-Notohikari (Table S4). Furthermore, while three additional variants of the *GS3* gene were found in LG-Notohikari, these variants were intron-variant and did not have any significant effect on gene product functions (Table S4). Additionally, other variants were found on chromosome 3 and 7. They were at the Notohikari segments in LG-Notohikari. Intron-variant one and two were detected in LG-Notohikari *PGL1* (Os03g0171300) and Notohikari *DEP2* (Os07g061600) genes, respectively (Table S4). Therefore, among the 43 previously identified genes, LG-Notohikari had a large-grain gene and a nonsense mutation in its *GS3* gene against Notohikari. Hence, it was used in further experiments.

LG-Notohikari had a grain length approximately 0.89 mm longer and 0.07 mm thicker than Notohikari. However, it was approximately 0.38 mm shorter and 0.10 mm thinner than Akita 63 (Table 1; Figure S2). Although LG-Notohikari was approximately 0.13 mm wider than Notohikari, no significant differences were observed in grain width between LG-Notohikari and Akita 63 (Table 1; Figure S2). Moreover, it has been reported that grain morphological size in rice is genetically constant irrespective of growth environments, including N nutrition (Makino, 2021; Yoshida, 1981). Thus, Table 1 only shows data for zero N application at the Kawatabi field in 2019.

3.2 | Increased yield and improved HI of LG-Notohikari

The field experiments in three locations (Kawatabi, Tsukuba, and Aobayama fields) in 2019 and 2020 showed that total dry matter

**TABLE 1** Grain (rough rice) morphological characteristics of Notohikari, LG-Notohikari and Akita 63 rice cultivars

Traits	Notohikari	LG-Notohikari	Akita 63
Grain length (mm)	7.53 ± 0.06 ^a	8.42 ± 0.06 ^b	8.80 ± 0.05 ^c
Grain width (mm)	3.38 ± 0.02 ^a	3.51 ± 0.02 ^b	3.47 ± 0.02 ^b
Grain thickness (mm)	2.23 ± 0.01 ^a	2.30 ± 0.01 ^b	2.40 ± 0.01 ^c

Note: The three rice cultivars of Notohikari, LG-Notohikari and Akita 63 were cultivated in the plots without N fertilizer at the Kawatabi field in 2019. Mean values ± standard errors of 30 independent seeds are indicated. A difference in the superscript letters a–c denotes a statistically significant difference in the grain length, grain width and grain thickness for Notohikari and LG-Notohikari ($P < 0.05$ according to one-way analysis of variance [ANOVA] followed by Fishers LSD test). “LG” stands for large-grain.

production of the above-ground part, panicle weights, and brown rice yields of LG-Notohikari tended to be greater than those of Notohikari because these mean values for LG-Notohikari were higher than those for Notohikari in all cultivation plots (Table 2). Total dry matter production and panicle weight per unit land area in LG-Notohikari tended to increase from 2% to 22% and from 5% to 32% in three experimental paddy fields (Table 2). The greatest total dry matter production and panicle weight exhibited 1610 and 880 g m⁻², respectively, in the plots of 15.0 g N m⁻² fertilization at Kawatabi field in 2019 (Table 2). Furthermore, no significant difference was observed in mean values for leaves and stems weight between Notohikari and LG-Notohikari for all N applications except with the 6.0 g N m⁻² application at the Aobayama field in 2020 (Table S5). Table 2 shows brown or rough rice yield in Kawatabi and Aobayama fields or Tsukuba fields. The brown rice yields per unit land area in LG-Notohikari significantly increased by up to 38% compared with those of Notohikari and 667 g m⁻² was the maximum value in the 15.0 g N m⁻² plots at Kawatabi field in 2019 (Table 2).

The panicle, leaves and stems weights were linearly correlated with the total dry matter of the above-ground parts (Figure 1a,b; Tables S6 and S7). LG-Notohikari exhibited a greater panicle weight than Notohikari at the harvesting stage for a given total dry matter production (Figure 1a; Tables S6 and S7). In contrast, leaves and stems weight was lower in LG-Notohikari than in Notohikari for a given total dry matter production (Figure 1b; Tables S6 and S7). Therefore, the HI of LG-Notohikari was approximately 5% higher than that of Notohikari (Figure 1c; Tables S6 and S7).

3.3 | Increased in plant N absorption of large-grain Notohikari during the ripening period

We examined the relationship between total plant N content of the above-ground parts per unit land area and the amount of N fertilization at each plot at the full-heading (Figure 2a) and harvesting stages (Figure 2b), including the relationship between total dry matter production of above-ground parts and plant N content at the full-heading (Figure 2c) and harvesting stages (Figure 2d). The plant N contents in two genotypes at both stages increased depending on the quantity of N fertilization (Figure 2a,b; Tables S8–S10). Although no difference in the plant N content was found between two genotypes at the full-heading stage (Figure 2a; Tables S8, S10,

and S11), LG-Notohikari exhibited a greater plant N content than Notohikari at the harvesting stage for the same N application plot (Figure 2b; Tables S9–S11). This indicates that the amount of N absorption in LG-Notohikari substantially increased from the full heading to the harvesting stage, in other words, during the ripening period. In contrast, total dry matter production of the above-ground part was linearly correlated with plant N content, and total dry matter production per plant N content did not differ between the two genotypes at either stage (Figure 2c,d; Tables 2, S8, S9, and S12–S14). These results indicate that greater dry matter production of LG-Notohikari at the harvesting stage was caused by higher N absorption during the ripening period.

3.4 | Relationship between yield, yield components, and plant N content at the harvesting stage

The single grain weight (brown or rough rice) in LG-Notohikari significantly increased from 14% to 26% compared with Notohikari (Table S15). However, no significant difference was observed in total spikelet numbers between Notohikari and LG-Notohikari grown in all N applications, except for the 6.0 g N m⁻² application at the Aobayama field in 2020 (Table S16). Also, no significant difference was observed in the ratio of filled or fertilized spikelets between the plants in all N applications, except for a 13.0 g N m⁻² application in the Aobayama field in 2020 (Table S17).

Relationships between yield, yield components, and plant N content per unit land area at harvesting stages were examined at the Kawatabi and Aobayama fields (Figure 3; Tables S13 and S14). The single grain (brown rice) weight remained fairly constant irrespective of plant N content in both varieties, and that was 16% larger in LG-Notohikari than in Notohikari (Figure 3a; Table S13). The total spikelet number relative to a given amount of plant N were approximately 10% lower in LG-Notohikari than in Notohikari (Figure 3b; Tables S13 and S14). Consequently, the sink capacity was approximately 6% greater in LG-Notohikari than in Notohikari for the same amount of plant N content because the increase in the single grain weight of LG-Notohikari exceeded the decrease in the total spikelet number (Figure 3c; Tables S13 and S14). Because there were no correlations between the ratio of filled spikelets and plant N contents at the harvesting stage (Table S13), the increase in grain (brown rice) yield of



TABLE 2 Total dry matter production of the above-ground section, panicle weight, grain (brown or rough rice) yield and harvest index of Notohikari and LG-Notohikari at the harvesting stage

N fertilization (g N m ⁻²)	Year	Location	Total dry matter (g m ⁻²)		Panicle weight (g m ⁻²)		Grain (brown or rough rice) yield (g m ⁻²)*		Harvest index	
			Notohikari	LG-Notohikari	Notohikari	LG-Notohikari	Notohikari	LG-Notohikari	Notohikari	LG-Notohikari
15.0	2019	Kawatatabi	1490 ± 23 (100) ^a	1610 ± 43 (108) ^b	794 ± 16 (100) ^a	880 ± 26 (111) ^b	601 ± 15 (100) ^a	667 ± 20 (111) ^b	0.53 ± 0.01 (100) ^a	0.55 ± 0.01 (104) ^b
13.0	2020	Aobayama	1222 ± 41 (100) ^a	1413 ± 37 (116) ^b	601 ± 26 (100) ^a	791 ± 20 (132) ^b	439 ± 24 (100) ^a	587 ± 16 (134) ^b	0.49 ± 0.01 (100) ^a	0.57 ± 0.00 (114) ^b
9.6	2019	Tsukuba	1194 ± 14 (100) ^a	1230 ± 15 (103) ^a	560 ± 18 (100) ^a	627 ± 15 (112) ^b	450 ± 31 (100) ^a	535 ± 21 (119) ^b	0.46 ± 0.01 (100) ^a	0.51 ± 0.01 (110) ^b
8.0	2019	Kawatatabi	1125 ± 27 (100) ^a	1223 ± 34 (109) ^b	578 ± 11 (100) ^a	646 ± 15 (112) ^b	455 ± 13 (100) ^a	507 ± 8 (111) ^b	0.52 ± 0.01 (100) ^a	0.53 ± 0.01 (103) ^a
6.0	2020	Aobayama	915 ± 36 (100) ^a	1062 ± 42 (116) ^b	419 ± 15 (100) ^a	515 ± 25 (123) ^b	284 ± 14 (100) ^a	392 ± 22 (138) ^b	0.46 ± 0.01 (100) ^a	0.49 ± 0.01 (106) ^a
4.8	2019	Tsukuba	997 ± 13 (100) ^a	1067 ± 9 (107) ^b	384 ± 20 (100) ^a	486 ± 10 (127) ^b	338 ± 17 (100) ^a	456 ± 26 (135) ^b	0.39 ± 0.02 (100) ^a	0.44 ± 0.01 (115) ^b
0	2019	Kawatatabi	862 ± 22 (100) ^a	859 ± 37 (100) ^a	445 ± 16 (100) ^a	468 ± 28 (105) ^a	338 ± 15 (100) ^a	362 ± 22 (107) ^a	0.52 ± 0.01 (100) ^a	0.52 ± 0.01 (101) ^a
0	2019	Tsukuba	764 ± 4 (100) ^a	781 ± 8 (102) ^a	307 ± 9 (100) ^a	335 ± 9 (109) ^a	269 ± 4 (100) ^a	287 ± 9 (107) ^a	0.41 ± 0.01 (100) ^a	0.43 ± 0.01 (105) ^b

Note: Mean values ± standard error of 9–15 independent plants are indicated. Statistical analysis was conducted by Student's *t*-test ($P < 0.05$). A difference in the superscript letters a and b denotes a statistically significant difference in each Notohikari factor and LG-Notohikari. Values in parentheses indicate the percentage of each factor of LG-Notohikari relative to that of Notohikari given the same treatment. "LG" stands for large-grain. *Values in Kawatatabi and Aobayama fields indicate the brown rice yield, and values in the Tsukuba field indicate the rough rice yield.

LG-Notohikari was also observed for a given plant N content, compared with Notohikari (Figure 3d; Tables S13 and S14).

3.5 | Higher efficiency of photosynthate translocation in LG-Notohikari

Grain filling in rice depends on (1) carbon assimilation in photosynthesis in leaves during the ripening period and (2) the translocation efficiency of photosynthates temporarily stored in leaves and stems during the ripening periods (Cock & Yoshida, 1972; Nagata et al., 2002). Figure 4 shows the correlations between total dry matter production of the above-ground part and contents of nonstructural carbohydrate (NSC), starch and sucrose plus glucose in the leaves and stems per unit land area in LG-Notohikari and Notohikari at the harvesting stage. The residual NSC and starch contents in the leaves and stems relative to total dry matter production were higher in Notohikari than in LG-Notohikari, respectively (Figure 4a,b; Tables S18 and S19). In contrast, there were no significant differences in sucrose plus glucose contents in the leaves and stems relative to total dry matter production (Figure 4c; Tables S18 and S19). As a result, the lower residual NSC, especially starch contents in the leaves and stems relative to total dry matter production of the above-ground parts of LG-Notohikari, coincided with higher HI in LG-Notohikari compared with Notohikari. Thus, LG-Notohikari had a higher photosynthate translocation efficiency than Notohikari.

4 | DISCUSSION

4.1 | The enlarged grain size of LG-Notohikari due to *gs3* originated from Akita 63

By replacing the *gs3* allele with a functional *GS3* gene originated from Koshihikari with a normal grain, the NIL in the genetic background of Akita 63 showed a decrease of 0.89 mm in grain length (Obara et al., 2022). Our results with NIL in the genetic background of Notohikari with the *gs3* allele from Akita 63, LG-Notohikari, indicated an enlarged grain size with 0.70 mm of grain length (Table 1). Thus, we concluded that *gs3* allele caused enlarged grain size of LG-Notohikari, although the substitution effect of *gs3* on grain length was slightly smaller in this study, compared with the results reported by Obara et al. (2022).

4.2 | The *gs3* allele originated from Akita 63 confers superior traits for yield production to Notohikari

In this study, LG-Notohikari increased grain yield with improved HI (Table 2) and NUE for yield production per plant N content (Figure 3d). An increase in yield can be achieved by increasing the total biomass production per unit land area, improving HI, or both

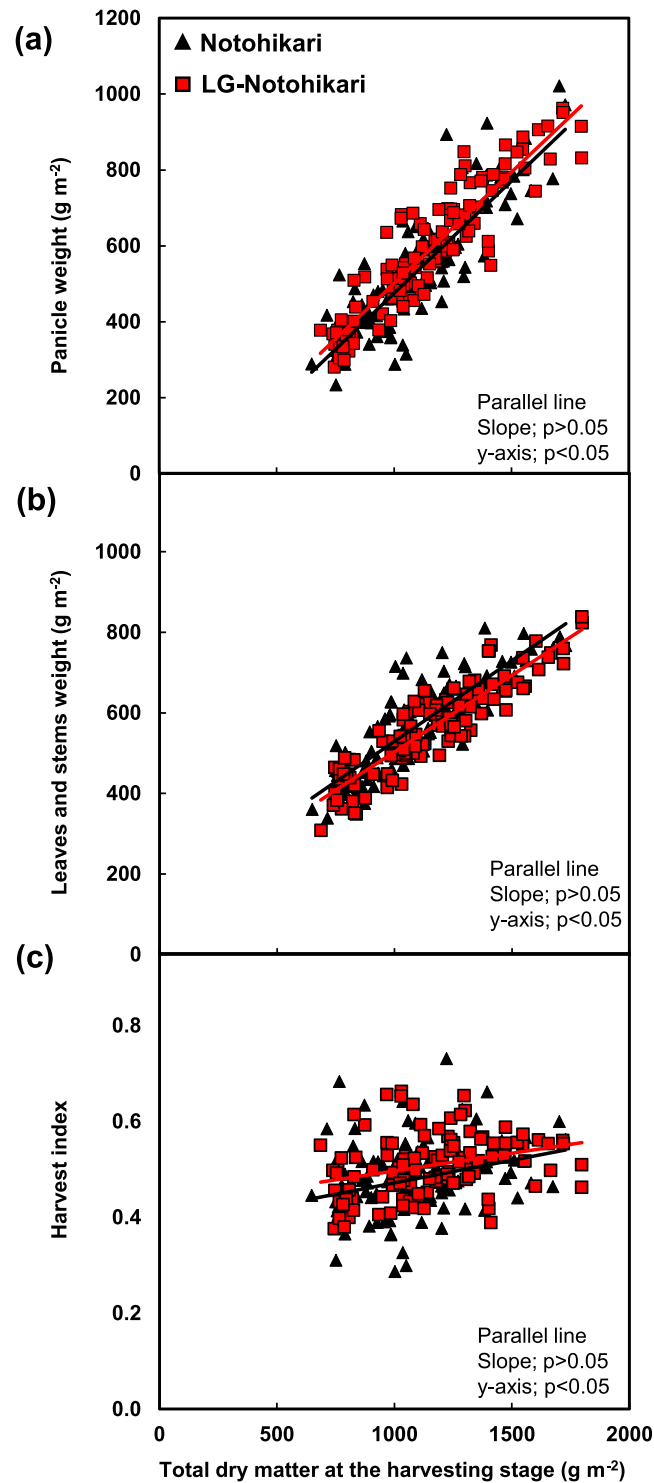


FIGURE 1 Relationships between panicle weight, leaves and stems weight and harvest index (HI) and total dry matter of the above-ground part per unit land area in Notohikari and LG-Notohikari rice plants at the harvesting stage. (a–c) Correlations between the total dry matter of the above-ground part of plants and the panicle weight per unit land area (a), leaves and stems weight (b) and HI (c) in two rice genotypes at the harvesting stage in Kawatabi, Aobayama, and Tsukuba fields. Dates of 9–15 independent plants in each N application in Kawatabi, Aobayama and Tsukuba fields are plotted (a–c). The regression lines, Pearson's product-moment correlation coefficients (R -value), and the correlation significance identified by Spearman's rank-order correlation (P -value) are presented in Table S6. Covariance analyses were performed between each parameter and the total dry matter of the above-ground part per unit land area in two rice genotypes at the harvesting stages. Significant differences (P -value) in the slope and y-axis intercept of two regression lines were shown in Table S7. Notohikari and large-grain Notohikari rice genotypes are represented by black triangles and red squares, respectively. 'LG' stands for large-grain

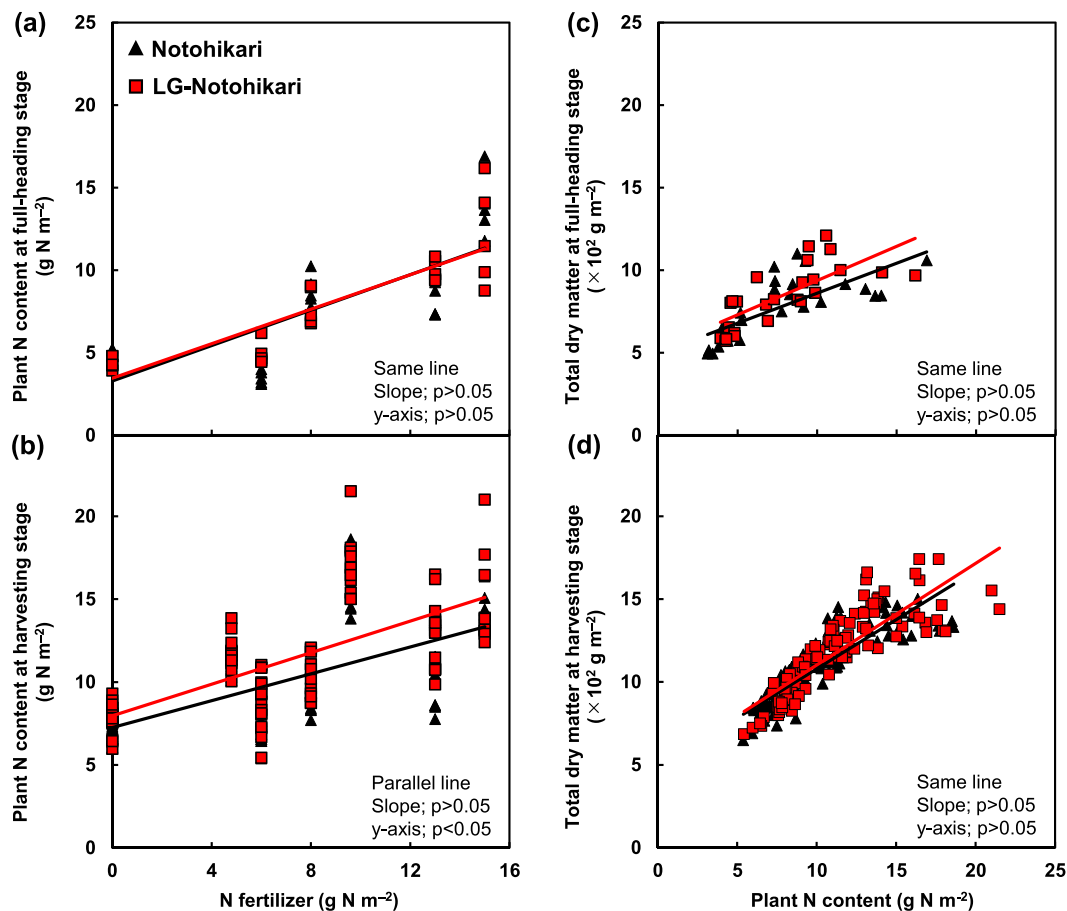


FIGURE 2 The effect of N fertilizer on the plant N content of the above-ground part of plants and the total dry matter of Notohikari and LG-Notohikari rice plants at the full-heading and harvesting stages. (a,b) Correlations between the plant N content and the amount of N fertilizer per unit land area in two rice genotypes at the full-heading in Kawatabi and Aobayama fields (a) and the harvesting stage (b) in Kawatabi, Aobayama, and Tsukuba fields. (c,d) Correlations between the total dry matter and the plant N content per unit land area in two rice genotypes at the full-heading in Kawatabi and Aobayama fields (c) and harvesting stages (d) in Kawatabi, Aobayama, and Tsukuba fields. The regression lines, Pearson's product-moment correlation coefficients (R -value), and the correlation significance identified by Spearman's rank-order correlation (P -value) are presented in Tables S10 and S13. Covariance analysis was performed between each parameter and the plant N content of the above-ground part per unit land area in two rice genotypes at the full-heading (a,c) and harvesting (b,d) stages. Significant differences (P -value) in the slope and y-axis intercept of two regression lines were shown in Tables S11 and S14. Notohikari and LG-Notohikari rice genotypes are represented by black triangles and red squares, respectively. 'LG' stands for large-grain

(Yoshida, 1981). In LG-Notohikari, the increased grain yields may have been caused by the above two factors: greater total dry matter and higher HI than those of Notohikari (Table 2). Also, the increase in N absorption during the ripening periods (Figure 2b) led to greater total dry matter and NUE improvements for yield production per plant N content (Figure 3d) and per amount of N fertilization (Table 2). In our previous study, transgenic rice plants overproducing ribulose 1,5-bisphosphate carboxylase-oxygenase, the CO₂ fixation enzyme of photosynthesis, exhibited increased yield production with an improvement in NUE per plant N content at harvesting stage and per amount of N fertilization for the yield under conditions of sufficient N fertilization (Yoon et al., 2020). These transgenic rice plants had greater N absorption during the ripening periods (Tanaka et al., 2022; Yoon et al., 2020), like LG-Notohikari (Figure 2a,b). It has been pointed out that the high amounts of N absorption during the ripening stage are

essential for high yields of rice plants and are commonly associated with crops having high HI (Sinclair, 1998; Wada et al., 1986). Recently, several studies have indicated that the G proteins, including GS3 are related to N uptake and assimilation and carbon metabolism in rice plants (Pandey, 2019; Sun et al., 2014; Xu et al., 2016; Zhang et al., 2019). However, it is unknown whether the G protein signaling pathway modified by the *gs3* allele controls the translocation efficiency of photosynthates and N absorptions during the grain-filling period in rice.

The analyses of yield components demonstrated that although the total spikelet number was a little smaller in LG-Notohikari than in Notohikari, greater single grain weight and a higher ratio of filled spikelets led to LG-Notohikari yield increased (Figure 3a). In contrast, the NIL in the genetic background of Akita 63 by replacing the *gs3* allele with the functional GS3 gene (Obara et al., 2022) was the

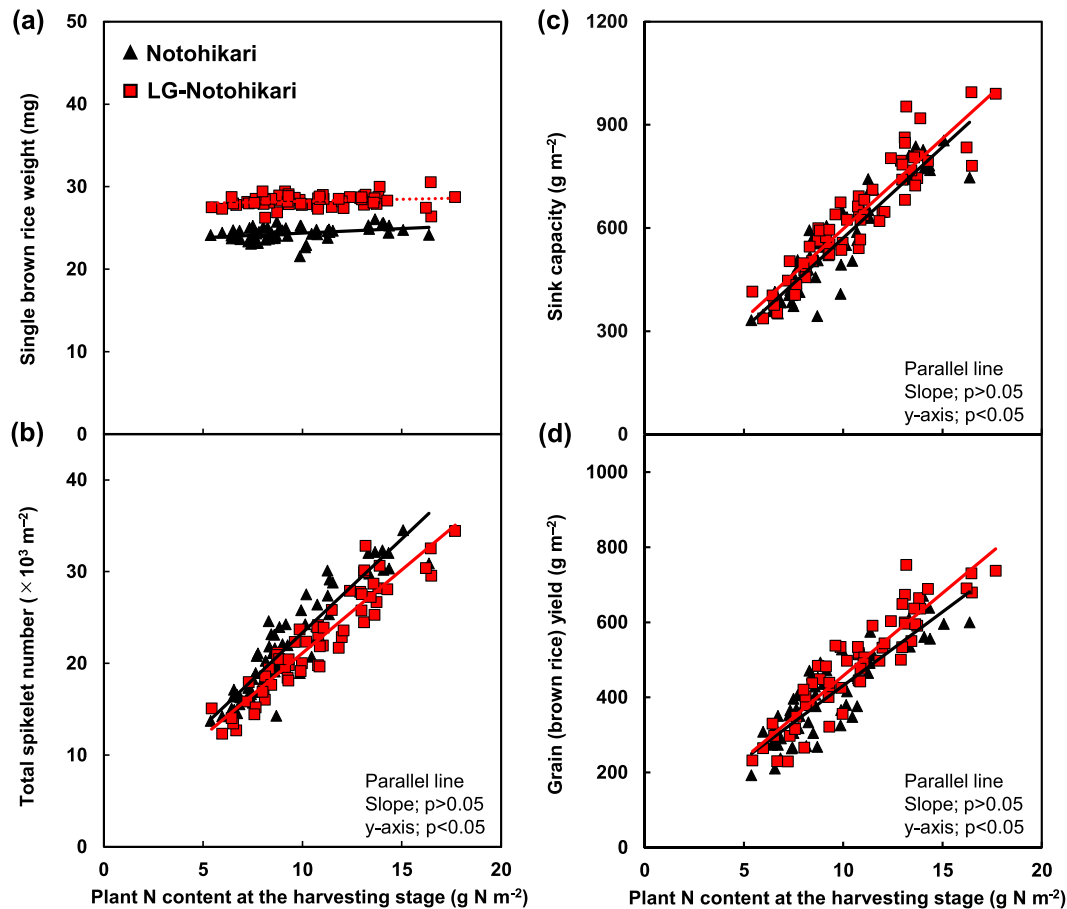


FIGURE 3 Relationships between single brown rice weight, total spikelet number, sink capacity, grain (brown rice) yields, and the plant N content of the above-ground part per unit land area in Notohikari and LG-Notohikari rice plants at the harvesting stage. (a–d) Correlations between the single grain (brown rice) weight (a), total spikelet number (b), sink capacity (number of total spikelets per unit land area [m^{-2}] \times single brown rice weight) (c), grain (brown rice) yield (d) in two rice genotypes at the harvesting stage in Kawatabi and Aobayama fields. The regression lines, Pearson's product-moment correlation coefficients (R -value), and the correlation significance identified by Spearman's rank-order correlation (P -value) are presented in Table S13. Correlation and noncorrelation are shown in solid and dashed lines, respectively. Covariance analysis was performed between each parameter and the plant N content of the above-ground part per unit land area in two rice genotypes at the harvesting stages. Significant differences (P -value) in the slope and y-axis intercept of two regression lines were shown in Table S14. Notohikari and LG-Notohikari plants are represented by black triangles and red squares, respectively. 'LG' stands for large-grain

opposite of our observation results. Because the increase in the total spikelet number did not overcome the decrease in single grain weight and the ratio of filled spikelet, the grain yields of the NIL in the genetic background of Akita 63 decreased compared with Akita 63 with the *gs3* allele (Obara et al., 2022).

In Akita 63, the single grain weight was about 35% larger, but the spikelet number for the same plant N content at the harvesting stage did not differ from the common japonica cultivars (Makino et al., 2020). Therefore, the large grain of Akita 63 directly contributed to an increase in yields (Mae et al., 2006). Alternatively, despite approximately 75% large single grain weight in the parental cultivar of Akita 63, Oochikara, a corresponding decrease in the number of grains per plant N content at the harvesting stage was observed, which did not increase yield (Makino et al., 2020). Thus, the high yield in Akita 63 was completely due to overcoming such a trade-off between single grain weight and the spikelet number. In this study, however, a slight

decrease in the spikelet number per plant N content at the harvesting stage was observed in LG-Notohikari (Figure 3b). These results indicate two possibilities: (1) That Akita 63 has an additional unknown gene(s) that restores the decrease in the spikelet number per plant N content at the harvesting stage, or (2) that a segment(s) of chromosome 3 from Akita 63 to LG-Noto contains an unknown gene(s) that reduces the spikelet number.

To achieve high yields, increasing the ratio of filled spikelets is also an essential factor. However, in many cases, high N fertilization decreased the ratio of filled spikelets (Matsushima & Tsunoda, 1957). This tendency was recognized in our previous studies (Makino et al., 2020; Yoon et al., 2020). Furthermore, in Akita 63, such a decrease in the ratio of filled spikelets with N fertilization was most noticeable (Makino et al., 2020). We considered that this was caused by the large sink, which resulted in a source limitation relative to the yield potential (Makino, 2011, 2021). In this study with LG-Notohikari,

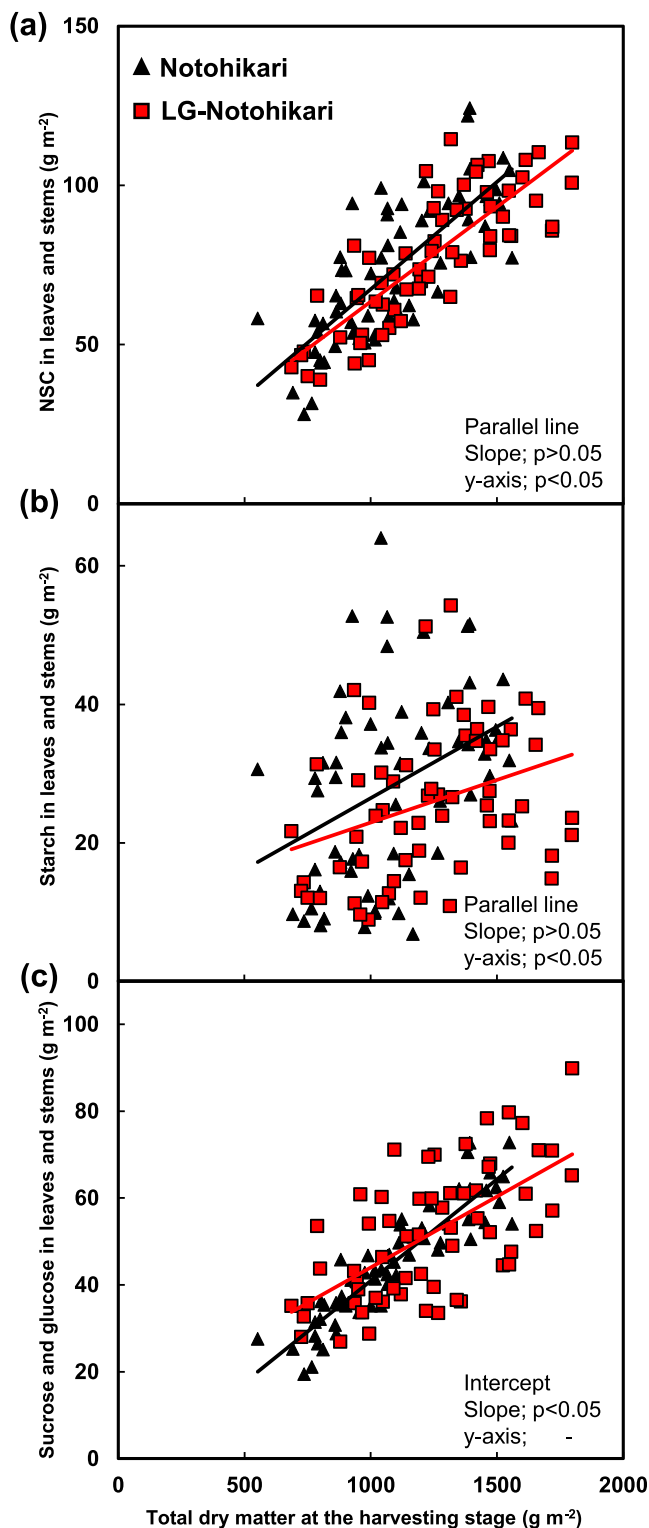


FIGURE 4 Correlation between NSC, starch, sucrose and glucose contents in leaves and stems and total dry matter of the above-ground part per unit land area of Notohikari and LG-Notohikari at harvesting stage. Correlations between NSC (starch + sucrose + glucose) (a), starch (b), sucrose and glucose contents (c) in leaves and stems and total dry matter of the above-ground part per unit land area in two rice cultivars at the harvesting stage in Kawatabi and Aobayama fields. The regression lines, Pearson's product-moment correlation coefficients (R -value), and the correlation significance identified by Spearman's rank-order correlation (P -value) are presented in Table S18. Covariance analysis was performed between grain (brown rice) yield per unit land area and each parameter in two rice cultivars at harvesting stages. Significant differences (P -value) in the slope and y-axis intercept of two regression lines were shown in Table S19. Notohikari and large-grain Notohikari rice cultivars are represented by black triangles and red squares, respectively. 'LG' stands for large-grain



however, despite the sink enlargement, there was no decrease in the ratio of filled spikelets with high N fertilization (Table S17). The sink capacity of Akita 63 exhibited approximately a 36% increase compared with the parental cultivar, Akita 39 (Makino et al., 2020). In contrast, the sink capacity of LG-Notohikari exhibited about a 6% increase compared with Notohikari (Figure 3c; Table S14) due to a slight decrease in the number of spikelets and lesser effect on the enlargement of grain size (Figure 3b; Table S14). Therefore, the lower increase in the sink capacity of LG-Notohikari may not have caused source limitation, resulting in no decrease in the ratio of filled spikelets. That means that developing a high yield potential of rice cultivars needs enlargement of sink capacity or enhancement of photosynthesis, such as in a previous study (Tanaka et al., 2022; Yoon et al., 2020).

5 | CONCLUSIONS

Our results with NILs of rice plants with large grains showed that the nonsense mutated GS3 gene, *gs3* allele from Akita 63, has superior traits for yield production with enlarged grain size and increased yield and improved HI and NUE for yields. In the past decades, there has been great research on the regulation of grain size in rice (Li et al., 2018). The grain size related to several signaling pathways was identified, such as G protein signaling, including the GS3 (Li & Li, 2016). From these facts, the *gs3* allele as a nonfunctional G γ subunit is a determinant for enlarging grain size and may function as positive effector for N uptake for the increase in yield in rice plants.

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CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

AUTHOR CONTRIBUTIONS

DKY, KI, TM, MO, and AM designed the research. MO produced near-isogenic line of Notohikari with large-grain. DKY, MS, KI, TK, MT, RN, DT, YS, and TM performed most of the experiments, as well as the growth, biomass and yield analyses. DKY, MS, and TK performed the biochemical and physiological experiments. DKY, MS, KI, HI, TM, and AM analyzed the data and DKY, KI, AM, and MO wrote most of the article.

DATA AVAILABILITY STATEMENT

Whole-genome resequencing datasets compared between genomic gene sequences of Notohikari and large-Notohikari to

Os-Nipponbare-Reference-IRGSP-1.0 reference genome are accessible through the NCBI PRJDB11789.

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SUPPORTING INFORMATION

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